Ministério da Saúde

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INSTITUTO OSWALDO CRUZ
Pós-Graduação em Biologia Celular e Molecular

NATIELE CARLA DA SILVA FERREIRA

Identificação de novos antagonistas para receptores purinérgicos P2

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutora em Biologia Celular e Molecular.

Orientador: Prof. Dr. Luiz Anastacio Alves

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ORIENTADOR: Prof. Dr. Luiz Anastacio Alves

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Dedico esse trabalho, fruto de quatro anos de
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familiares e amigos que sempre
acreditaram em mim.
Essa conquista também é de vocês!

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"Tempo rei, ó tempo rei, ó tempo rei
Transformai as velhas formas do viver
Ensinai-me, ó Pai, o que eu ainda não sei
Mãe Senhora do Perpétuo socorrei"

(Tempo Rei, Gilberto Gil)

Que os conhecimentos e os
títulos adquiridos jamais
removam de meu coração
a humildade e a consciência
de que a felicidade
reside nas coisas mais
simples da vida.

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IDENTIFICAÇÃO DE NOVOS ANTAGONISTAS PARA RECEPTORES PURINÉRGICOS P2

RESUMO

TESE DE DOUTORADO

Natiele Carla da Silva Ferreira

Os receptores purinérgicos P2 são receptores expressos na membrana plasmática de diversos tipos celulares humanos, nos quais exercem importantes funções fisiológicas. Eles são divididos em duas classes, de acordo com a estrutura apresentada. A classe P2X compreende os subtipos de receptores ionotrópicos que são ativados fisiologicamente pelo ATP. Dentre os receptores P2X, o P2X7 destaca-se por suas funções associadas à dor e à inflamação. Já a classe P2Y compreende os subtipos de receptores metabotrópicos, os quais são ativados por diferentes nucleotídeos extracelulares. Nessa última classe, o subtipo P2Y2 destaca-se pelos seus papeis desempenhados na inflamação e no câncer, enquanto o P2Y4 se sobressai pela escassez de informações acerca de suas funções fisiológicas devido a limitações de caráter farmacológico. Apesar de serem considerados importantes alvos-terapêuticos, ainda não existem fármacos com atuação sobre esses receptores que sejam aprovados para uso clínico, o que encoraja a busca por novas moléculas com atividade antagonista. Nesse contexto, o objetivo deste estudo foi identificar novos antagonistas para receptores P2 (P2X7, P2Y2 e P2Y4) e averiguar se eles exercem algum papel na atenuação da dor e inflamação. Na primeira parte desse trabalho foi identificada a atividade antagonista do extrato galhos de *Joannesia princeps* Vell. após a realização de uma mini-campanha de triagem. Utilizando a técnica de mensuração de cálcio intracelular, foi demonstrado que esse extrato foi capaz de inibir a mobilização de cálcio induzida por UTP de forma concentração-dependente, sendo que esse efeito não foi provocado por citotoxicidade ou fenômeno *quenching*. Além disso, esse extrato também foi capaz de inibir parcialmente a mobilização de cálcio induzida por UDP. Com isso, os resultados sugerem uma possível seletividade quanto à ação antagonista desse extrato sobre os receptores P2Y ativados por nucleotídeos derivados da uridina. Enquanto isso, na segunda parte do trabalho foi identificada a atividade antagonista da molécula CS-15 sobre o receptor P2X7 durante a realização de uma mini-campanha de triagem. A molécula CS-15, um triterpeno isolado da planta *Clusia studartiana* C. M. Vieira & Gomes da Silva, foi capaz de inibir a captação do corante YO-PRO-1 em ensaios de permeabilização celular de forma concentração-dependente e apresentou um IC_{50} na faixa de nanomolar. Ademais, essa molécula não demonstrou toxicidade *in vitro* e ainda foi capaz de aliviar os sintomas de nocicepção em modelo experimental animal. Coletivamente, os resultados apontam para a descoberta de novos antagonistas para receptores P2 de origem natural, os quais podem contribuir no futuro para o escasso campo da terapia associada aos receptores purinérgicos.

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IDENTIFICATION OF NEW ANTAGONISTS FOR P2 PURINERGIC RECEPTORS

ABSTRACT

TESE DE DOUTORADO

Natiele Carla da Silva Ferreira

P2 purinergic receptors are receptors expressed on the plasma membrane of several human cell types, in which they exert important physiological functions. They are divided into two classes, according to the structure presented. The P2X class comprises the ionotropic receptors subtypes that are physiologically activated by ATP. Among P2X receptors, P2X7 stands out due to its functions associated with inflammation and pain. The P2Y class comprises the metabotropic receptors subtypes, which are activated by different extracellular nucleotides. In this latter class, the P2Y2 subtype stands out for its roles in inflammation and cancer, while P2Y4 stands out because of the scarce information about its physiological functions due to pharmacological limitations. Despite being considered important therapeutic targets, there are still no approved drugs for clinical use acting on these receptors, which encourages the search for new molecules with antagonistic activity. In this context, the objective of this study was to identify new antagonists for P2 receptors (P2X7, P2Y2, and P2Y4) and to verify if they play any role in inflammation and pain attenuation. In the first part of this work the antagonistic activity of *Joannesia princeps* Vell. stem extract was identified after we carried out a mini-screening campaign. Using the intracellular calcium measurement technique, it was demonstrated that this extract was able to inhibit UTP-induced calcium mobilization in a concentration-dependent manner and that this effect was not triggered by cytotoxicity or quenching phenomenon. In addition, this extract partially inhibited UDP-induced calcium mobilization. Therefore, the results suggest a possible selectivity for the antagonistic action of this extract on the P2Y receptors activated by uridine-derived nucleotides. Meanwhile, in the second part of the work, the antagonist activity of the compound CS-15 on the P2X7 receptor was identified during a mini-screening campaign. The CS-15 compound, a triterpene isolated from the *Clusia studartiana* C. M. Vieira & Gomes da Silva plant, inhibited the YO-PRO-1 dye uptake in a concentration-dependent manner on cell permeabilization assays and showed an IC_{50} in the nanomolar range. In addition, this compound did not demonstrate toxicity *in vitro* and was able to alleviate nociception symptoms in an experimental animal model. Collectively, the results point to the discovery of new antagonists for P2 receptors from natural origin, which may contribute in the future to the scarce field of therapy associated with purinergic receptors.

LISTA DE ABREVIATURAS

2-MeSADP - 2-metiltioadenosina difosfato

2-thio-UTP - 2-thiouridina-5'-trifosfato

α-SMA - α-actina do músculo liso

AC - adenilato ciclase

ADA - adenosina deaminase

ADP - adenosina 5'-difosfato

AdeR - receptor ativado por adenina

AM - acetoximetil

AMP - adenosina 5'-monofosfato

ANVISA - Agência Nacional de Vigilância Sanitária

ATP - adenosina 5'-trifosfato

BAPTA - 1,2-bis(2-aminofenóxi)etano-N,N,N′,N′-ácido tetra acético

BBG - *Brilliant Blue G*

BzATP - 2′(3′)-O-(4-benzoilbenzoil) adenosina 5′-trifosfato

CaCC - canal de cloro ativado por cálcio

cAMP - adenosina 3',5'-monofosfato cíclico

CFTR - condutibilidade transmembrana da fibrose cística

COX-2 - ciclooxigenase-2

DAG - diacilglicerol

EC_{50} - *half maximal effective concentration*

EGFR - receptor do fator de crescimento epidérmico

EGTA - etileno glicol-bis(2-aminoetiléter)-N,N,N′,N′-ácido tetra acético

ELISA - *enzyme-linked immunosorbent assay*

EMA - *European Medical Agency*

ENaC - canal de sódio epitelial

EP3 - receptor de prostaglandina E3

EROS - espécies reativas de oxigênio

FDA - *Food and Drug Administration*

FRET - transferência de energia de ressonância Förster

HDL - lipoproteína de alta densidade (*high density lipoprotein)*

HIV - vírus da imunodeficiência humana

HMGB1 - proteína de alta mobilidade do grupo de caixa 1

HPLC - cromatografia líquida de alta performance (*high performance liquid chromatography*)

HTS - triagem de alto desempenho (*high-throughput screening*)

IC_{50} - *half maximal inhibitory concentration*

IL-1β - interleucina-1β

IL-18 - interleucina-18

iNOS - óxido nítrico sintase induzida

IP_3 - inositol trifosfato

IP_3R - receptor para o inositol trifosfato

IUPHAR - União Internacional de Farmacologia Básica e Clínica

KO – nocaute (*knockout*)

MAPK - proteínas quinases ativadas por mitógenos

NCE - novas entidades químicas (*new chemical entities*)

oATP - ATP oxidado

OMS - Organização Mundial de Saúde

ORCC - canal de cloro retificador externo

oxLDL - lipoproteína de baixa densidade oxidada

PCR - reação em cadeia de polimerase

PGE_2 - prostaglandina E_2

PIP_2 - fosfatidilinositol 4,5-bifosfato

PLC - fosfolipase C

PLCβ - fosfolipase C β

PPA - proteína precursora amilóide

PPADS - piridoxal fosfato-6-azo(benzeno-2,4-ácido disulfônico)

RB2 - *Reactive Blue-2*

TNF-α - fator de necrose tumoral-α

TNP-ATP - 2′,3′-O-(2,4,6-trinitrofenil) adenosina 5′-trifosfato

UDP - uridina5’-difosfato

UTP - uridina5’-trifosfato

WT - *wild-type*

VCAM-1 - proteína de adesão celular vascular 1

SUMÁRIO

I. INTRODUÇÃO

1. Breve histórico sobre a descoberta dos receptores purinérgicos

A história dos receptores purinérgicos iniciou-se com a descoberta de seus principais ligantes fisiológicos, as purinas e pirimidinas por Ludwig Kossel no fim do século XIX. Em 1878, Kossel publicou um de seus primeiros trabalhos, no qual ele descreve o isolamento das bases hipoxantina e xantina do conteúdo nuclear de leveduras [1]. Já no ano de 1885, Kossel descreveu pela primeira vez o isolamento da adenina em leveduras e no pâncreas humano, bem como delineou a transformação química dessa base em hipoxantina [1]. Em 1887, ele isolou e determinou a estrutura química da teofilina extraída de folhas de chá e determinou a sua relação com a cafeína, a qual é um reconhecido antagonista natural dos receptores purinérgicos P1. Finalmente, entre os anos de 1891 e 1901, Kossel publicou os seus resultados sobre o isolamento do ácido fosfórico e das bases adenina, guanina, timina, citosina e uracila [1]. Simultaneamente, dois químicos alemães, Emil Fischer e Adolf Pinner, se dedicaram ao estudo das estruturas químicas dessas bases nitrogenadas recém-descobertas e denominaram-nas purinas e pirimidinas [2]. Posteriormente, Phoebus Levene descreveu o açúcar que era conjugado a essas bases, caracterizando os nucleosídeos (bases nitrogenadas contendo açúcar) e nucleotídeos (bases nitrogenadas contendo açúcar e fosfato(s)) [2,3].

Até esse momento da história, acreditava-se que as atividades dessas moléculas restringiam-se ao núcleo celular. Entretanto, em 1929, Alan Drury e Albert Szent-Györgyi ao isolarem substâncias do tecido cardíaco identificaram que a adenosina 5'-monofosfato (AMP) era a responsável por alterar o ritmo cardíaco, diminuir a pressão arterial e inibir movimentos intestinais em cobaias. Eles também observaram que a adenosina era mais potente do que o AMP em produzir um bloqueio cardíaco [2,4]. Esses experimentos foram repetidos por diversos autores como Gillespie (1934), que apontou os diferentes efeitos biológicos promovidos pela adenosina e seus derivados. Ele observou que a adenosina 5'-trifosfato (ATP) era mais potente em aumentar a pressão arterial e o tônus uterino em cobaias, enquanto que o AMP e a adenosina produziam efeitos antagônicos. Ele descreveu ainda que a perda da pentose causava um detrimento da atividade biológica das moléculas [5].

Posteriormente, no final da década de 1940, surgiram os primeiros estudos relacionando os efeitos da adenosina e seus derivados no sistema nervoso. Buhthal et al. observaram que a injeção de ATP na medula espinhal de cobaias produzia contrações musculares [2]. Durante a década de 1950, Holton e Holton caracterizaram pela primeira vez o papel do ATP na neurotransmissão. No experimento de 1954, eles observaram que a injeção de ATP produzia vasodilatação na orelha desnervada de coelhos [6]. Já no experimento de

1959, Pamela Holton estudando ainda o papel do ATP na orelha de coelhos observou que este era liberado após a estimulação do nervo auricular ou da pele do animal, confirmando assim, a sua participação na neurotransmissão [7]. Nos anos seguintes, diversos trabalhos que demonstravam os efeitos do ATP e seus derivados no sistema nervoso foram publicados. Até que em 1970, Geoffrey Burnstock ao observar que o estímulo dos nervos causava a liberação do ATP em órgãos do sistema digestório de cobaias, propôs que o mesmo poderia atuar como um neurotransmissor, além de co-transmissor [8,9].

Assim, com a evidente descoberta da atividade do ATP e seus derivados em sistemas fisiológicos, Burnstock propôs em 1978 a primeira classificação para os "purinoceptores", isto é, receptores ativados por purinas e pirimidinas. Com isso, os receptores ativados pela adenosina foram nomeados de P1, enquanto os receptores ativados por ATP/ADP foram denominados P2 [10]. Nos anos seguintes, foram realizados diversos estudos farmacológicos e em 1979, van Calker et al. propuseram uma subdivisão para os receptores P1 em A1 e A2, uma vez que eles mediavam a inibição ou estimulação do AMP cíclico, respectivamente [11]. Já na década de 1980, Burnstock e Kennedy também propuseram uma divisão para os receptores P2 em duas classes: P2X e P2Y de acordo com critérios farmacológicos e efeitos biológicos que apresentavam [12].

Durante o início da década de 1990, diversos trabalhos acerca de clonagem dos subtipos de receptores purinérgicos foram publicados. Em 1994, um grupo de pesquisadores se reuniu e oficialmente estabeleceu uma nomenclatura e classificação para os receptores purinérgicos, a qual foi reconhecida pelo Comitê da União Internacional de Farmacologia Básica e Clínica (IUPHAR) sobre Nomenclatura de Receptores e Classificação de Medicamentos. Essa classificação seguiu critérios farmacológicos e estruturais e manteve a distinção das duas famílias: P1 e P2. A família dos receptores P1 apresentava quatro subtipos (A1, A2A, A2B e A3). Enquanto isso, a família dos receptores P2 foi dividida em cinco subtipos P2X, P2Y, P2Z, P2U e P2T [13]. Contudo, nesse mesmo ano, Abbracchio e Burnstock propuseram manter a classificação em P2X e P2Y e agruparam os subtipos P2U (atual P2Y2) e P2T (extinto P2Y3) na classe P2Y, e o P2Z (atual P2X7) na classe P2X [14]. Nos anos seguintes, finalmente foram reconhecidos e caracterizados os sete subtipos de receptors P2X (P2X1-P2X7) e os oito subtipos de receptores P2Y (P2Y1,2,4,6,11-14) [15,16].

Recentemente, foram descobertos um grupo de receptores são ativados por adenina (AdeR), os quais foram nomeados de receptores P0. No entanto, esses receptores ainda não foram caracterizados em humanos. Apenas se tem registro sobre a sua expressão em ratos, camundongos e hamsters [17].

2. Receptores purinérgicos

Os receptores purinérgicos são receptores conhecidos pela sua expressão na membrana plasmática em diferentes tipos celulares em mamíferos, onde são ativados por purinas e pirimidinas extracelulares. Todavia, já se tem informações na literatura sobre a expressão desses receptores na membrana de organelas celulares, tais como mitocôndria e núcleo celular [18,19], embora esses achados não tenham sido aprofundados pelos demais grupos de pesquisa da área.

A expressão de formas primitivas desses receptores já foi observada em outros seres vivos, tais como os seguintes invertebrados: o helminto *Schistossoma mansoni* que possui um receptor ativado fisiologicamente por ATP denominado *Sch*P2X; o caracol de lagoa *Lymnaea stagnalis* que expressa o receptor *Lym*P2X; o carrapato *Boophilus microplus* que possui o receptor *Bm*P2X; e o tardígrado *Hypsibius dujardini* que expressa o receptor *Hd*P2X. Esses receptores também estão presentes em protistas como o *Dictyostelium discoideum* (ameba), que expressa cinco tipos de receptores P2X (*Dd*P2X$_{A-E}$), e *Ostreococcus tauri* (alga), a qual possui um receptor capaz de ser ativado por ATP denominado *Ot*P2X [20].

Os receptores purinérgicos expressos em humanos são divididos em duas famílias: a família P1, que agrupa os membros ativados fisiologicamente por adenosina, e a família P2 que abriga os receptores ativados por diferentes nucleotídeos. Além disso, essa última família é subdivida em duas classes, de acordo com a estrutura apresentada. Portanto, existe a classe P2X, que agrupa os receptores que formam canais iônicos cátion-seletivos, e a classe P2Y que abriga os receptores acoplados à proteína G [14]. No quadro 2.1 estão descritos os subtipos de receptores purinérgicos de acordo com a classe a que pertencem.

Quadro 2.1 – Classificação dos receptores purinérgicos em humanos

Famílias	Classes	Receptores	Agonista(s) fisiológico(s)
P1	-	A1, A2A, A2B, A3	Adenosina
P2	P2X	P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7	ATP
	P2Y	P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14*	ADP, ATP, UDP, UDP-glicose ou UTP

*O intervalo na sequência dos números desses receptores se deve ao fato de que alguns foram caracterizados incorretamente, e após investigação, ficou constatado que eles não pertenciam a essa classe.

2.1 – Receptores P1

Os receptores P1 são receptores acoplados à proteína G e possuem quatro subtipos: A1, A2A, A2B e A3. Esses receptores possuem sete domínios transmembranas (TM1-7), além dos sítios N-terminal extracelular e C-terminal intracelular [21]. Os receptores A2A e A2B são acoplados à proteína Gs (estimulatória), a qual está envolvida com a estimulação da adenilato ciclase (AC) e com a produção de adenosina 3',5'-monofosfato cíclico (cAMP). Já os subtipos A1 e A3 estão associados à proteína Gi (inibitória) e inibição da AC. Além disso, o receptor A2B também pode se associar à proteína Gq (quiescente), favorecendo o aumento de cálcio intracelular a partir da via de sinalização da fosfolipase C (PLC) e inositol trifosfato (IP_3), conforme salientado na Figura 2.1.1 [22].

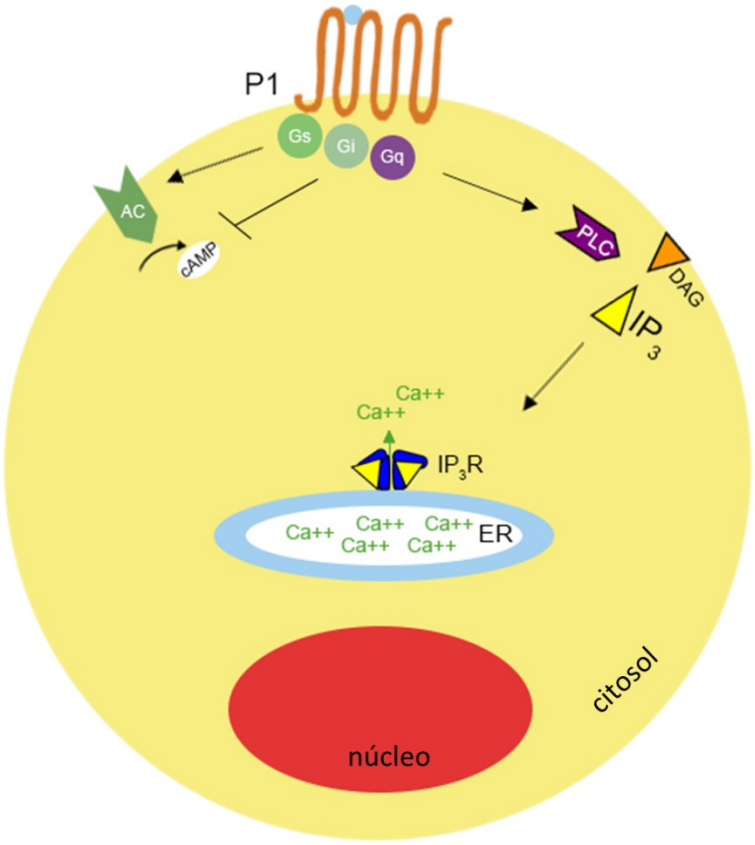
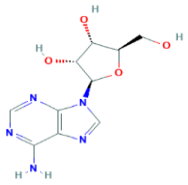
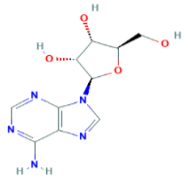
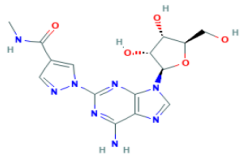
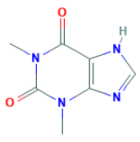
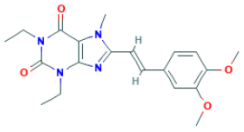


Figura 2.1.1: Receptores P1 e suas proteínas G associadas. Os receptores purinérgicos P1 podem se associar a três diferentes tipos de proteínas G. Os receptores A2A e A2B estão acoplados a proteína Gs, que ativa a enzima adenilato ciclase (AC), produzindo o cAMP a partir da catálise do ATP. Já os receptores A1 e A3 estão acoplados à proteína Gi, a qual possui ações antagônicas à Gs. O receptor A2B também pode acoplar-se à proteína Gq, a qual promove a ativação da fosfolipase C (PLC), que cliva o fosfatidilinositol 4,5-bifosfato (PIP_2) em diacilglicerol (DAG) e uma molécula solúvel denominada inositol trifosfato (IP_3). A IP_3 por sua vez, ativa um receptor próprio (IP_3R) expresso na membrana do retículo endoplasmático, permitindo um influxo de cálcio para o citosol.

Esses receptores são expressos em diferentes órgãos em humanos, tais como cérebro, medula espinhal, testículos, coração, terminais nervosos autonômicos, pulmões, baço, intestino, bexiga e fígado [21]. Esses receptores são ativados fisiologicamente pela adenosina. Entre os seus antagonistas não seletivos encontram-se a cafeína, a teofilina, entre outras xantinas de ocorrência natural. Alguns fármacos similares à adenosina ou à teofilina já se encontram aprovados para uso terapêutico no tratamento da taquicardia e asma, respectivamente [22]. Apesar disso, já foram desenvolvidos diversos agonistas e antagonistas seletivos (sintéticos e semissintéticos) para cada um dos subtipos desses receptores, sendo que alguns deles já se encontram em diferentes fases de ensaios clínicos. O subtipo A2A, por sua vez, é o único dentre os receptores P1 que apresenta agonista e antagonista seletivo aprovado para uso clínico [22]. O quadro 2.1.1 apresenta os ligantes dos receptores P1 aprovados para uso clínico.

Quadro 2.1.1 – Agonistas e antagonistas dos receptores P1 aplicados na terapia

Fármaco	**Mecanismo de ação**[1]	**Aplicação**[1]	**Empresa responsável**	**Estrutura química**
Adenocard	Ativação de receptores P1	Taquicardia paroxismal ventricular	Astellas	
Adenoscan	Ativação de receptores P1	Imageamento de perfusão miocárdica	Astellas	
Regadenoson (Lexiscan)	Agonista seletivo do receptor A2A	Imageamento de perfusão miocárdica	Astellas/ Gilead	
Teofilina	Antagonista de receptores P1	Asma e doença obstrutiva crônica pulmonar	-	
Istradefilina (KW-6002)	Antagonista seletivo do receptor A2A	Doença de Parkinson	Kyowa Hakko	

[1]Referências: [22,23]; [2]Estruturas químicas extraídas da Base Livre de Dados Químicos (PubChem): (https://pubchem.ncbi.nlm.nih.gov).

2.2 – Receptores P2Y

Os receptores P2Y partilham da mesma estrutura que os receptores P1: possuem sete domínios transmembrana (TM1-7), além dos domínios N-terminal extracelular e C-terminal intracelular, conforme demonstrado na Figura 2.2.1.

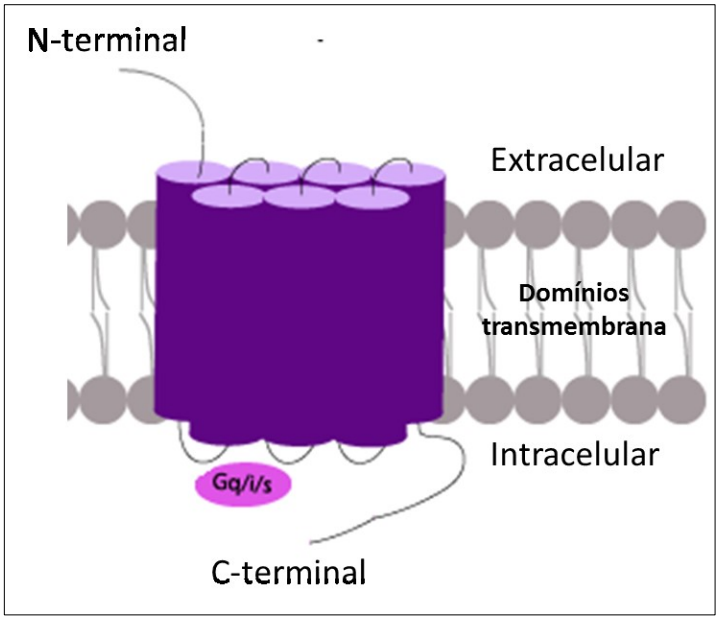


Figura 2.2.1 – Topologia básica dos receptores P2Y. Os receptores P2Y possuem a topologia básica dos receptores metabotrópicos: sete domínios transmembrana, um domínio N-terminal extracelular e um domínio C-terminal intracelular. As caudas intracelulares que conectam domínios transmembranas adjacentes são responsáveis pela interação com a proteína G (q/i/s).

Os subtipos P2Y1, P2Y2, P2Y4, P2Y6 e P2Y11 estão acoplados à proteína Gq, embora o último também se acople à proteína Gs. Já os subtipos P2Y12, P2Y13 e P2Y14 estão acoplados à proteína Gi. Esses receptores são ativados fisiologicamente por diferentes nucleotídeos, tais como ADP (P2Y1, P2Y12 e P2Y13), ATP (P2Y11 e P2Y2), UDP (P2Y6 e P2Y14), UDP-glicose (P2Y14) e UTP (P2Y2 e P2Y4) [22]. Um resumo das principais características dos receptores P2Y está descrito no quadro 2.2.1

Quadro 2.2.1 – Principais características farmacológicas dos receptores P2Y

Receptor P2Y	Proteína G associada	Principais efetores	Agonista fisiológico	Agonistas seletivos	Antagonistas seletivos
P2Y1	Gq	Ativação da PLC, Rac e Rho	ADP	MRS2365	MRS2179 MRS2279 MRS2500 MRS2950
P2Y2	Gq	Ativação da PLC, Rac e Rho	UTP e ATP	MRS2768 2-thio-UTP	ARC-118925
P2Y4	Gq	Ativação da PLC	UTP	MRS4062	-
P2Y6	Gq	Ativação da PLC e Rho	UDP	PSB-0474 MRS2693 MRS2782 INS48823 5-O-metil-UDP	MRS2578
P2Y11	Gq; Gs	Ativação da PLC e AC	ATP	NF546 AR-C67085	NF340
P2Y12	Gi	Inibição da AC e ativação da PLC e Rho	ADP	-	PSB-0739 ticlopidina clopidogrel prasugrel ticagrelor elinogrel
P2Y13	Gi	Inibição da AC e ativação da PLC e Rho	ADP	CT1007900	MRS2211
P2Y14	Gi	Inibição da AC e ativação da PLC	UDP e UDP-glicose	MRS2690 MRS2802	PPTN

PLC: fosfolipase C; AC: adenilato ciclase.

O receptor P2Y1 é expresso em células epiteliais, endoteliais, imunes, plaquetas e osteoclastos [21]. Apesar de o ADP ser o seu agonista fisiológico, o 2-MeSADP é um dos agonistas mais potentes e o MRS2365 é o agonista seletivo. Já existem antagonistas caracterizados para esse receptor, tais como o MRS2179, MRS2279, MRS2500 e MRS2950 [21,24]. Esses antagonistas vêm sendo utilizados em diversos trabalhos para a elucidação de funções associadas a esse receptor, como nocicepção, hiperalgesia e dor inflamatória [25–27], além da hiper-reatividade de astrócitos na doença de Alzheimer [28], quimiotaxia [29] e agregação plaquetária [30–35].

O receptor P2Y2 é expresso em células do sistema imune, células epiteliais e endoteliais, além de túbulos renais e osteoblastos [21]. O P2Y2 é ativado fisiologicamente pelo UTP e ATP, mas também apresenta agonistas seletivos como o MRS2768 e o 2-thio-UTP. Entretanto, apenas um antagonista seletivo é descrito para esse receptor, o ARC-118925 [22]. O P2Y2 demonstrou estar envolvido no desenvolvimento da dor neuropática [36,37], no câncer [38–42] e na inflamação [43–48].

O receptor P2Y4 é expresso no intestino, cérebro, pulmão, coração, entre outros tipos celulares [49]. O P2Y4 é ativado fisiologicamente pelo UTP e seletivamente pela molécula MRS4062 [22]. Devido à inexistência de antagonistas seletivos para esse receptor, poucas funções fisiológicas são de fato reconhecidas como provenientes de sua ativação.

O receptor P2Y6 é expresso em células epiteliais, linfócitos T, placenta e timo [21]. Fisiologicamente, esse receptor é ativado pelo UDP, entretanto, já foram desenvolvidos diversos agonistas seletivos para esse receptor, tais como PSB-0474, MRS2693, MRS2782, INS48823 e 5-O-metil-UDP, assim como o antagonista seletivo MRS2578 [22,24]. Trabalhos recentes vêm demonstrando a participação desse receptor na dor [27,50–52], no câncer [53] e na inflamação [54–58], sendo que o tratamento com o antagonista MRS2578 é capaz de atenuar esses efeitos. Todavia, essa molécula ainda não avançou para estudos em seres humanos devido às suas limitações quanto à reatividade e hidrofobicidade [59].

O receptor P2Y11 é expresso no baço, no intestino e em granulócitos [21]. O P2Y11 é ativado fisiologicamente pelo ATP e os seus agonistas seletivos são NF546 e AR-C67085. O NF-340 é o antagonista seletivo para esse receptor, uma vez que não interage com outros receptores P2Y e P2X. Devido à inexistência de sequência para o receptor P2Y11 em camundongos, muitas funções fisiológicas desse receptor ainda não foram completamente caracterizadas [24,60]. Apesar disso, alguns trabalhos apontam a participação desse receptor no desenvolvimento da dor em modelos animais, porém, a existência de possíveis efeitos colaterais que possam ser responsáveis por essa atividade deve ser investigada [27,51].

O receptor P2Y12 é expresso principalmente em plaquetas e células da glia [21]. O P2Y12 é ativado fisiologicamente pelo ADP, e até o momento, não possui nenhum agonista seletivo [22]. Contudo, esse é o primeiro e único subtipo de receptor da família P2 que possui antagonistas empregados na terapia na prevenção da agregação plaquetária. Entre os antagonistas seletivos para o P2Y12 encontram-se o clopidogrel, o prasugrel, o ticagrelor, o cangrelor, o PSB-0739 e a ticlopidina, sendo os quatro primeiros aprovados para uso clínico [24]. Além do importante papel desempenhado na agregação plaquetária, estudos recentes demonstraram a participação do P2Y12 na dor [24], no câncer [61,62] e na inflamação [63,64].

O receptor P2Y13 é expresso no cérebro, no baço, nos linfonodos e na medula óssea [21]. Esse receptor é ativado fisiologicamente pelo ADP, e recentemente foi descrita a ação agonista de uma molécula, a CT1007900, em animais [65]. O MRS2211 é o único antagonista seletivo para o P2Y13. Apesar disso, existem poucos estudos mencionando papéis fisiológicos associados a esse receptor. Entre eles encontra-se um estudo que mostra a importância da ativação do P2Y13 no aprimoramento do metabolismo do colesterol HDL [65], além de estudos descrevendo a participação do receptor na produção de citocinas inflamatórias no fígado [66] e no desenvolvimento da dor neuropática [50].

O P2Y14 é expresso na placenta, no tecido adiposo, no estômago, no intestino e em algumas regiões do cérebro [21]. O P2Y14 é ativado fisiologicamente pelo UDP e UDP-glicose e as moléculas MRS2690, MRS2802 e α,β-metileno-2-thio-UDP são os seus agonistas seletivos [24]. Recentemente, foi descrito um antagonista seletivo para esse receptor denominado PPTN, o qual foi importante para a realização de estudos que demonstram a participação do P2Y14 na inflamação [67,68].

2.3 – Receptores P2X

Os receptores P2X são canais iônicos que quando ativados por ligantes, permitem a passagem seletiva dos cátions Na^+, Ca^{2+} e K^+, de acordo com o gradiente eletroquímico. O P2X5 é o único subtipo que permite a passagem do íon cloreto (Cl^-), além dos cátions citados [15].

Atualmente, existem sete subtipos de receptores pertencentes a essa classe: P2X1-P2X7. Esses receptores são triméricos, isto é, são formados por três subunidades, as quais possuem domínios N e C-terminais intracelulares, sendo que esse último apresenta sítios de ligação para proteínas quinases. Eles também possuem dois domínios transmembranas (TM1

e TM2), os quais estão envolvidos com a formação do canal iônico e do poro, respectivamente, além de uma cauda extracelular que possui resíduos de cisteína que atuam na formação de pontes dissulfeto [21]. A Figura 2.3.1 demonstra a topologia dos receptores P2X.

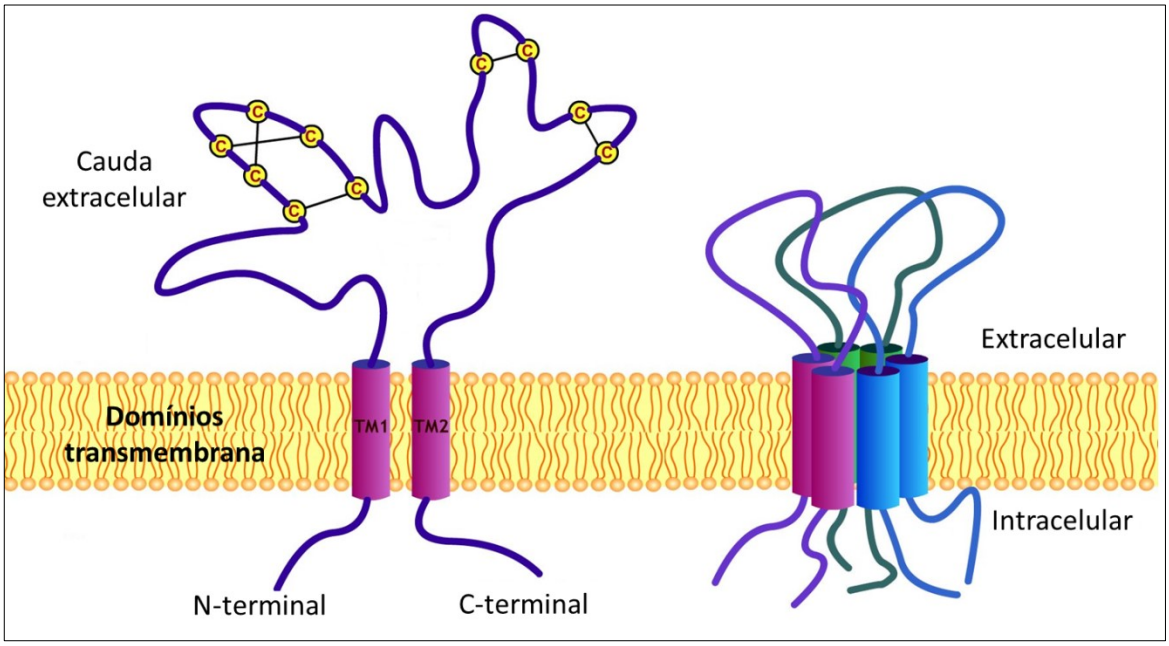


Figura 2.3.1 – Topologia básica dos receptores P2X. Cada subunidade do receptor P2X (à esquerda) é composta por uma cauda extracelular com domínios ricos em cisteína, que se unem para formar pontes dissulfeto, além de dois domínios transmembrana (TM1 e TM2) que são responsáveis pela formação do canal iônico, bem como os sítios N e C-terminais intracelulares. Três subunidades de iguais (representadas pela mesma cor) ou diferentes (representadas por cores diferentes) subtipos de receptor P2X se agrupam para formar um receptor homo ou heteromultimérico, respectivamente (à direita). Imagem adaptada de: Drug Design Team da Universidade de Camerino (https://sites.google.com/a/unicam.it/diego-dal-ben/activities-and-collaborations).

Os receptores P2X podem ser homomultiméricos, isto é, todas as subunidades formadas correspondem à sequência de um único receptor (ex: P2X1, P2X2, P2X3, P2X4, PX5, P2X6 e P2X7), ou heteromultiméricos, quando um canal iônico é formado pela associação entre subunidades de diferentes P2X (ex: P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6). O P2X6 é um exemplo de receptor que funciona apenas na forma de heteromultímero, enquanto o P2X7 só funciona como homomultímero [21].

O agonista fisiológico dos receptores P2X é o ATP. Todos os membros dessa classe são ativados pelo ATP entre 0,1 a 10 μM, com exceção do P2X7, cuja ativação ocorre em concentrações superiores a 100 μM [15]. Além dessa particularidade, o P2X7 também é o único que possui a habilidade de formar um poro membranar, o qual permite a passagem de

moléculas de até 900 Da, incluindo íons, água, ATP, corantes, entre outros [69]. Já está descrito na literatura que outros subtipos de receptores P2X, como o P2X2 e o P2X4, também possuiriam essa habilidade de formar poros membranares similares ao P2X7 [70,71], contudo, há controvérsias sobre a formação desses poros. Apesar disso, a origem do poro membranar ainda é uma incógnita. Alguns autores acreditam que a abertura do poro membranar esteja relacionada com a dilatação do canal iônico, enquanto outros acreditam que a hipótese mais plausível seja de que outra proteína membranar funcione como um poro, sendo essa ativada através de segundos mensageiros derivados da ativação dos receptores P2X [72–74]. O quadro 2.3.1 resume as principais características dos receptores P2X.

Quadro 2.3.1 - Principais características farmacológicas dos receptores P2X

Receptor P2X	Heteromultímeros formados	Agonista fisiológico (EC_{50})	Antagonista(s) seletivo(s)
P2X1	P2X1/2 P2X1/4 P2X1/5	ATP (100 a 700 nM)	NF279 NF449
P2X2	P2X1/2 P2X2/3 P2X2/6	ATP (2 a 8 μM)	PSB-1011 PSB-10211 NF770 NF778
P2X3	P2X2/3	ATP (1 μM)	A-317491 MK-390 AF-219 RO3 RO4
P2X4	P2X1/4 P2X4/6	ATP (1 a 10 μM)	5-BDBD PSB-12054 PSB-12062 BX-430
P2X5	P2X1/5	ATP (0,5 a 4 μM)	-
P2X6	P2X2/6 P2X4/6	-	-
P2X7	-	ATP (> 100 μM)	A-438079 A-740003 A804598 AACBA AZD9056 AZ10606120 AZ11645373 CE-224,535 JNJ-47965567 oATP

O P2X1 é expresso na bexiga, intestinos, vasos deferentes, artérias, plaquetas, entre outros tipos celulares [75]. Esse receptor é o que possui maior afinidade pelo ATP dentre os P2X, possuindo EC_{50} entre 100 a 700 nM [75]. O P2X1 também possui antagonistas seletivos, como o NF279 e o NF449, os quais são capazes de inibir a atividade de outros P2X em concentrações superiores ao IC_{50} [76]. Alguns trabalhos apontam que a ativação do P2X1 pode ter associação com o desenvolvimento da doença de Parkinson e a formação de trombos [77,78]. Além disso, já foi observada a importância desse receptor na função reprodutiva masculina e Mulryan et al. (2000) até propuseram o uso de antagonistas do P2X1 como uma pílula contraceptiva masculina [79].

O P2X2 é expresso em células dos tecidos nervoso, esquelético, cardíaco, muscular, endotelial, epitelial, entre outros [75]. Assim como os demais P2X, o receptor P2X2 é ativado fisiologicamente pelo ATP e apresenta um EC_{50} entre 2 a 8 μM [75]. Entre os antagonistas seletivos para esse receptor estão: PSB-1011, PSB-10211, NF770 e NF778 [75,76]. As funções fisiológicas do P2X2 ainda são pouco estudadas. Apesar disso, já foi observado que o P2X2 está associado ao bom funcionamento da audição e à fertilidade dos espermatozoides [80,81].

O P2X3 possui uma expressão restrita a neurônios sensoriais, além de medula espinhal e tecido cardíaco [15,75]. Ele é ativado fisiologicamente pelo ATP na concentração de 1 μM [75]. Além disso, já estão descritos na literatura alguns antagonistas seletivos para esse receptor, que incluem o A-317491, MK-390 e o AF-219. Existem também outros antagonistas pertencentes à classe de diaminopirimidina, tais como o RO3 e RO4, que se ligam ao sítio alostérico do canal do P2X3. A molécula AF-219 é o único antagonista do receptor P2X3 que se encontra em etapa de ensaios clínicos para o tratamento da tosse crônica e fibrose pulmonar idiopática [76]. Uma vez que a ativação do P2X3 está associada com a dor [82,83], já foi demonstrado experimentalmente que a sua inibição produz efeitos analgésicos [84–87].

O P2X4 é expresso nos sistemas imune, nervoso e reprodutor masculino, entre outros [15,75]. Esse receptor é ativado fisiologicamente pelo ATP na faixa de 1 a 10 μM [75]. Até o momento já se tem descrito na literatura os seguintes antagonistas seletivos: 5-BDBD, PSB-12054, PSB-12062 e BX-430, cujos IC_{50}s estão na faixa de 0,189 a 1,6 μM em humanos [88]. Há evidências de que o P2X4 contribui para o desenvolvimento de diversas doenças, incluindo a dor neuropática [89–92] e doenças inflamatórias, como artrite reumatóide, asma e neuroinflamação [93–96].

O P2X5 também é encontrado no sistema nervoso, além de vasos sanguíneos, coração, músculo esquelético, rins e glândula adrenal [75]. Ele é fisiologicamente ativado pelo ATP na faixa de 0,5 a 4 μM [75]. Todavia, não existe até o momento um antagonista seletivo para

esse receptor e enquanto isso, antagonistas não seletivos de receptores P2 como Suramina, PPADS, TNP-ATP e Brilliant Blue G (BBG) são utilizados em experimentos para prevenir a sua ativação [97]. Apesar das suas funções serem pouco conhecidas, recentemente Kim et al. (2017) demonstraram a sua participação na diferenciação de osteoclastos [98].

O P2X6 está presente em algumas células do sistema nervoso, além de células do útero, ovário, epitélio brônquico, timo e glândula salivar [15,75]. Uma vez que o P2X6 não funciona como um homomultímero, dados referentes à sua farmacologia e funções fisiológicas são escassos [15,21].

Já o P2X7 é vastamente expresso em células do sistema imune, bem como em células epiteliais e glandulares, glia, fibroblastos, osteoblastos e hepatócitos [15,75]. Como mencionado anteriormente, o receptor P2X7 é o que possui menor afinidade pelo ATP, sendo ativado em concentrações superiores a 100 μM [15]. Em diversos trabalhos, o P2X7 é estimulado com o BzATP, um agonista análogo ao ATP e mais potente que este. Entretanto, o BzATP também possui atividade agonista sobre o P2X4 e funciona como agonista parcial dos receptores P2X1, P2X2, P2X3 e P2X5 [75]. O P2X7 possui diversos antagonistas seletivos descritos na literatura. Entre eles encontram-se: AACBA, A-438079, A-740003, A804598, AZ10606120, AZ11645373, AZD9056, CE-224,535, JNJ-47965567 e ATP oxidado (oATP) [99–101]. Além do papel na inflamação [102], o P2X7 também participa no desenvolvimento de doenças neurológicas e neurodegenerativas [99–101,103–112], câncer [113–117] e dor [118–122].

3. Desenvolvimento de fármacos com atividade sobre os receptores P2

Apesar da descoberta dos receptores P2 ter ocorrido durante as décadas de 1980 e 1990, seus papéis fisiológicos começaram a ser caracterizados a partir do fim dos anos 1990. Embora tenha aumentado de modo expressivo o conhecimento dos receptores P2 no contexto patológico, o grande desafio dos últimos anos é desenvolver fármacos com atividade sobre esses receptores que sejam eficazes. Nesse cenário, diversos antagonistas seletivos para os receptores P2 foram desenvolvidos, todavia, a maioria deles apresenta alguma limitação que não os permitiu evoluir para a fase de terapia em humanos.

Até o momento, o receptor P2Y12 é o primeiro caso de sucesso entre os receptores P2 com relação à aplicação clínica. Ele possui um antagonista, o clopidogrel, que é aplicado na terapia desde a década de 1990. O clopidogrel é antagonista irreversível do P2Y12 em plaquetas. Esse fármaco funciona como uma pró-droga, que necessita do metabolismo da enzima hepática CYP450 para produzir o seu metabólito ativo, um tiol reativo que apresenta

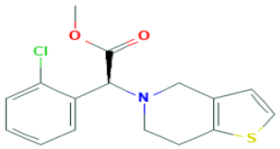
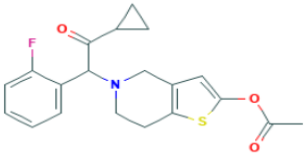
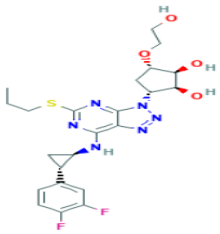
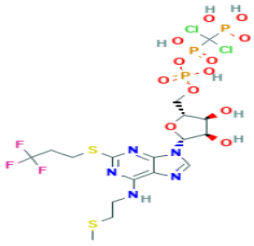
propriedades anti-agregantes e antitrombóticas. Atualmente, ele é administrado em conjunto com a aspirina na prevenção de trombose em pacientes com síndrome aguda coronária ou que foram submetidos a uma intervenção coronária percutânea [123]. Um estudo realizado com mais de 12 mil pacientes demonstrou que essa associação foi responsável pela diminuição de 2,1% de mortes em decorrência de eventos cardiovasculares durante o período avaliado [124].

Além do clopidogrel, outros fármacos com ação antagonista sobre o P2Y12 foram desenvolvidos. Entre eles, o prasugrel, que possui estrutura similar ao clopidogrel, funcionando também como uma pró-droga. Ele demonstrou ser mais eficaz que o seu antecessor sobre a diminuição de mortes em decorrência de eventos cardiovasculares, no entanto, ele está associado a um maior risco de hemorragias [125,126].

Posteriormente foi desenvolvido o ticagrelor, o primeiro antagonista reversível e seletivo do P2Y12 com atividade intrínseca. Apesar de ter auxiliado na sobrevivência de 90,2% de pacientes com risco de morte em decorrência de eventos cardiovasculares, esse antagonista provocou um número maior de efeitos colaterais do que o clopidogrel na população estudada [127,128].

O mais recente antagonista aprovado para uso clínico é o cangrelor. Ele é um antagonista do P2Y12 que está disponível apenas na forma intravenosa, sendo utilizado, portanto, em emergências médicas [129]. Entretanto, o cangrelor não é seletivo para o P2Y12, uma vez que também inibe o receptor P2Y13 [24]. O quadro 3.1 fornece um resumo dos fármacos com atividade sobre o receptor P2Y12 que são aprovados para uso terapêutico.

Quadro 3.1 – Antagonistas do receptor P2Y12 aprovados para uso clínico

Fármaco	Ano de aprovação[1]	Empresa responsável[1]	Estrutura química[2]
Clopidogrel	1997	Sanofi	
Prasugrel	2009	Eli Lilly and Co	
Ticagrelor	2011	AstraZeneca	
Cangrelor	2015	The Medicines Company	

[1]Informações extraídas do *website* do Departamento de Administração de Alimentos e Fármacos dos Estados Unidos (FDA): (https://www.fda.gov); [2]Estruturas químicas extraídas da Base Livre de Dados Químicos (PubChem): (https://pubchem.ncbi.nlm.nih.gov).

3.1 – O receptor P2Y2 como alvo terapêutico

O P2Y2 é um receptor fisiologicamente ativado pelo UTP e ATP extracelular. Ele é expresso em diversos órgãos tais como pulmão, coração, baço e rins, além de células do sistema imune como linfócitos e macrófagos [24,130]. Esse receptor possui importantes funções relacionadas ao fluxo de íons. O P2Y2 expresso nas células epiteliais da córnea, conjuntiva e vias aéreas estimula a secreção de cloro através da ativação do canal de cloro retificador externo (ORCC), promovendo a lubrificação das superfícies e hidratação do muco. Além disso, esse receptor é capaz de inibir a absorção de sódio e estimular o batimento ciliar, bem como a secreção de muco [131]. A Figura 3.1.1 demonstra as principais funções do receptor P2Y2 sobre o fluxo iônico em células epiteliais.

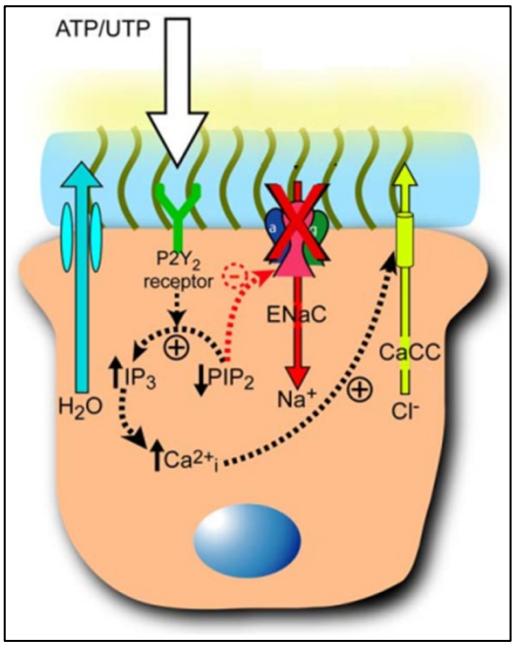


Figura 3.1.1 – Transporte iônico promovido pelo receptor P2Y2 em células epiteliais das vias aéreas. O P2Y2 é ativado fisiologicamente pelo ATP e UTP extracelular. Uma vez ativado, a proteína Gq associada estimula a enzima fosfolipase C a clivar o PIP_2 em diacilglicerol e inositol trifosfato (IP_3). Este último ativa o receptor IP_3R no retículo endoplasmático, promovendo a liberação do Ca^{2+} dos estoques intracelulares. Esse aumento de cálcio promove a abertura dos canais de cloro ativados por cálcio (CaCC), favorecendo o efluxo de Cl^- e o transporte de água. Além disso, a hidrólise do PIP_2 inibe os canais de sódio epiteliais (ENaC), diminuindo a reabsorção do Na^+. Adaptado de [132].

Essas importantes funções relacionadas ao fluxo de íons, podem contribuir para a lubrificação e hidratação de tecidos epiteliais, especialmente em doenças em que essas atividades estejam comprometidas, como a fibrose cística e a doença do olho seco.

A fibrose cística é uma doença genética, caracterizada pela mutação do gene que codifica a proteína reguladora da condutância transmembrana na fibrose cística (CFTR), a qual está envolvida na regulação do cloro, sódio e bicarbonato na superfície aérea. Com o fluxo iônico comprometido e o líquido superficial reduzido, os pacientes sofrem com um aumento da suscetibilidade de infecções e inflamações e com danos progressivos nas vias aéreas devido à limitação da depuração mucociliar [133].

Tendo em vista que a ativação do P2Y2 poderia corrigir o transporte iônico de forma independente do genótipo CFTR, a molécula Denufosol (INS37217) foi desenvolvida pela empresa Inspire Pharmaceuticals no início dos anos 2000. Essa molécula foi formulada como uma solução inalatória que ativa o receptor P2Y2, mimetizando a ação dos nucleotídeos ATP/UTP. Ela é um dinucleotídeo derivado de uridina, que estimula a secreção de cloro e mucina, o batimento ciliar, além de ser resistente à ação das ectonucleotidases (embora seja capaz de ativar outros receptores P2Y, como o P2Y4 e o P2Y6) [134]. O Denufosol foi

testado em 178 pacientes em ensaios clínicos de fase 3. Esses pacientes receberam três doses diárias de 60 mg de Denufosol ao longo de 24 semanas. Apesar de ter sido bem tolerado, a molécula não apresentou eficácia significativa em promover uma melhora da função pulmonar e redução de incidências de exacerbações pulmonares. Mesmo em uma segunda etapa dentro da fase 3, que incluía um número maior de pacientes (n= 233) e tempo mais longo de tratamento (48 semanas), ela também falhou em demonstrar eficácia [132,133,135]. Essa molécula chegou a ser estudada para o tratamento de descolamento de retina, edema macular e uveíte, entretanto, esses estudos foram terminados ou abandonados desde 2006, de acordo com informações depositadas no banco de dados Clinical Trials (clinicaltrials.gov).

A doença do olho seco é uma doença multifatorial associada com a instabilidade e aumento da osmolaridade do filme lacrimal, bem como inflamação da superfície ocular. Os seus sintomas incluem sensação de corpo estranho, secura, irritação, coceira e sensibilidade à luz [136]. Entre os agentes farmacológicos desenvolvidos para o tratamento desta doença, encontra-se o Diquafosol, uma solução oftálmica a 3%, comercialmente conhecida como Diquas®, elaborado pela empresa japonesa Santen Pharmaceutical conforme apresentado na Figura 3.1.1.1. O Diquafosol foi lançado no ano de 2010 e é aprovado para uso apenas no Japão, Coreia do Sul, Tailândia e Vietnã [137]. Ele funciona como um agonista do receptor P2Y2, o qual é expresso em células da superfície ocular. Uma vez ativado, esse receptor estimula a secreção de fluidos e mucina, promovendo a hidratação da superfície ocular. Apesar de ter demonstrado eficácia e segurança, esse fármaco ainda não foi aprovado pelo órgão americano FDA, que alegou a necessidade de execução de mais ensaios clínicos antes de conceder a sua aprovação [136,138]. Recentemente, um estudo conduzido pela Universidade de Yonsei, na Coreia do Sul vem pesquisando a importância de utilizar o Diquafosol como forma de aliviar os sintomas do olho seco após a cirurgia de catarata e teria previsão de término para janeiro de 2019 (NCT03640351), de acordo com informações depositadas no banco de dados Clinical Trials (clinicaltrials.gov).

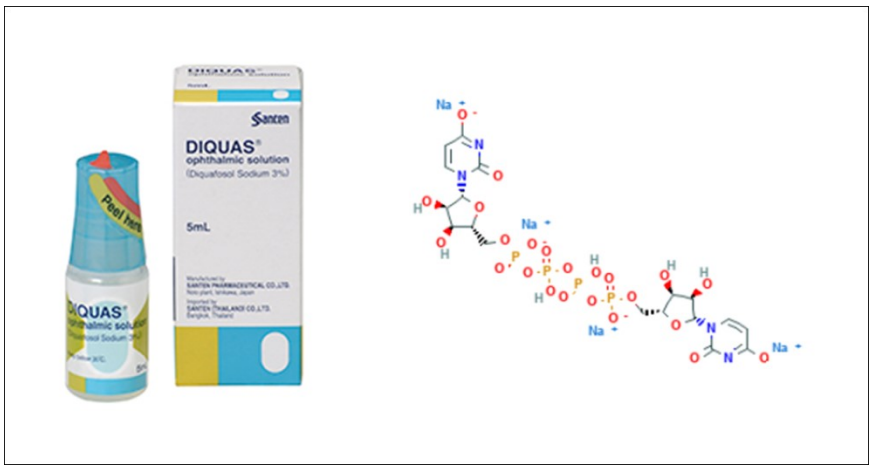


Figura 3.1.2 – Fármaco com ação sobre o receptor P2Y2. O fármaco Diquas® é um agonista do receptor P2Y2 aprovado para o tratamento da doença do olho seco em alguns países asiáticos. À esquerda observa-se a sua formulação farmacêutica e à direita, a sua estrutura química. Imagens extraídas do site do fabricante, Santen Pharmaceuticals e da Base Livre de Dados Químicos (PubChem): (https://pubchem.ncbi.nlm.nih.gov).

Além dessas funções que são importantes para o tratamento da fibrose cística e doença do olho seco, outros efeitos promovidos pelo receptor P2Y2 foram capazes de melhorar a reconstituição da glândula salivar e a cicatrização, tratar a doença crônica renal e infecção por leishmaniose, além de conferir neuroproteção. A ativação do P2Y2 promoveu a agregação das células epiteliais salivares, além da proliferação e migração *in vitro* de células progenitoras cardíacas humanas [139,140]. Em modelo experimental de cicatrização, Jin et al. (2014) observaram que os animais $P2Y2^{-/-}$ levaram mais tempo para que a ferida cicatrizasse, possivelmente devido a uma diminuição da expressão de proteínas de matriz extracelular [141]. O P2Y2 também parece exercer papeis benéficos no controle da progressão da doença crônica renal, uma vez que animais $P2Y2^{-/-}$ submetidos ao procedimento de nefrectomia sobreviveram menos tempo que os animais selvagens e apresentaram aumento da pressão sistólica e de ureia no soro após a cirurgia [142]. A ativação do P2Y2 confere proteção contra agentes infecciosos como a *Leishmania amazonensis* uma vez que o tratamento intralesional com o UTP (lesão na pata) diminuiu a carga parasitária nos sítios de infecção nos animais [143]. Em doenças neurodegenerativas o receptor P2Y2 executa ações protetoras. Na doença de Alzheimer, já foi observado que o P2Y2 contribuiu com o processamento da proteína precursora amilóide (PPA), além de induzir a sua fagocitose e degradação [144–147]. Ajit et al. (2014) também observaram que a deleção do P2Y2 promoveu um acúmulo da placa de β-amilóide no córtex cerebral e hipocampo em camundongos, bem como provocou um aumento do déficit neurológico [148].

Apesar da ativação do P2Y2 conferir benefícios para o tratamento de várias doenças, a sua inibição também pode ser útil para tratar doenças inflamatórias, dor e câncer. O P2Y2 pode ser considerado um alvo terapêutico para o tratamento de doenças inflamatórias, uma vez que a sua ativação estimulou a produção de mediadores inflamatórios, bem como a migração de células inflamatórias para o sítio de lesão [48,149], contribuindo para a inflamação intestinal, pulmonar e hepática [43–45,47]. A ativação do P2Y2 também causou recrutamento de macrófagos e hiperplasia arterial, contribuindo para o desenvolvimento de lesões arteriais [150].

Recentemente, Merz et al. (2018) demonstraram a possível participação do P2Y2 no controle de doenças metabólicas. Animais $P2Y2^{-/-}$ apresentavam um número reduzido de macrófagos no tecido adiposo, menor ganho de peso após 15 semanas, ausência de sinal de esteatose hepática e diminuição dos níveis de colesterol plasmático [151]. Já foi observado também que animais $P2Y2^{-/-}$ parecem apresentar proteção contra a inflamação [152–154]. Além disso, a inibição desse receptor promoveu analgesia em modelo experimental de dor neuropática no nervo trigêmeo [37].

Os antagonistas do receptor P2Y2 também podem funcionar como agentes antimetastáticos. Uma vez que os nucleotídeos podem ser encontrados em altas concentrações no sítio tumoral [155], já foi observado que o P2Y2, ao ser ativado pelo ATP ou UTP, pode promover proliferação, migração, invasão e metástase de linhagens tumorais. Entre os tipos de câncer analisados estão tumores de mama e próstata, carcinoma hepatocelular humano e células epiteliais cancerosas do ducto pancreático [38–42,156]. O aumento da expressão do receptor P2Y2 ainda está relacionado com um mau prognóstico no adenocarcinoma do ducto pancreático [157].

Apesar de serem bastante promissores, muitos estudos referentes à função do P2Y2 precisam ser avaliados com cautela. Primeiramente, na maioria dos trabalhos o P2Y2 é estimulado apenas com ATP e UTP, sendo que ambos são capazes de ativar outros receptores P2. Além disso, esses nucleotídeos podem ser degradados pelas ectonucleotidases, gerando novas moléculas, como ADP e UDP, por exemplo, que são capazes de ativar diferentes subtipos de receptores P2, ocasionalmente afetando a caracterização do efeito biológico estudado. Somado a isso, são raros os trabalhos que utilizam agonistas seletivos para esse receptor, como o 2-thio-UTP, MRS2698 e o MRS768, apesar desse último apresentar estabilidade limitada em meio biológico [49].

O antagonista seletivo do P2Y2 (AR-C118925), o qual é utilizado com frequência na execução de experimentos, também possui algumas limitações. O AR-C118925 foi desenvolvido pela empresa AstraZeneca e chegou a ser avaliado em formulação tópica para o

tratamento da psoríase crônica, embora tenha demonstrado ineficácia. Apesar de ter sido uma ferramenta muito útil para a execução de experimentos *in vitro* e *in vivo*, essa molécula não avançou em testes com humanos, devido a algumas propriedades farmacológicas indesejadas, que incluem alta polaridade e baixa biodisponibilidade através da via oral [49]. Portanto, essa lacuna na farmacologia do receptor P2Y2 abre margem para a descoberta e/ou desenvolvimento de novas moléculas com ação antagonista sobre esse receptor. A Figura 3.1.3 resume os principais papeis do P2Y2 e a sua associação a diferentes contextos fisiológicos.

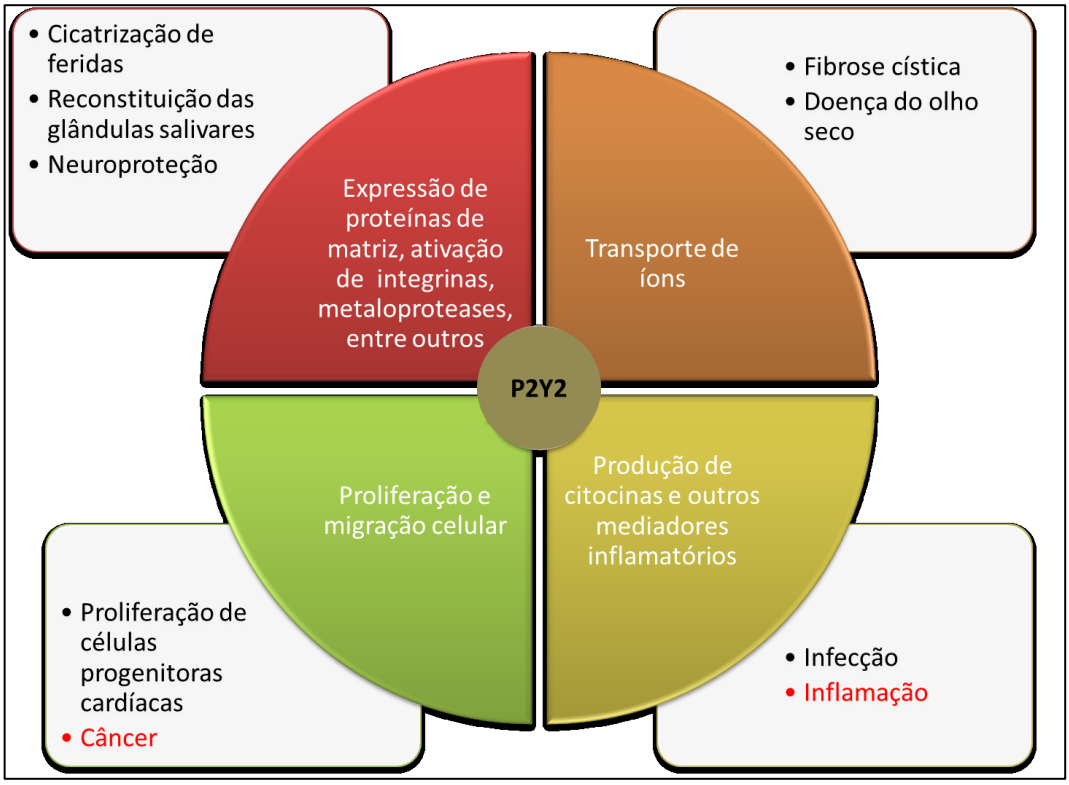


Figura 3.1.3 – Principais efeitos da ativação do P2Y2. A figura resume os principais efeitos da ativação do receptor P2Y2 e a sua implicação em diversos contextos fisiológicos, os quais podem ser aprimorados através do uso de agonistas desse receptor (redigidos em preto) ou ainda atenuados através do uso de seus antagonistas (redigidos em vermelho).

3.2 - O receptor P2Y4 como alvo terapêutico

O receptor P2Y4 é ativado fisiologicamente pelo UTP extracelular. Em ratos e camundongos, ele também pode ser ativado pelo ATP extracelular [130]. O P2Y4 é expresso em diversos órgãos tais como intestino, cérebro, pulmão, coração, próstata, pele e baço [49]. Por ser semelhante estruturalmente e farmacologicamente ao receptor P2Y2 e por vezes, ser expresso nos mesmos tipos celulares que ele, a caracterização dos efeitos fisiológicos do

P2Y4 tem se tornado um grande desafio [49]. O desenvolvimento de agonistas seletivos para cada um desses receptores tem permitido certo avanço nesse campo da Farmacologia dos receptores P2. Assim, já se tem registros na literatura de que três moléculas são capazes de ativar seletivamente o receptor P2Y4. São eles: MRS2927, MRS4062 e N(4)-(feniletóxi)-CTP, sendo que essas duas últimas foram desenvolvidas inicialmente como versões aprimoradas de agonistas para o receptor P2Y2 [24,49].

Apesar das limitações farmacológicas, algumas funções fisiológicas do P2Y4 já foram elucidadas. O P2Y4 assim como o P2Y2 exerce um papel na regulação do transporte de íons. Ghanem et al. (2005) demonstraram que o P2Y4 promove a secreção de Cl^- na mucosa intestinal, efeito esse que foi significativamente reduzido em animais $P2Y4^{-/-}$ [158]. O P2Y4 também é expresso em células epiteliais na membrana de Reissner (situada na cóclea), onde controla a homeostase do Na^+ na endolinfa a partir da inibição de canais ENaC [159].

O P2Y4 exerceu um papel neuroprotetor em modelo experimental de Alzheimer, no qual ele está envolvido na indução da pinocitose do peptídeo β-amilóide [160]. Ward et al. (2008) demonstraram que a expressão do P2Y4 em células cones e bastonetes na retina sofre alteração após a adaptação à luz e ao escuro, sugerindo que esse receptor estaria envolvido na modulação da atividade interna da retina [161]. O P2Y4 ainda exerce importantes funções cardíacas que podem conferir cardioproteção. Horckmans et al. (2012) demonstraram que animais $P2Y4^{-/-}$ apresentavam microcardia após 12 semanas de vida [162] e que a deleção desse receptor também está associada a uma menor resistência durante o exercício físico [163]. Lemaire et al. (2017) observaram que animais $P2Y4^{-/-}$ apresentaram menor tamanho da área de infarto comparado aos animais selvagens [164]. Por outro lado, em modelo experimental de isquemia a deleção desse receptor conferiu cardioproteção. Horckmans et al. (2015) observaram que animais $P2Y4^{-/-}$ apresentavam menor área de infarto, necrose, fibrose e diminuição do recrutamento de neutrófilos [165]. Zizzo et al. (2012) demonstraram que um antagonista para o P2Y4 poderia ser útil no tratamento da constipação, uma vez que esse receptor parece estar envolvido na inibição da atividade contrátil espontânea do músculo longitudinal do íleo através da via de sinalização da fosfolipase C/IP_3 [166].

Apesar disso, a ausência de antagonistas seletivos para esse receptor ainda dificulta a caracterização do papel do P2Y4 no desenvolvimento das doenças relacionadas à sua ativação. Nesse sentido, a molécula RB-2 vem sendo utilizada como um antagonista para esse receptor. Entretanto, devido a sua capacidade de inibir outros receptores P2, deve-se tomar cautela na análise dos resultados. Contudo, ela constitui um modelo estrutural para a síntese e desenvolvimento de novos antagonistas seletivos para o P2Y4 [49]. Nesse contexto, a descoberta de novos antagonistas para o receptor P2Y4 permitirá uma caracterização mais

fidedigna de funções fisiológicas associadas a esse receptor *in vitro* e *in vivo*, além de possibilitar a sua implementação na terapia clínica. A Figura 3.2.1 resume as principais funções do receptor P2Y4 e a sua associação a diferentes contextos fisiológicos.

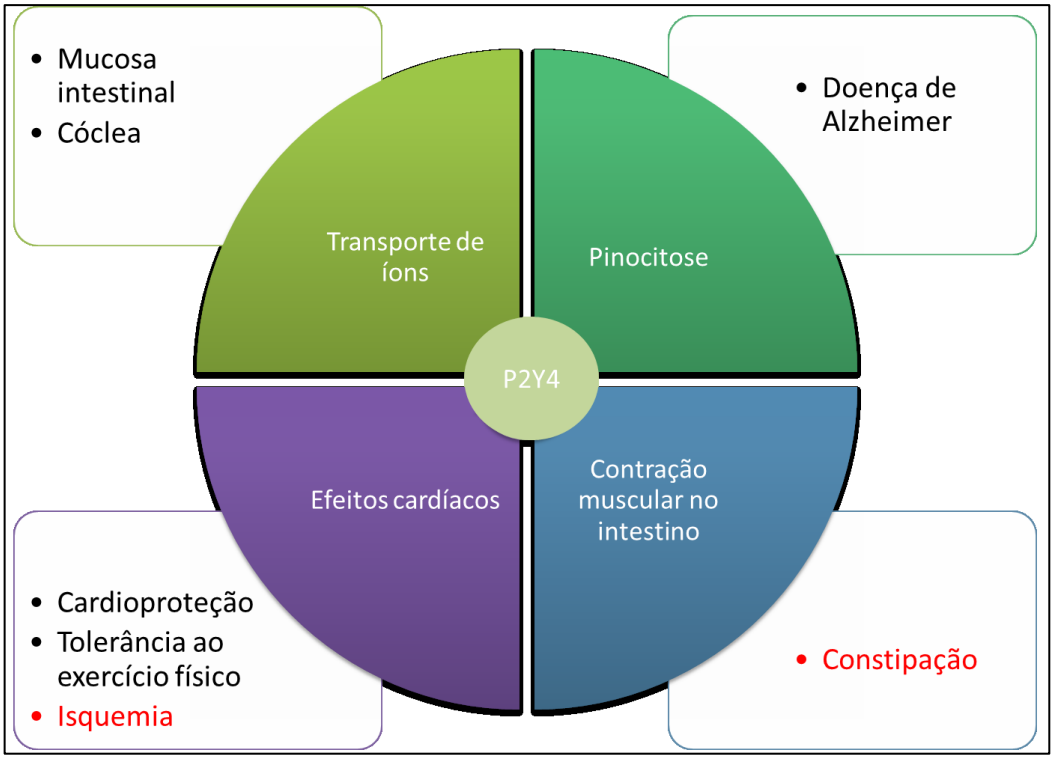


Figura 3.2.1 – Principais efeitos da ativação do P2Y4. A figura resume os principais efeitos da ativação do receptor P2Y4 e a sua implicação em diversos contextos fisiológicos, os quais podem ser aprimorados através do uso de agonistas desse receptor (redigidos em preto) ou ainda atenuados através do uso de seus antagonistas (redigidos em vermelho).

3.3 – O receptor P2X7 como alvo terapêutico

O receptor P2X7 é expresso em células do sistema imune, tais como mastócitos, eritrócitos, monócitos, macrófagos, células dendríticas e linfócitos, além de células da glia [167]. Ele é ativado fisiologicamente pelo ATP extracelular em uma concentração em torno de 100 μM, a qual é aproximadamente 10 vezes maior do que a necessária para a ativação dos demais receptores P2X [15]. Por essa razão, alguns autores consideram que a sua ativação seja um indicativo de "sinal de perigo", uma vez que esses altos níveis de ATP extracelular são alcançados fisiologicamente, por exemplo, através de citólise [102]. Esse receptor apresenta a peculiar capacidade de formar um poro membranar em situações de exposição prolongada ao ATP. Esse poro membranar permite a passagem de moléculas de até 900 Da, incluindo íons (Na^+, Ca^{2+}, K^+), água, ATP e corantes como iodeto de propídeo (668 Da),

brometo de etídeo (394 Da) e YO-PRO-1 (629 Da), que são comumente utilizados para o estudo da atividade do P2X7 [69,168].

A ativação do P2X7 incita uma série de respostas celulares com perfil pró-inflamatório, que incluem a formação do inflamassomo, a ativação de caspases, fosfolipases (A_2 e D), proteínas quinases ativadas por mitógenos (MAPK) e fatores de transcrição como o NF-κB, bem como a transcrição de genes pró-inflamatórios como COX-2 e iNOS. A sua ativação ainda promove a liberação de citocinas como IL-1β, IL-18 e TNF-α, a geração de espécies reativas de oxigênio (EROS), além de morte celular [102,169–172]. Devido a essas funções desempenhadas pelo P2X7, diversos trabalhos da literatura já descreveram a sua participação no desenvolvimento da inflamação, dor, neurodegeneração, isquemia e hipóxia, câncer e infecção.

Existem diversos trabalhos na literatura demonstrando que os antagonistas do P2X7 apresentam efeitos anti-inflamatórios. O tratamento prévio de animais com A-438079 e BBG (antagonistas do P2X7) foi capaz de suprimir a ativação do inflamassomo e a liberação de citocinas, diminuindo a severidade de nefrotoxicidade renal induzida por cisplatina e nefrite [173,174]. Os antagonistas BBG, A-740003 e A-438079 foram capazes de prevenir a colite em ratos e inibir a infiltração de neutrófilos na orelha de camundongos [175–177]. Em modelo experimental de lesão hepática e fibrose, o antagonista A-438079 diminuiu a necrose, a inflamação, o acúmulo de colágeno, bem como a liberação de citocinas pró-inflamatórias [178]. Além disso, o P2X7 é expresso no tecido sinovial inflamado e já foi observado que o tratamento com antagonistas foi capaz de aliviar a inflamação nas articulações de animais [119,179,180]. Recentemente, foi desenvolvida uma espécie de anticorpos em escala de nanômetros, chamados de "nanocorpos" com reconhecimento do P2X7. Eles possuem administração sistêmica e podem atenuar a ativação do P2X7 em células T e macrófagos, aliviando os sintomas da glomerulonefrite experimental e dermatite de contato [181]. A ativação do P2X7 também está associada com a hiperalgesia inflamatória, a qual pode ser atenuada com o tratamento prévio com antagonistas desse receptor [118–122].

Em modelos experimentais de doenças neurodegenerativas já foi observado um aumento da expressão do P2X7 em alguns tipos celulares no cérebro, o que geralmente está associado com o agravamento dessas enfermidades. Os antagonistas do P2X7 foram capazes de aliviar a severidade das convulsões e os danos neuronais em camundongos epiléticos [99–101]. Em modelos de esclerose lateral amiotrófica, foi observado que os antagonistas BBG, oATP e KN-62 reduziram a morte de neurônios motores e a gliose, resultando em um aprimoramento do desempenho motor dos animais [109–112]. O P2X7 também está relacionado com a diminuição da atividade da enzima α-secretase, a qual é responsável pela

clivagem da proteína precursora amilóide (PPA) [182]. Essa atividade produz um acúmulo de placas amilóides em modelos *in vivo* de doença de Alzheimer, o que foi revertido através do tratamento prévio com antagonistas desse receptor, os quais também auxiliaram na prevenção do déficit cognitivo [106,183].

A ativação do P2X7 na isquemia parece ser prejudicial. Estudos de privação de oxigênio-glicose *in vitro* demonstraram que o uso de antagonistas do P2X7 reduziram os danos mitocondriais, a produção de EROS e a viabilidade celular após o estímulo isquêmico [184–186]. Já em modelos *in vivo* de isquemia, os antagonistas do P2X7 aumentaram a sobrevivência de neurônios, além de reduzirem a mortalidade dos animais, a ativação da glia e a transcrição de citocinas [187,188]. Por outro lado, alguns trabalhos demonstraram que a inibição do P2X7 também pode ser danosa. Anagisawa et al. (2008) observaram que o tratamento com o oATP resultou na perda de neurônios e no desempenho motor dos animais [189]. Brinda et al. (2014) demonstraram que o BBG aboliu o efeitos neuroprotetores produzidos pelo pós-condicionamento como a memória e o desempenho motor [190]. Já Kaiser et al. (2015) observaram que animais $P2X7^{-/-}$ apresentaram maior área de edema cerebral comparado aos animais controles [191]. Esses achados controversos sugerem que as ações do P2X7 na isquemia podem ser resultado da severidade e duração do evento isquêmico, influenciando assim, o papel do receptor para um perfil de proteção ou de agressão [192].

No câncer, assim como na isquemia, a ativação do P2X7 demonstra um duplo papel, podendo favorecer o crescimento das células tumorais ou mesmo a sua morte. Em determinados tipos de câncer como neuroblastoma e glioma, a ativação do P2X7 está envolvida na proliferação celular, o que foi atenuado através da administração intravenosa de BBG [193]. A ativação do P2X7 também estimula a proliferação, migração e invasão de células de adenocarcinoma ductal pancreático e de algumas linhagens de câncer de próstata, pulmão, mama e melanoma, sendo essas atividades revertidas com o tratamento de antagonistas desse receptor [113–117]. Apesar disso, curiosamente, Fang et al. (2013) observaram que tanto o tratamento com o BBG quanto o silenciamento do P2X7 através de RNA de interferência (RNAi) aumentaram o crescimento tumoral e a angiogênese em células de glioma C6 [194].

Nas doenças infecciosas, o P2X7 também possui uma função dupla. A sua ativação pode exercer papéis protetores ou deletérios para o hospedeiro. De acordo com Savio et al. (2018), o favorecimento de um desses dois efeitos depende do tipo de patógeno, da virulência e da severidade da infecção [195]. A participação do P2X7 já foi caracterizada em diversas doenças infecciosas virais, bacterianas, fúngicas, bem como em protozooses. Na tuberculose,

a ativação do P2X7 está envolvida com a apoptose de células infectadas com a cepa H37Rv de *Mycobacterium tuberculosis* [196]. Na toxoplasmose, a ativação do P2X7 promove a eliminação do *Toxoplasma gondii* através da produção de EROS [197]. Já na infecção por clamídia, a eliminação acontece através da ativação da fosfolipase D, uma enzima com atividade antimicrobicida [198]. Recentemente, Graziano et al. (2015) demonstraram a participação do P2X7 na liberação de virions do HIV em macrófagos humanos de pacientes soropositivos e células D-U1 infectadas, sendo que essa atividade foi inibida pelo antagonista oATP [199]. Em modelo experimental de sepse, Santana et al. (2015), demonstraram que os animais $P2X7^{-/-}$ apresentaram maior taxa de sobrevivência e menores números de unidades formadoras de colônia, leucócitos e citocinas pró-inflamatórias no peritônio comparados aos animais controle [200].

Apesar dos resultados serem bastante promissores, alguns cuidados devem ser tomados ao se avaliar os estudos com o receptor P2X7. Primeiramente, muitos estudos utilizam o ATP exógeno para estimular o receptor P2X7, contudo, como a concentração necessária para ativar esse receptor é alta (>100 μM), outros receptores P2X e P2Y podem ser ativados, provocando algum tipo de interferência nas respostas. Além disso, a expressão de ectonucleotidases no tipo celular ou tecido estudado ainda pode direcionar parte da resposta para receptores P2Y e P1 [195]. Em alguns trabalhos, os autores optam por utilizar o BzATP, entretanto, ele não é seletivo para o P2X7, uma vez que possui afinidade para os receptores P2X1 e P2X3 [59]. O BBG, utilizado em muitos experimentos como antagonista do P2X7, também é capaz de inibir o receptor P2X4 e canais de sódio ativados por voltagem [76]. O ATP oxidado, outro antagonista do P2X7, também possui outros alvos moleculares além desse receptor [201]. Além disso, os animais $P2X7^{-/-}$ produzidos pelas empresas GlaxoSmithKline e Pfizer, expressam o receptor em células T, embora ele seja funcional apenas nos camundongos da primeira empresa [195].

Apesar da perspectiva quanto ao uso de antagonistas do P2X7 na terapia, ainda não existem fármacos na clínica que sejam utilizados para o tratamento de doenças relacionadas a esse receptor. Baseado nos resultados observados em modelos animais de artrite reumatoide, alguns grupos de pesquisa estudaram a aplicação de antagonistas do P2X7 nessa doença. Utilizando os parâmetros do Colégio Americano de Reumatologia sobre o alívio dos sintomas, os autores observaram que o tratamento de pacientes com o AZD9056 e CE-224,535 não demonstrou eficácia superior em relação ao grupo placebo. Além disso, os pacientes tratados com esses antagonistas relataram diversos efeitos colaterais, que incluíam náusea, dor abdominal, diarreia e dor de cabeça [202–204]. Devido a essas falhas na eficácia, as pesquisas com ambos as moléculas se encerraram na fase 2 de ensaios clínicos. Assim, essa

lacuna na terapia associada ao receptor P2X7 estimula a pesquisa e o desenvolvimento de fármacos com atuação sobre este promissor alvo-terapêutico. A Figura 3.3.1 resume as principais funções do receptor P2X7 e a sua associação a diferentes contextos fisiológicos.

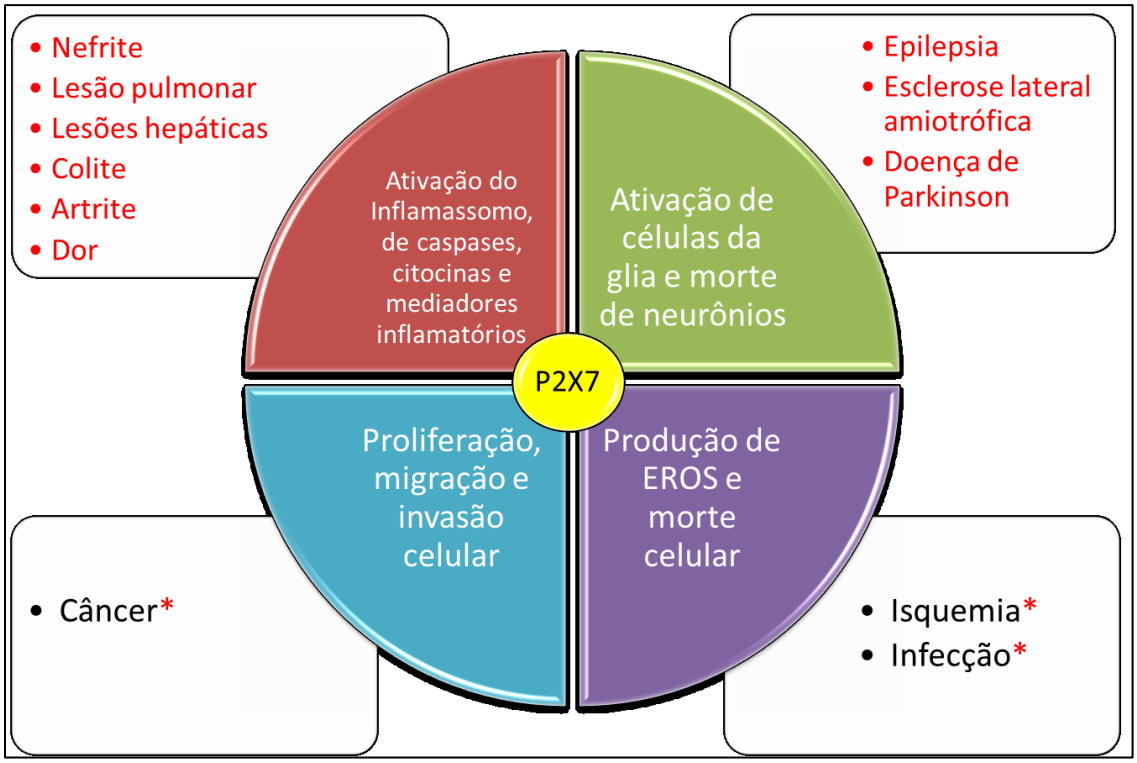


Figura 3.3.1 – Principais efeitos da ativação do P2X7. A figura resume os principais efeitos da ativação do receptor P2X7 e a sua implicação em diversos contextos fisiológicos, os quais podem ser aprimorados através do uso de agonistas desse receptor (redigidos em preto) ou ainda atenuados através do uso de seus antagonistas (redigidos em vermelho ou marcados com um asterisco vermelho).

4- Os produtos naturais como uma fonte para a descoberta de novos antagonistas

"Presentes esplêndidos: A natureza produz constantemente moléculas que não foram pensadas pelos seres humanos." (Satoshi Omura)

Atualmente no campo de desenvolvimento de fármacos, o maior desafio é encontrar candidatos que sejam capazes de tratar uma determinada doença com eficácia e apresente um mínimo de efeitos colaterais [205]. Nesse contexto, os produtos naturais emergem como uma importante fonte para a descoberta inovadora de novos fármacos devido à sua enorme diversidade estrutural e química. Essas características foram adquiridas ao longo de milhares de anos de evolução e ainda continuam inspirando novas descobertas nas áreas de Química, Biologia e Medicina [205,206].

Tradicionalmente, os fármacos à base de produtos naturais eram oriundos de plantas, minerais e animais, mas hoje já são estudadas moléculas presentes em seres marinhos, fungos, bactérias, entre outros [207]. Esses seres vivos estão agrupados dentro de cinco reinos biológicos: Animalia, Plantae, Fungi, Protista e Monera, que juntos abrigam uma imensa biodiversidade de espécies, representando assim, um grande número de amostras para testes.

Mora et al. (2011) analisaram bancos de dados nos quais estavam depositadas informações sobre o número de espécies pertencentes a um determinado reino biológico, e chegaram a uma estimativa de mais de 10 milhões de espécies eucarióticas habitantes da terra e dos oceanos. Apesar disso, se tem dados catalogados referentes à apenas cerca de 10% dessas espécies, que estão agrupadas dentro de quatro dos cinco reinos dos seres vivos (Animalia, Plantae, Fungi e Protista). Além desses, o reino Monera abriga aproximadamente 11 mil espécies de bactérias procariontes [208]. No Brasil, um estudo similar foi realizado por Lewinsohn e Prado (2005), no qual eles estimaram que existissem mais de 200 mil espécies classificadas dentro desses cinco reinos [209]. No quadro 4.1 estão especificados os números de espécies catalogadas e estimadas para cada um dos reinos de seres vivos.

Quadro 4.1 - Estimativa acerca da riqueza biológica mundial e brasileira

Reino	Mundo[1]		Brasil[2]
	Número de espécies catalogadas	Número de espécies estimadas	Número de espécies estimadas
Animalia	1.124.516	9.920.000	103.780–136.990
Plantae	224.244	314.600	43.020–49.520
Fungi	44.368	616.320	13.090–14.510
Protista	34.128	107.700	7.650–10.320
Monera	11.513	11.420	800-900
Total	**1.438.769**	**10.970.040**	**168.340-212.240**

[1]Dados extraídos do trabalho de [208]. [2]Dados extraídos do trabalho de [209].

Apesar dessa riqueza, apenas 20% das plantas foram investigadas quanto ao seu uso medicinal em todo o mundo. No Brasil, por exemplo, que abriga cerca de 10% da flora mundial, menos de 1% das espécies já foram investigadas nos campos químico e farmacológico [210]. Apesar da baixa exploração, o mercado de fitoterápicos atual apresenta um faturamento anual de aproximadamente 14 bilhões de dólares, correspondendo a cerca de 5% do mercado farmacêutico [210].

Além disso, Newman e Cragg (2016) realizaram uma pesquisa no banco de dados do FDA para investigar a quantidade de novas entidades químicas (*new chemical entities* -

NCEs) baseadas em produtos naturais que surgiram entre os anos de 1981 e 2014. Eles observaram que das 1562 NCEs: a) 16% eram de origem biológica, isto é, consistiam em peptídeos (> 50 resíduos) ou proteínas isoladas ou sintetizadas de um organismo ou linhagem celular; b) 4% eram oriundas de produtos naturais que não sofreram alteração em sua estrutura, embora possam ser produzidos de forma sintética ou semissintética; c) 1% eram oriundas de espécies botânicas, isto é, consistem em medicamentos contendo material vegetal, cujo princípio ativo ainda não foi identificado; d) 21% eram derivados de produtos naturais, isto é, sofreram algum tipo de modificação semissintética; e) e 4% foram produzidos através de síntese, porém apresentavam farmacóforo similar a um produto natural. Esses fármacos possuem vasta aplicação na terapia, incluindo o tratamento de doenças neurodegenerativas, cardíacas, metabólicas, infecciosas, inflamatórias, entre outras [211]. Cabe ressaltar que até o ano de 2007, ao menos 91 moléculas derivadas de plantas encontravam-se em etapa de ensaios clínicos em todo o mundo para o tratamento de diversas enfermidades [212].

Nesse contexto, a Organização Mundial de Saúde (OMS) estima que mais de 80% da população mundial depende da medicina tradicional para as suas necessidades em cuidados primários. Deve-se salientar que estão descritas aproximadamente 200 mil plantas medicinais, as quais são utilizadas em 91 países, incluindo Brasil, China, França, Alemanha e Reino Unido [213,214].

A descoberta de moléculas oriundas de produtos naturais que apresentam importante aplicação na terapia já premiou pesquisadores com o Prêmio Nobel, como Alexander Fleming, Ernest Chain e Howard Florey em 1945 pela descoberta da penicilina; Selman Waksman em 1952 pela descoberta da estreptomicina; William Campbell e Satoshi Omura em 2015 pela descoberta da avermectina; e Youyou Tu em 2015 pela descoberta da artemisinina [215].

Apesar das vantagens oferecidas, algumas pesquisas em desenvolvimento de fármacos a partir de produtos naturais foram substituídas por estudos com bibliotecas sintéticas [216]. Esse fato se deve a algumas razões. A maior delas é em decorrência da necessidade de se realizar diversos processos até a determinação da molécula ativa, uma vez que as amostras para teste muitas vezes consistem em extratos ou frações. Muitas pesquisas estacionam na etapa anterior à purificação da molécula ativa. Isto se deve à alta complexidade das misturas moleculares, sendo que algumas vezes, a atividade terapêutica encontrada em extratos pode ser oriunda de uma ação sinérgica e simultânea de diversas moléculas. Além disso, moléculas isoladas muitas vezes não estão disponíveis em quantidades suficientes para uso durante processos de triagem de alto desempenho. A falta de seletividade também pode ser considerada um obstáculo à pesquisa devido à capacidade de diferentes moléculas presentes

nos extratos serem capazes de se ligar a diversos alvos celulares [205,207,215]. Por fim, limitações de caráter legal ainda podem impactar a pesquisa com produtos naturais, tais como a certificação necessária para utilizar informações a respeito da biodiversidade em pesquisa, bem como a dificuldade de gerar patentes com produtos naturais em alguns países [216].

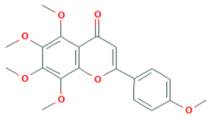
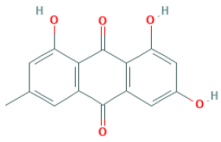
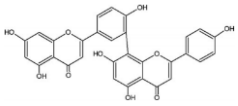
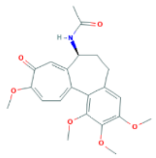
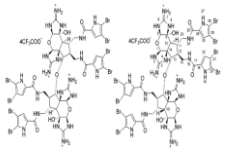
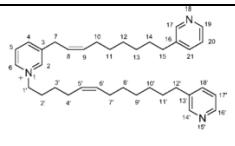
Todavia, a evolução da bioinformática em conjunto com tecnologias analíticas tem revolucionado o campo de pesquisa com produtos naturais, ao permitir uma rápida detecção de *hits* através de *screening* virtual, além de facilitar o isolamento e a elucidação estrutural de moléculas ativas [215]. Entre as tecnologias que mais tem contribuído para o avanço da química medicinal, encontram-se: a cromatografia líquida de alta-desempenho, a ressonância magnética nuclear espectroscópica, a espectrometria de massas, a microfluídica e os algoritmos computacionais. Técnicas de pré-fracionamento e procedimentos de extração mais eficientes, como os que utilizam tecnologia de separação por membrana, extração fluída, bem como a extração assistida por enzima, ultrassom ou micro-ondas também podem colaborar para tornar mais fácil e ágil o processo de *screening* e caracterização da molécula ativa [205].

Outras áreas das Ciências Médicas podem contribuir significativamente para oferecer maior qualidade para a pesquisa com produtos naturais. A Genômica pode auxiliar na correta identificação das espécies utilizadas, conferindo ainda uma autenticação aos produtos à base de plantas. A Transcriptômica pode auxiliar na observação dos genes que estão sendo expressos, enquanto a Proteômica pode colaborar para o estudo das moléculas presentes nos produtos naturais. Já a Metabolômica pode auxiliar na identificação e quantificação das moléculas associadas ao metabolismo desses materiais. Além disso, as ferramentas de bioinformática podem permitir o uso dos dados obtidos através dessas técnicas na elucidação dos efeitos patofisiológicos, na especificidade ao alvo, nos efeitos moleculares, bem como na determinação dos parâmetros farmacodinâmicos, farmacocinéticos e toxicológicos [205].

4.1 – Receptores P2 e produtos naturais

Devido ao vasto campo de aplicação dos produtos naturais e à necessidade de se buscar novas moléculas com ação antagonista sobre receptores P2, alguns grupos de pesquisa empenharam esforços para encontrar novas moléculas de origem natural que apresentem essa característica. Faria et al. (2012) realizaram um levantamento na literatura para investigar as pesquisas com produtos naturais com atuação sobre os receptores P2 e encontraram mais de 30 moléculas com ação agonista, antagonista ou moduladora sobre esses receptores [206]. O quadro 4.1.1 resume as principais características de algumas moléculas com ação antagonista sobre os receptores P2Y2 e P2X7.

Quadro 4.1.1 - Produtos naturais com ação antagonista sobre os receptores P2Y2 e P2X7

Receptor P2	Espécie biológica	Molécula	Peso molecular	Estrutura	Referência
P2Y2	*Citrus reticulata*	Tangeretina	372.37 g/mol		[217]
P2Y2/ P2Y4	*Joannesia princeps* Vell.	ND	ND	ND	[218]
P2Y2/ P2Y4	*Peixotoa* A. Juss	ND	ND	ND	[218]
P2X7	*Rheum officinale* Baill	Emodina	270,24 g/mol		[219–221]
P2X7	*Rheedia longifolia* Planch & Triana	Amentoflavona	ND		[222,223]
P2X7	*Colchicum autumnale*	Colchicina	399.443 g/mol		[224]
P2X7	*Stylissa flabellata* Ridley & Dendy 1886	Estalissadinas A e B	ND		[225]
P2X7	*Callyspongia* sp.	Nifatoxina C	ND		[226]
P2X7	*Vishniacozyma victoriae*	ND	ND	ND	[227]
P2X7	*Metschnikowia australis*	ND	ND	ND	[227]
P2X7	*Ascomycota* sp.	ND	ND	ND	[227]

(ND) significa não determinada.

A tangeretina é uma flavona extraída da tangerina (*Citrus reticulata*) que foi capaz de inibir a mobilização de cálcio intracelular induzida pelo UTP de forma concentração-dependente. Ela foi descoberta através da realização de ensaios de *screening* de 42 substâncias isoladas. Após a execução de novos experimentos, os pesquisadores observaram que ela funciona como um antagonista não competitivo do receptor P2Y2 [217].

Recentemente, Ferreira et al. (2018) demonstraram a atividade antagonista do extrato de galhos de *Joannesia princeps* Vell. e extratos de folhas e flores de *Peixotoa* A. Juss, após a realização de uma campanha de triagem com a finalidade de descobrir novos antagonistas para os receptores P2Y2 e P2Y4. Essas espécies vegetais, que são encontradas no Brasil, inibiram de forma concentração-dependente a mobilização de cálcio intracelular induzida pelo UTP sem promover citotoxicidade. Entretanto, as moléculas responsáveis por essa atividade ainda não foram caracterizadas [218].

A emodina é uma antraquinona derivada do ruibarbo *Rheum officinale* Baill. No trabalho de Liu et al. (2010) a emodina demonstrou capacidade de inibir a morte celular causada pela ativação do P2X7 de forma concentração-dependente, além de prevenir a captação do corante brometo de etídeo através do poro membranar formado por esse receptor. A emodina também inibiu a mobilização de cálcio intracelular bem como a corrente iônica promovida pelo P2X7 [219]. Além disso, a emodina ainda foi capaz de inibir a proliferação de linhagem tumoral de câncer de mama, assim como a ativação do inflamassomo NLRP3 [220,221].

O extrato bruto e frações das folhas da planta arbórea *Rheedia longifolia* Planch & Triana apresentaram atividade antagonista sobre o receptor P2X7. Santos et al. (2011) demonstraram que essas amostras foram capazes de inibir a captação do iodeto de propídeo através do poro membranar formado pelo P2X7, bem como as suas correntes iônicas [223]. Além disso, as frações desse extrato também apresentaram atividade analgésica e anti-inflamatória, e a molécula ativa isolada foi uma amentoflavona [222].

A colchicina, um alcaloide extraído da planta *Colchicum autumnale*, que é utilizada no tratamento de doenças inflamatórias, também inibe as funções do P2X7, como a captação do YO-PRO-1 e brometo de etídeo. Essa molécula também diminuiu a formação de EROS, nitrito e a liberação da IL-1β [224].

Buchanan et al. (2007) também identificaram a atividade antagonista de dois alcaloides da esponja do mar australiana *Stylissa flabellata* Ridley & Dendy 1886 após a execução de uma campanha de triagem de alto desempenho em parceria com a empresa AstraZeneca. Essas moléculas inibiram a formação do poro membranar associado ao receptor P2X7 de forma concentração-dependente [225]. Durante essa campanha de *screening*, que

testou mais de 62 mil amostras, o grupo também observou uma atividade antagonista de mais um alcaloide presente no extrato de outra esponja do mar australiana, a *Callyspongia* sp. Esse alcaloide, a nifatoxina C, também inibiu a captação de corante via poro associado ao receptor P2X7 de forma concentração-dependente [226].

A atividade antagonista de espécies fúngicas também foi relatada sobre o receptor P2X7. Soares-Bezerra et al. (*in press*) demonstraram que os fungos antárticos *Vishniacozyma victoriae*, *Metschnikowia australis*, bem como o fungo algícola *Ascomycota* sp. foram capazes de inibir a captação de corantes associado ao poro membranar do P2X7 e sua corrente iônica, além de prevenir a morte celular induzida pela ativação do receptor, a liberação da citocina IL-1β e a formação de nitrito e EROS. Contudo, a caracterização das moléculas ativas ainda não foi realizada [227].

5- Triagem de alto desempenho aplicada à descoberta de novos antagonistas

A procura por novos fármacos é uma questão centenária, que se fortificou a partir da evolução da Química e o estabelecimento da Farmacologia como Ciência. A primeira campanha de *screening* foi realizada por Paul Ehrlich no início do século XX, quando este buscava moléculas para o tratamento da sífilis. Com isso, ele encontrou a molécula número 606, denominada Salvarsan (diaminodióxi-arsenobenzeno), o qual foi lançada no ano de 1910, causando uma grande euforia e sendo reconhecida como uma "droga milagrosa" [228].

Com a evolução da Bioquímica ao longo do século XX, chegou-se a conclusão de que as enzimas e os receptores eram os alvos farmacológicos. Atualmente, já se tem conhecimento de que os fármacos atuam sobre várias biomoléculas, incluindo enzimas, transportadores, receptores acoplados à proteína G (como os receptores P2Y), bem como canais iônicos (como os receptores P2X) [228].

O processo de triagem de alto desempenho (*high throughput screening* - HTS) como é conhecido atualmente, surgiu no início dos anos 1990 e ainda é empregado rotineiramente, especialmente pela indústria farmacêutica e centros de pesquisa, como uma importante tecnologia para a descoberta de novos fármacos. Ele funciona como um método eficiente para a obtenção de dados acerca da atividade biológica de um grande número de amostras testadas a partir ensaios de alta vazão [228]. O processo intermediário entre a triagem e a descoberta de uma molécula ativa é denominado "*hit-to-lead*", em que a primeira etapa consiste na identificação das moléculas que apresentam a atividade almejada (denominados "*hits*"), enquanto que as etapas posteriores consistem na confirmação do efeito e determinação da potência ("*leads*"), eliminando assim possíveis falsos positivos [228].

Atualmente, existem diversas estratégias para a execução dos ensaios de *screening*. Uma delas envolve a realização de ensaios bioquímicos, que geralmente consistem em reações de enzimas/substratos, ligação a receptor, interações entre proteínas, entre outros. Alguns exemplos de ensaios que adotam essa estratégia são: cintilação, transferência de energia de ressonância Förster (FRET), ensaio de imunoabsorção enzimática (ELISA) e reação em cadeia de polimerase (PCR) quantitativo. Outra estratégia envolve os ensaios baseados em células que mimetizam o contexto fisiológico. Eles são utilizados para mensurar diversos parâmetros celulares, que incluem crescimento celular, transcrição, morfologia, metabolismo, ativação de mensageiros intracelulares e mudança no potencial de membrana. Alguns ensaios que estão alinhados a essa estratégia são: ensaios de mensuração de cálcio intracelular, eletrofisiologia, análise do potencial de membrana, entre outros. Essa última estratégia é capaz de classificar a molécula testada como agonista, antagonista, agonista inverso e modulador alostérico [228].

O tipo de ensaio mais comum para analisar a resposta de ativação/ inibição dos receptores P2Y ativados por UTP (P2Y2 e P2Y4) é a mensuração dos níveis de cálcio intracelular ($[Ca^{2+}]_i$), uma vez que o Ca^{2+} funciona como um segundo mensageiro destes receptores, cuja liberação no citoplasma é mediada pela via da PLC/IP_3. Já para o receptor P2X7 são relatados ao menos três tipos de ensaios possíveis: a) mensuração de $[Ca^{2+}]_i$, uma vez que a ativação desse receptor está associada ao influxo desse íon; b) ensaios de *patch-clamp*, os quais tem por objetivo a mensuração das correntes geradas a partir da abertura do canal iônico; c) ensaios de permeabilização celular que consistem análise da captação de corante através do poro membranar após a estimulação do P2X7 com altas concentrações de ATP.

5.1 – Ensaios para mensuração dos níveis de cálcio intracelular

A mensuração da mobilização intracelular de cálcio é uma técnica comumente utilizada para estudar a atividade dos receptores P2Y, mas também pode ser empregada para o estudo de receptores P2X. Atualmente, existem diversas ferramentas para o estudo do cálcio intracelular, empregando métodos físicos, biológicos ou químicos para a detecção desse íon.

Os microeletrodos sensíveis ao cálcio são um exemplo de método físico de detecção do íon. Eles consistem em micropipetas de vidro com tamanho inferior a 2 μm que podem ser inseridas no interior da célula ou no meio extracelular para mensurar os níveis de cálcio intra ou extracelular, respectivamente. Esse sistema mede os níveis de cálcio quando acoplado a um aparato de eletrofisiologia [229,230].

Os métodos biológicos consistem no uso de fotoproteínas como a aequorina e obelina, por exemplo, que são capazes de reagir com o cálcio e emitir luz [231]. Essas proteínas são inseridas nas células através de microinjeção ou transfecção. Elas foram utilizadas durante muitos anos, mas foram substituídas por indicadores de cálcio químicos [232,233].

Os métodos químicos incluem os indicadores de cálcio metalocrômicos, como o arsenazo III e antipilazo III, que absorvem luz de forma dependente de cálcio. Entretanto, devido à sua impermeabilidade, eles necessitam ser introduzidos nas células através de microinjeção ou permeabilização celular [234]. Todavia, o mais famoso e popular método químico consiste no uso de indicadores fluorescentes de cálcio baseados na estrutura dos quelantes de cálcio EGTA (etileno glicol-bis(2-aminoetiléter)-N,N,N′,N′-ácido tetra acético) e BAPTA (1,2-bis(2-aminofenóxi)etano-N,N,N′,N′-ácido tetra acético). Esses indicadores possuem afinidade pelo cálcio e ao se ligarem a esse íon emitem fluorescência. Além disso, a conjugação de um radical de acetóximetil (AM) confere a eles uma natureza permeável, permitindo o carregamento de diversas células simultaneamente. Entre esses indicadores, os mais conhecidos são Fura-2 AM, Fluo-3 AM e Fluo-4 AM [234]. O quadro 5.1.1 fornece uma síntese dos principais métodos de mensuração do cálcio intracelular.

Quadro 5.1.1 – Principais ferramentas de mensuração dos níveis de cálcio intracelular

Ferramenta	Principal vantagem	Principal desvantagem	Referência
Microeletrodos sensíveis ao cálcio	Detectam um aumento da resposta de cálcio em segundos	Não detectam respostas rápidas e transientes	[229,230]
Fotoproteínas	Distribuição intracelular seletiva	Necessitam ser introduzidas no citosol através de microinjeção ou transfecção	[232,233]
Sondas de cálcio metalocrômicas	Absorvem luz de maneira específica e dependente de cálcio	São impermeáveis à membrana, sendo introduzidas através de microinjeção	[234]
Indicadores fluorescentes	São associados ao radical acetóximetil (AM), permitindo um carregamento simples e rápido de um grande número de células	Difusão do corante para algumas organelas como a mitocôndria e o retículo endoplasmático	[234–236]

Os principais equipamentos utilizados no monitoramento do sinal de cálcio incluem microscópios confocal e de fluorescência, bem como fluorímetro e citômetro de fluxo. Apesar do uso de microscopia permitir a visualização da fluorescência de um determinado grupo de células, o que é conhecido como imageamento de cálcio, essa técnica só permite a análise de uma amostra por vez, tornando-a inviável para a execução de *screening* em larga escala. Contudo, foram desenvolvidos microscópios automatizados que são capazes de realizar a análise de placas de 6 a 384 poços que é conhecido como triagem de alto conteúdo (*high content screening* - HCS) [237]. Na citometria de fluxo limitação similar quanto à aquisição das amostras também existiu, no entanto, com a modernização e automatização dos citômetros de fluxo, atualmente é possível realizar o procedimento de triagem em alto desempenho utilizando esses equipamentos.

Em todo caso, os instrumentos denominados fluorímetros são os mais populares na execução de triagem de alto desempenho. Eles são capazes de analisar um grande número de amostras simultaneamente preparadas em placas de 96, 384 ou até 1536 poços, de forma eficiente através ensaios baseados na fisiologia celular. Esses equipamentos, que por vezes funcionam como leitores multimodais, tornaram possível a implementação de ensaios de cálcio como protocolo para *screening* em larga escala [237]. Leitores de microplacas multimodais como o FlexStation III (Molecular Devices), o FLUOstar Omega (BMG Labtechnologies) e o Synergy (Biotek), por exemplo, atuam como plataformas que integram as funções de leitura e adição de reagentes na placa de ensaio. Eles possuem um sistema de pipetagem automatizado que permite a adição dos reagentes e a leitura dos sinais em tempo real [238]. Essa habilidade é importante em ensaios de cálcio, por exemplo, uma vez que a resposta referente à mobilização desse íon é dependente do tempo, podendo ser observada entre 10 e 30 segundos após a ativação dos receptores metabotrópicos [239]. Além dessas vantagens, o usuário pode ajustar vários parâmetros para otimizar o teste, tais como: uso de placas de 96, 384 ou 1536 poços, tipo de leitura (fluorescência, absorbância ou luminescência), tempo de leitura, tempo e velocidade em que os reagentes serão adicionados, número de reagentes a serem adicionados, seleção de ponteiras e placas para a leitura, dentre outras. Além disso, esses leitores multimodais podem ser integrados a uma variedade de sistemas robóticos para realizar novas funções [238].

5.2 – Ensaios de *patch-clamp*

Uma segunda técnica que é utilizada para investigar a ativação de receptores P2, especificamente os ionotrópicos (P2X), é o *patch-clamp*. O *patch-clamp* é a técnica

considerada padrão-ouro na investigação de canais iônicos. Ela foi desenvolvida na década de 1950 e permite o estudo de um grupo ou mesmo de um único canal iônico individualmente. Através do *patch-clamp* ("*patch*"= fragmento; "(*to) clamp*"= prender, fixar) é possível fixar o potencial da membrana em uma faixa predeterminada e assim, mensurar a corrente iônica promovida pelo fluxo de íons através dos canais [240,241].

Apesar de um único canal iônico permitir a passagem de até 10 milhões de íons por segundo, a corrente iônica gerada encontra-se na faixa de picoampères. Para realizar a mensuração dos canais iônicos de um fragmento de membrana, são utilizadas pipetas de vidro ou de quartzo contendo um microeletrodo, cuja ponta mede em torno de 1 μm. Elas também são utilizadas para "fazer contato" (selagem) com a membrana plasmática [265]. Primeiramente, uma leve sucção é aplicada para a obtenção de um selamento firme entre a pipeta e a membrana. Essa configuração, conhecida como *cell-attached*, permite que a membrana plasmática permaneça intacta, sendo muito utilizada para o registro da atividade de um único ou de um pequeno grupo de canais iônicos (Figura 5.2.1.A). Se a pipeta for retraída, um fragmento da membrana poderá ser separado da célula, permitindo que a face citoplasmática da membrana fique exposta. Essa configuração, conhecida como *inside-out*, é utilizada para estudar a atividade de um único canal (Figura 5.2.1B). Caso seja aplicada uma segunda e forte sucção, a membrana plasmática poderá ser rompida e com isso, a pipeta ganha acesso ao citoplasma. Essa configuração é conhecida como *whole-cell* e é a mais utilizada, sendo empregada no estudo do potencial elétrico ou de correntes iônicas da célula por inteiro. Dentro dessa modalidade, ainda é possível optar por um dos dois modos de registro o *voltage-clamp* ou o *current-clamp*, sendo a voltagem mantida constante para a mensuração da corrente, ou vice-versa (Figura 5.2.1C). Dentro da configuração de *whole-cell*, ainda existe a variação *perforated-cell*, na qual a segunda sucção necessária para romper as células é substituída pelo uso de antibióticos ou antifúngicos (ex: anfotericina B, nistatina e gramicidina), os quais produzem poros na membrana celular. Essa configuração vem sendo empregada na mensuração de potenciais de ação. Por fim, se a pipeta for retraída, dois fragmentos de membrana que foram deslocados podem se reconectar e formar uma espécie de vesícula, deixando a superfície extracelular exposta. Essa configuração, conhecida como *outside-out*, é empregada na análise de estímulos extracelulares sobre o canal iônico (Figura 5.2.1D) [240–242]. A Figura 5.2.1 ilustra as quatro configurações da técnica de *patch-clamp*.

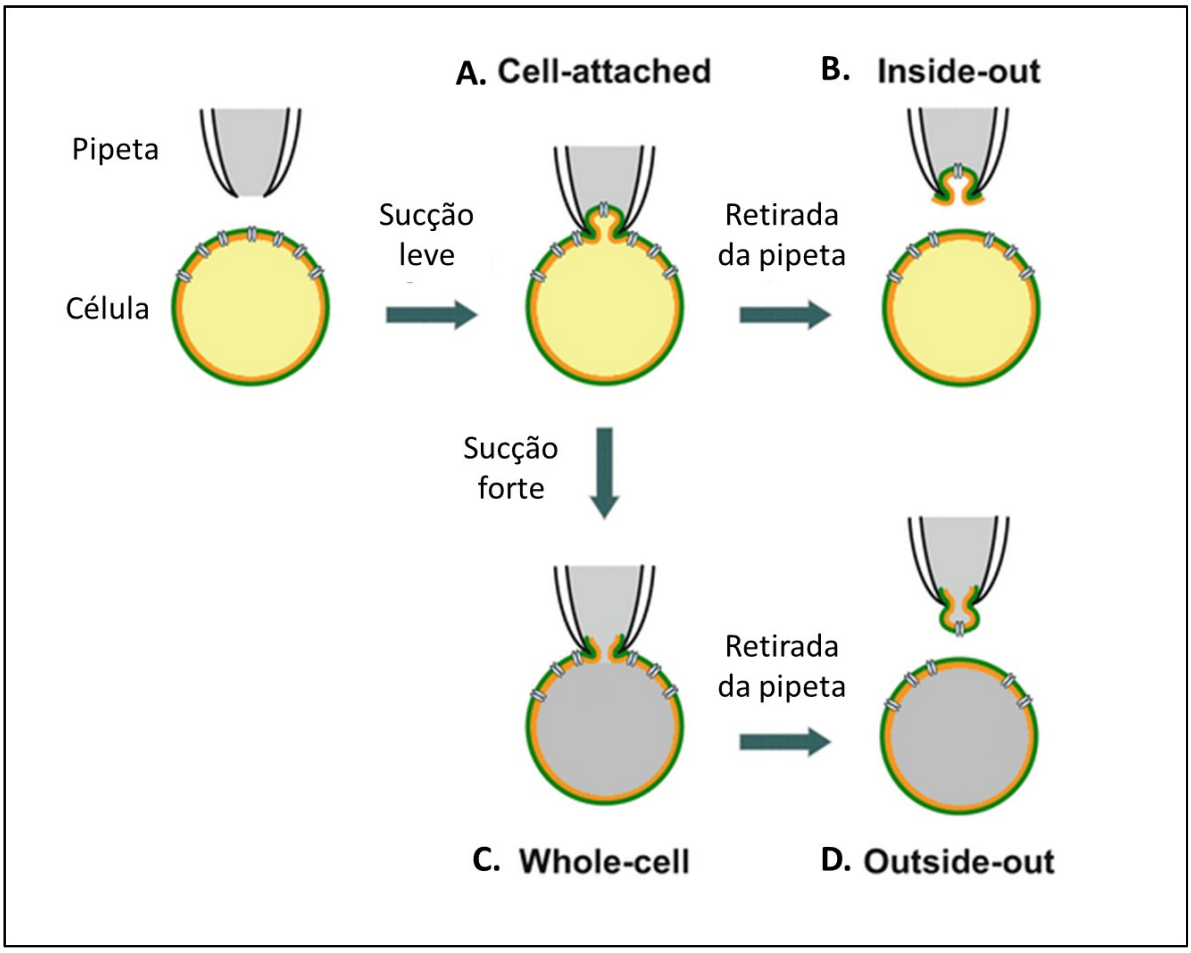


Figura 5.2.1 – Configurações da técnica de *patch-clamp*. Nesta técnica, uma pipeta de vidro contendo um microeletrodo é aproximada da célula e uma sucção leve, mas suficiente para "selar" a célula, é promovida definindo a configuração *cell-attached* (A). A retirada da pipeta pode promover o deslocamento de um pequeno fragmento de membrana, definindo a configuração *inside-out* (B). Caso uma segunda sucção mais forte seja promovida, a pipeta poderá ser contínua à célula, configurando a modalidade *whole-cell* (C). Após o estabelecimento desta configuração, a pipeta também poderá ser retirada, carregando consigo dois fragmentos de membrana que podem se unir e formar uma vesícula, modalidade conhecida como *outside-out* (D). Imagem adaptada de [243].

No que tange aos ensaios de *patch-clamp* relacionados à atividade do receptor P2X7, duas configurações já foram utilizadas. Na configuração *cell-attached*, já foi reportado uma corrente de aproximadamente 10 pS, entretanto, a maioria dos estudos utilizam a configuração *whole-cell*. Nessa configuração, as correntes macroscópicas são divididas em dois componentes. O primeiro componente está relacionado com a abertura do canal iônico, enquanto que o segundo está relacionado a um estado de alta condutância, o qual pode caracterizar a formação do poro membranar [69].

A técnica de *patch-clamp* pode ser considerada a melhor técnica para avaliação dos receptores P2X, uma vez que apresenta sensibilidade e acuracidade. Entretanto, ela é laboriosa, uma vez que só permite a análise de uma célula por vez, além de não poder ser

aplicada a todos os tipos celulares e exigir habilidade técnica e treinamento do operador [244]. Esses desafios quanto à aplicação da técnica de *patch-clamp* como metodologia de *screening* tem sido superados com o desenvolvimento de aparato de eletrofisiologia automatizado, como o equipamento IonFlux da empresa Molecular Devices.

5.3 – Ensaios de permeabilização celular

Os ensaios de permeabilização celular ou de captação de corantes avaliam a função do poro associado ao P2X7 [169,172]. Esse fenômeno é considerado como o segundo estágio de permeabilização membranar relacionado à ativação desse receptor. A formação do poro ocorre após a exposição prolongada a altas concentrações de ATP (< 100 μM) e tende a aumentar ao longo do tempo. Apesar da origem desse poro ainda ser debatida, já se sabe que ele permite a passagem de íons (Na^+, Ca^{2+}, K^+), corantes, água e ATP, conforme mostrado na Figura 5.3.1 [168].

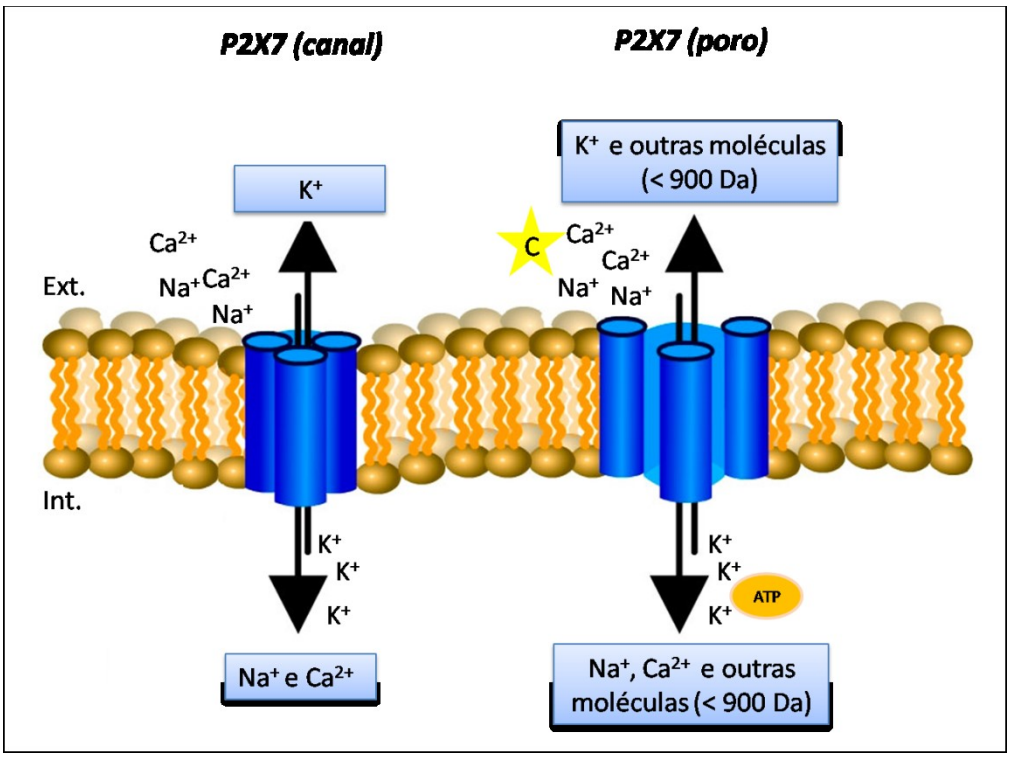


Figura 5.3.1 – Canal e poro membranar do P2X7. A ativação do receptor P2X7 promove a abertura do canal cátion-seletivo, permitindo o fluxo dos íons Na^+, Ca^{2+} e K^+ de acordo com o gradiente eletroquímico. Em situações de ativação prolongada ou na presença de altas concentrações do ATP (faixa de milimolar), esse receptor induz a formação de um poro membranar que permite a passagem de moléculas de até 900 Da, incluindo os íons Na^+, Ca^{2+} e K^+, ATP (simbolizada pela elipse laranja) e corantes (simbolizados pela estrela amarela) como o YO-PRO-1, iodeto de propídeo, brometo de etídeo, amarelo de lúcifer, entre outros que apresentam peso molecular inferior ao *cut-off* descrito. (Ext.) significa meio extracelular e (Int.) significa meio intracelular. Imagem adaptada de [192].

A permeabilização celular associada ao receptor P2X7 (antigo receptor P2Z) foi caracterizada no fim da década de 1980 em macrófagos da linhagem celular J774, utilizando o corante amarelo de lúcifer [245]. Nas décadas seguintes, outros corantes passaram a ser utilizados, incluindo brometo de etídeo, iodeto de propídeo e YO-PRO-1, os quais emitem fluorescência ao serem incorporados ao DNA [246]. As propriedades desses corantes estão descritas no quadro 5.3.1.

Quadro 5.3.1 – Principais características dos corantes mais populares no estudo da formação do poro membranar associado ao receptor P2X7[1]

Corante	Peso Molecular (Da)	Diâmetro (Å)	Carga
Amarelo de lúcifer	444	13	2-
Brometo de etídeo	394	11	+
Iodeto de propídeo	668	11,5	2+
YO-PRO-1	629	8	2+

[1]Referência [168].

Inicialmente esses ensaios eram realizados através de técnicas de microscopia, o que permitia apenas a análise de um grupo de células por vez, o que os tornava inviáveis para ensaios de alto desempenho. Entretanto, diversos grupos de pesquisa padronizaram a execução desses ensaios em formato de placa, empregando leitores multimodais na aquisição dos dados. Com isso, esse tipo de ensaio passou a ser aplicado no campo de descoberta de fármacos. [244,247,248].

5.4 – Descobertas recentes através de campanhas de HTS e perspectivas

Empregando a técnica de mensuração dos níveis de cálcio, Ito et al. (2017) testaram mais de 140 mil moléculas, identificando 43 *hits* que apresentaram atividade inibitória para o receptor P2Y6. Dentre elas, a molécula TIM-38 destacou-se pela inibição da resposta de cálcio induzida pelo UDP, além de atenuar a liberação da IL-8 [249]. Recentemente, Ferreira et al. (2018) também empregaram o ensaio de cálcio em uma mini-campanha de *screening* (100 amostras) para a descoberta de novos antagonistas para os receptores P2Y2 e P2Y4 (Artigo 1 da tese). Eles identificaram três extratos provenientes de duas espécies botânicas, *Joannesia princeps* Vell. e *Peixotoa* A. Juss, que inibiram a mobilização de cálcio induzida pelo UTP de forma concentração-dependente [218].

Utilizando a técnica de cálcio, Fischer et al. (2016) testaram 800 moléculas oriundas de produtos naturais e identificaram três delas (teniposida, agelasina e ácido garcinólico) como

moduladores da atividade do P2X7 [250]. Enquanto isso, Soares-Bezerra et al. (*in press*) empregaram a técnica de permeabilização celular para testar cerca de 1800 amostras oriundas de produtos naturais. Como resultado, eles identificaram a atividade antagonista de três extratos fúngicos sobre o receptor P2X7 [227].

Outra metodologia que vem ganhando espaço no cenário de *screening* de novos antagonistas para os receptores P2 é a análise *in silico* de moléculas adquiridas de bancos de dados virtuais através da técnica de *docking* molecular. Entre as vantagens dessa última metodologia podem ser listadas a economia de tempo, uma vez que é possível realizar o teste de um grande número de amostras em um curto espaço temporal, a redução de gastos com o uso de reagentes e materiais consumíveis de laboratório, além de permitir uma análise seletiva quanto à interação da molécula-teste e o alvo-farmacológico [251]. Além disso, já existem diversas bibliotecas virtuais em que se encontram cadastradas milhares de moléculas de origem sintética e natural. Alguns bancos de dados ofertam inclusive, moléculas provenientes de plantas utilizadas na Medicina Tradicional Chinesa ou Africana, como por exemplo, o *The Traditional Chinese Medicine Integrated Database* (TCMID) e *African medicinal plants database* (AfroDb) [207].

II. OBJETIVOS

Objetivo Geral

Realizar a triagem de alto desempenho de extratos e moléculas isoladas de plantas pertencentes à flora brasileira a fim de identificar novos candidatos a antagonistas de receptores P2.

Objetivos específicos

1. Identificar candidatos a antagonistas de receptores P2 através de triagem de alto desempenho;
2. Avaliar a citotoxicidade desses candidatos;
3. Identificar o IC_{50} dos extratos e moléculas candidatas e caracterizá-los com parâmetros farmacológicos;
4. Avaliar a atividade dos extratos e moléculas candidatas na atenuação da inflamação e da dor em modelos *in vivo*;
5. Realizar a caracterização química dos extratos e moléculas candidatas.

III. RESULTADOS

- **Os resultados da tese estão contidos nos seguintes trabalhos, os quais serão apresentados a seguir:**

1. New insights in purinergic therapy: Novel antagonists for uridine 5'-triphosphate-activated P2Y receptors from Brazilian flora;

2. The antagonistic effect of *Joannesia princeps* Vell. stem on UTP-activated receptors;

3. Discovery of antagonistic activity of CS-15 compound on P2X7 receptor;

4. Potential therapeutic applications of P2 receptor antagonists: from bench to clinical trials.

- **Título do artigo 1: <u>New insights in purinergic therapy: Novel antagonists for uridine 5'-triphosphate-activated P2Y receptors from Brazilian flora</u>**

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- **Situação:** Publicado

- **Descrição:** O presente artigo foi desenvolvido parcialmente durante o meu Mestrado, contudo, a sua conclusão só ocorreu ao longo do Doutorado. Nesse artigo, apresentamos o passo-a-passo para a otimização do ensaio de cálcio no leitor multimodal FlexStation III. O objetivo dessa padronização era realizar a triagem de extratos vegetais pertencentes a diferentes biomas brasileiros como Amazônia, Cerrado, Caatinga, Pantanal, Mata Atlântica e Pampa, com o intuito de identificar potenciais antagonistas para os receptores P2Y ativados por UTP, isto é, P2Y2 e P2Y4. Para isso, utilizamos células da linhagem J774.G8, as quais expressam esses receptores, como um modelo para a execução do *screening*. Posteriormente, os extratos considerados promissores "*hits"* foram testados novamente em células primárias (macrófagos peritoneais) a fim de validarmos o modelo previamente estabelecido. A partir do teste com 100 extratos, descobrimos que as espécies *Joannesia princeps* Vell. e *Peixotoa* A. Juss apresentaram um efeito inibitório concentração-dependente sobre a mobilização de cálcio induzida pelo UTP. Cabe ressaltar que esse efeito inibitório não foi causado pela citotoxicidade do extrato de galhos de *J. princeps* Vell. e dos extratos de folhas e flores de *Peixotoa* A. Juss. Esse trabalho atende aos objetivos específicos 1, 2 e 3 da tese.

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New Insights in Purinergic Therapy: Novel Antagonists for Uridine 5′-Triphosphate-Activated P2Y Receptors from Brazilian Flora

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ABSTRACT P2Y2 and P2Y4 receptors are physiologically activated by uridine 5′-triphosphate (UTP) and are widely expressed in many cell types in humans. P2Y2 plays an important role in inflammation and proliferation of tumor cells, which could be attenuated with the use of antagonists. However, little is known about the physiological functions related to P2Y4, due to the lack of selective ligands for these receptors. This can be solved through the search for novel compounds with antagonistic activity. The aim of this study was to discover new potential antagonist candidates for P2Y2 and P2Y4 receptors from natural products. We applied a calcium measurement methodology to identify new antagonist candidates for these receptors. First, we established optimal conditions for the calcium assay using J774.G8, a murine macrophage cell line, which expresses functional P2Y2 and P2Y4 receptors and then, we performed the screening of plant extracts at a cutoff concentration of 50 μg/mL. ATP and ionomycin, known intracellular calcium inductors, were used to stimulate cells. The calculated EC_{50} were 11 μM and 103 nM, respectively. These cells also responded to the UTP stimulation with an EC_{50} of 1.021 μM. Screening assays were performed and a total of 100 extracts from Brazilian plants were tested. *Joannesia princeps* Vell. (stem) and *Peixotoa* A. Juss (flower and leaf) extracts stood out due to their ability to inhibit UTP-induced responses without causing cytotoxicity, and presented an IC_{50} of 32.32, 14.99, and 12.98 μg/mL, respectively. Collectively, our results point to the discovery of potential antagonist candidates from Brazilian flora for UTP-activated receptors.

KEYWORDS: • ***calcium*** • ***natural products*** • ***P2Y2*** • ***P2Y4*** • ***screening***

INTRODUCTION

P2YR ARE G PROTEIN-coupled receptors expressed in many cell types in humans. Currently, eight P2Y subtypes have been cloned in mammals: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14.[1] These subtypes are divided into two groups according to the signaling pathway promoted by G protein. P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 subtypes are coupled to G_q protein and activate phospholipase Cβ (PLCβ) that forms diacylglycerol (DAG) and inositol 1,4,5,triphosphate (IP_3). IP_3 induces an increase of intracellular calcium through IP_3 receptor activation in the endoplasmic reticulum. Meanwhile, P2Y12, P2Y13, and P2Y14 subtypes are coupled to G_i protein and inhibit adenylyl cyclase, (AC) causing a decrease in cyclic adenosine monophosphate (cAMP) formation.[1,2]

P2Y2 and P2Y4 receptors are physiologically activated by uridine 5′-triphosphate (UTP) and promote an increase in intracellular calcium through PLC/IP_3.[2] They are expressed in many cell types, such as epithelial cells, monocytes, macrophages, neutrophils, cardiomyocytes, and organs, including the brain, heart, kidneys, liver, spleen, and muscle.[3] They have effects on chloride secretion in epithelial cells from the airway and eyes, and also induce water secretion in bowel epithelial cells. These functions are important to promote surface lubrification and mucus hydration, which can improve disease treatment, such as dry eye disease and chronic constipation.[2,4]

P2Y2R also plays a role in neuroprotection, chemokinesis, and in the proliferation of hepatocytes and tumor cells.[5–8] P2Y2 is also involved in the production of inflammatory modulators, such as cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE_2), and neutrophil accumulation in

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lungs and liver during inflammation.[9–13] Thus, P2Y2 antagonists might alleviate inflammation signals.

P2Y4R participates in Na^+, K^+, and Cl^- regulation processes, but its functions are not completely characterized, as it is expressed in the same tissues and cell types as P2Y2R, and activated by the same physiological ligand, UTP.[14–17] Therefore, the lack of selective ligands impairs the discovery of new functions associated with these receptors. However, this problem can be solved by the search for novel compounds with antagonistic activity on P2Y2 and P2Y4 receptors.

Natural products are an important source for the discovery of new molecules with antagonistic activity. They offer the advantage of millenary use in the treatment of several diseases, such as inflammation, parasitoses, pain, and recently, cancer, constituting the basis of traditional oriental medicines.[18–20] Among the 13 drugs approved for global marketing between 2005 and 2007, five were classified as natural products, whereas the others were semisynthetic or derived, which reinforces natural product importance for clinical use.[21] In addition, the literature reports that some biological species express compounds with antagonistic activity on P2 receptors, such as emodin from *Rheum officinale* Bail and amentoflavone from *Rheedia longifolia* Planch & Triana on P2X7R.[22,23] However, due to the large number of samples for testing, implementation of high-throughput screening (HTS) methodologies is necessary to meet this demand, since they have the advantage of evaluating many compounds in a short period of time.[24,25]

In this scenario, intracellular calcium measurement techniques emerge as a tool for the screening of new antagonists, since P2Y2 and P2Y4 receptors are coupled to the calcium signaling pathway.[26] Kaulich *et al.* tested a series of 40 flavonoids using calcium assays to discover novel lead antagonists for P2Y2R. They found that certain flavonoids could inhibit P2Y2R and suggest using their structures to develop new compounds with antagonistic activity.[27] Recently, Ito *et al.* applied an intracellular calcium measurement protocol through a HTS campaign to search for novel antagonists for the P2Y6 receptor and found one compound, TIM-38, with potential activity.[28]

Thus, the aim of this study was to improve a protocol based on intracellular calcium measurements and apply it in the discovery of natural product compounds with antagonist activity on P2Y2 and P2Y4 receptors.

MATERIALS AND METHODS

Reagents

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), including ATP, UTP, ADP, UDP, $\alpha\beta$-meATP, Suramin, DMEM, and RPMI culture mediums, NaCl, KCl, Na_2HPO_4, KH_2PO_4, $MgCl_2$, $CaCl_2$, Glucose, DMSO, MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium, Probenecid, and Ionomycin. Fetal bovine serum was obtained from Cultilab (Campinas, SP, Brazil) and Fluo-4 AM was obtained from Life Technologies (CA, USA).

Extracts

Extracts used in this study were collected from Brazilian biomes (Amazon, Cerrado, Caatinga, Atlantic Forest, Pantanal, and Pampas), obtained through methanol extraction, and deposited at Bioprospecting Platform from the Chemistry of Natural Products Laboratory.

The stems from *Joannesia princeps* Vell. (blind code: JA2) were collected in the Atlantic biome, in Linhares, in the state of Espírito Santo, Brazil. The stems were crushed and mixed with a 1:1 mixture of CH_2Cl_2-CH_3OH to obtain the extract deposited at voucher number EX294. *Peixotoa* A. Juss were collected in the Cerrado biome, in Tiradentes, located in the state of Minas Gerais, Brazil. *Peixotoa A. Juss* flower (blind code: RA3) and leaf (blind code: RB3) extracts are deposited at voucher numbers EX9355 and EX9356, respectively. A botanical specimen was deposited at the Federal University of São João del Rei Herbarium under code HUFSJ1103. The flowers and leaves were crushed and homogenized with ethanol to obtain the crude extracts. For the experiments, they were resuspended in a 1 mL Dulbecco's PBS solution containing 0.5% DMSO. The extracts were tested using a single-blind method, that is, the professional responsible for performing screening experiments did not know the name of the biological species that originated the samples.[29] Because of this, they were identified by a code.

Cell culture

J774.G8 is a murine macrophage cell line that expresses P2 receptors. The J774.G8 cell line was kindly provided by Dr. Vinícius Cotta from the Laboratory on Thymus Research at the Oswaldo Cruz Institute, Rio de Janeiro, Brazil. The cells were routinely maintained in culture with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere. The medium was changed every 3 days and the cells were adjusted to a concentration of 2×10^6 cells per 150-cm^2 cell culture flask (Corning, NY, USA). Cell viability was analyzed by the Trypan Blue assay. For this, 10 μL of cell sample was diluted 1:100 in 0.04% Trypan Blue solution. After dilution, 10 μL of the sample were added into the Neubauer chamber and observed under optical microscope Nikon Eclipse TE 2000-S (Nikon, Tokyo, Japan) to count viable cells. Calcium assays were conducted only when the viability range was above 90%.

Calcium assays

J774.G8 cells were plated at a concentration of 2×10^5 cells/well in a 96-well black-wall, clear-bottom plate (Corning, NY, USA), and maintained in culture with DMEM supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24 h. Subsequently, the medium was replaced by 100 μL of Dulbecco's PBS (DPBS) (136 mM NaCl, 2.68 mM KCl, 8 mM Na_2HPO_4, 1.46 mM

KH_2PO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5.55 mM Glucose, and 2.5 mM Probenecid–pH 7.4) containing Fluo-4 AM [2 μM] and the cells were incubated for 60 min at 37°C in a 5% CO_2 atmosphere. Extracellular Fluo-4AM was then removed by three consecutive washes with 200 μL DPBS and the cells received a final volume of 150 μL DPBS. Subsequently, cells were treated with extracts [50 μg/mL] and PPADS [300 μM], Suramin [300 μM], and Reactive Blue-2 (RB-2) [20 μM] antagonists for 30 min. Finally, the assay plate was placed into the FlexStation III equipment (Molecular Devices, CA, USA), which performed the agonist addition to each well, diluted in 50 μL DPBS. The mobilization of intracellular calcium was measured in real-time prior, during, and after the addition of the agonists at λ (excitation) of 485 nm and λ (emission) of 525 nm. Total measurement runtime was 90 sec and agonists were added at the 20-sec mark. Readings were carried out every 1.52 sec and a total of 60 readings were obtained for each well. The ΔFU, that is, the difference between the fluorescence peak associated with the maximum concentration of intracellular calcium ($[Ca^{2+}]_i$) and basal fluorescence of calcium was determined according to the protocol of Hansen and Bräuner-Osborne (2009).[26] The wells whose signal did not return to approximately 30% of the baseline after stimulation were discarded as quality control.

Cell loading observation under a fluorescence microscope

J774.G8 cells were loaded with Fluo-4AM as described previously and observed under a Nikon Eclipse TE 2000-S fluorescence microscope (Nikon, Tokyo, Japan), using 20× objectives. Fluo-4 was excited using a mercury lamp and a set of filters: exciter: 480/30 and barrier: 535/40. Ionomycin was added after 40 sec of observation and pictures were captured with an Infinity 3 Microscope Digital Camera (Lumenera, Ontario, Canada).

RNA extraction, Reverse transcriptase polymerase chain reaction, and electrophoresis

mRNA was isolated from the J774.G8 cell line from a concentration of 5×10^6 cells using TRIzol (Life Technologies, CA, USA), according to the manufacturer's specifications. Then, 20 μL of mRNA was reverse transcribed at 42°C for 1 h and at 70°C for 5 min using 1 μL of reverse transcriptase enzyme. Next, 4 μL of cDNA samples were used with 4 μL of a primer sequence specific for each P2 receptor subtype, as shown in Table 1. These samples were used for the RT-PCR analysis in a 50 μL reaction volume containing 5 μL of Buffer 10×, 4 μL Mg^{2+}, 0.4 μL dNTP, 0.2 μL Taq polymerase, and 4 μL of water. Cycling conditions were: 94°C for 45 sec, followed by 35 cycles at 54°C (P2Y11), 55°C (P2Y6), 57°C (P2X1), 58°C (P2X3, P2Y14, and P2Y13), 60°C (P2Y2, P2X2, P2X5, P2X6, P2Y12, P2Y1, P2Y4, and GAPDH) or 65°C (P2X4 and P2X7) for 1 min and 72°C for 45 sec. PCR products were submitted to electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining under a transilluminator.

Table 1. Primer Sequence of P2 Receptors Used in RNA Extraction, Reverse Transcriptase Polymerase Chain Reaction, and Electrophoresis Experiments

P2 receptor subtype	*Primer sequence*
P2X1	Forward: ATGTCCTCGGCATATTTGAA
	Reverse: ATTGTGCAGAGAACCCAGAA
P2X2	Forward: TTCATCGTGCAGAAAAGCTACC
	Reverse: TGGATGCTGTTCTTGATGAGGA
P2X3	Forward: CTTCACCTATGAGACCACCAAG
	Reverse: CGGTATTTCTCCTCACTCTCTG
P2X4	Forward: TGCTCATCCGCAGCCGCAAAGT
	Reverse: AGTGGTCGCATCTGGAATCTCGG
P2X5	Forward: ACTTCCCTGCAGAGTGCTGT
	Reverse: GGCAGCTTTATCAAGGTCACA
P2X6	Forward: TATGTGATGACCAGGAACTG
	Reverse: CTCCAGATCTCACAGGTCCT
P2X7	Forward: GGCAGTTCAGGGAGGAATCATGG
	Reverse: AAGCGCCAGGTGGCATAGCTC
P2Y1	Forward: CTGTTGAGACTTGCTAGACCTC
	Reverse: ATGTTCAATTTGGCTCTGGC
P2Y2	Forward: GTCGCTTCAACGAGGACTTC
	Reverse: GCCAGGAAGTAGAGCACAGG
P2Y4	Forward: TGGCATTGTCAGACACCTTG
	Reverse: AAGCAGACAGCAAAGACAGTC
P2Y6	Forward: CGCTTCCTCTTCTATGCCAA
	Reverse: GTAGGCTGTCTTGGTGATGTG
P2Y11	Forward: CAGCGTCATCTTCATCACC
	Reverse: GCTATACGCTCTGTAGGC
P2Y12	Forward: CTGCCAAGTCATTTTCTGGA
	Reverse: AGCATGCTCATCAAGGAATTT
P2Y13	Forward: AGGGGTTTTGTGTGCACTTT
	Reverse: CTGACTGCTGTGGTGCTCAT
P2Y14	Forward: GCTGTCCCCAACATCATTCT
	Reverse: GTTTTGGGGTAACTCGCAGA
GAPDH	Forward: ATCACCATCTTCCAGGAGCG
	Reverse: CCTGCTTCACCACCTTCTTG

Animals

The experiments with animals were approved by Oswaldo Cruz Institute Ethics Committee on the use of Animals, under identification code L-037/2017, approved on November 24, 2017, and followed the ethical principles in animal experimentation from the Brazilian College of Animal Experimentation. Healthy male C57BL/6 mice weighing between 25 and 30 g were obtained from the Oswaldo Cruz Central Bioterium. The animals were housed under 12-h light/12-h dark cycle conditions with free access to food and water.

Peritoneal macrophage isolation

Animals were euthanized by CO_2 asphyxiation. Subsequently, 5 mL of RPMI medium was injected into the peritoneal cavity. Peritoneal cells were collected and macrophages were isolated by centrifugation at 378 *g* for 10 min. Cell viability was analyzed by the Trypan Blue assay. Peritoneal macrophages were plated at a concentration of 4×10^5 cells/well in a 96-well black-wall, clear-bottom plate and maintained in RPMI culture supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24 h. Calcium assays were carried out using the same conditions described previously.

Cell viability measurement

J774.G8 cells were plated at a concentration of 2×10^5 cells/well in a 96-well plate (Corning, NY, USA), and maintained in culture with DMEM supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24 h. Next, cells were treated with extracts [50 μg/mL] and the antagonists, PPADS [300 μM] and RB-2 [20 μM] for 1, 6, or 24 h. Cells treated with Triton-X (0.1%) were considered negative viability controls, whereas positive controls were obtained with cells that did not receive any treatment. Subsequently, the medium was replaced by 180 μL of DMEM without phenol red and each well received 20 μL of MTT solution [100 μg/well]. The plate was then incubated for 3 h at 37°C in a 5% CO_2 atmosphere and then centrifuged at 312 *g* for 1 min. Finally, supernatants were collected and formazan crystals were dissolved in 100 μL DMSO.[30] The absorbance of the wells was measured on a FlexStation III equipment, using λ: 570 nm.

Data analyses

Each sample was measured in triplicate and all experiments were performed on at least 3 independent days. All data are presented as mean ± SDM, whereas the curves are presented as mean ± SEM. To test if samples follow a Gaussian distribution, the D' Agostino and Pearson normality test was used. If data followed a Gaussian distribution, an appropriate parametric test was applied, if not, an appropriate nonparametric test was used. The applied tests were specified in figure legends. *P* values of .05 or less were considered significant. Graphs and statistical analyses were performed using the GraphPad Prism version 7 software (GraphPad Software, San Diego, CA, USA). The quality of the calcium assay was assessed by calculating the z′-factor. The z′-factor is a valuable tool to evaluate the robustness and suitability of HTS assays. z′-factor values above 0.5 consider the assay as excellent and equal to 1, an ideal assay, whereas values below 0.5 consider that the assay should be reformulated.[31] This parameter was calculated using the following equation:

$$Z' = 1 - \frac{(3\text{SD of the positive control} + 3\text{SD of the negative control})}{(\text{mean of the positive control} - \text{mean of the negative control})}$$

where cells stimulated with UTP at [10 μM] were considered positive control (concentration used in screening experiments) and cells stimulated with DPBS only were considered negative control.

RESULTS

Establishment of a protocol for the detection of intracellular calcium mobilization

Our primary aim was to discover new antagonists for P2Y receptors activated by UTP, so a protocol for the detection of intracellular calcium mobilization was first established. Fluo-4 is a calcium indicator widely applied in HTS assays of metabotropic receptors that induce increase in intracellular calcium.[32] The literature states a range of Fluo-4 used in calcium assays, between 2 and 4 μM.[33–36] To use low doses of the dye, the first step was to optimize Fluo-4 concentration in our assay conditions, making it more economical for HTS. In this context, we investigated concentrations ranging from 1 to 8 μM, using ATP [100 μM] to stimulate the cells due to its activity as a main physiological P2 receptor agonist, widely expressed in J774.G8 cells. As shown in Figure 1A, no significant difference in the quantification of calcium responses for concentrations between 1 to 4 μM was observed. Concentrations of 6 and 8 μM have shown significant difference only compared with 1 μM.

A similar result was obtained in cell stimulation with ionomycin, a calcium ionophore, which promotes a massive transport of this ion from the extracellular to intracellular medium. All concentrations evaluated have a similar profile for calcium response quantification (Fig. 1B). Thus, the selected Fluo-4 concentration to use in our experiments was 2 μM, as calcium responses in this concentration did not differ from higher concentrations, and is cited in the literature.

As we could observe in Figure 1C, cells loaded with Fluo-4 at 2 μM shown a homogenous dye scattering in their cytosol and, after stimulus with ionomycin, become more fluorescent.

Next, two assays were performed to verify Fluo-4 sensibility at the established concentration (2 μM). Cells were stimulated with increasing ATP and ionomycin concentrations, and Fluo-4 fluorescence increased in a concentration-dependent manner, as shown in Figure 1D and E. EC_{50} for ATP and ionomycin were 11 μM and 103 nM, respectively.

Therefore, in this primary step, Fluo-4 concentrations at 2 μM were ideal for our assay, since cells presented homogenous dye loading and sensibility in responding to calcium stimuli in increasing concentrations.

Quality assessment of calcium responses induced by P2 receptors

To characterize the quality of calcium responses induced by P2 receptors through ATP stimulation acquired on the FlexStation III equipment, we analyzed original records. Original records indicated that DPBS addition alone did not cause signal variations, as expected (Fig. 2A). ATP stimulus, on the other hand, increased intracellular calcium levels, as seen by the fluorescence peak shown in Figure 2B. This effect was reversed with the addition of EGTA, a calcium chelating agent, indicating that the fluorescence is

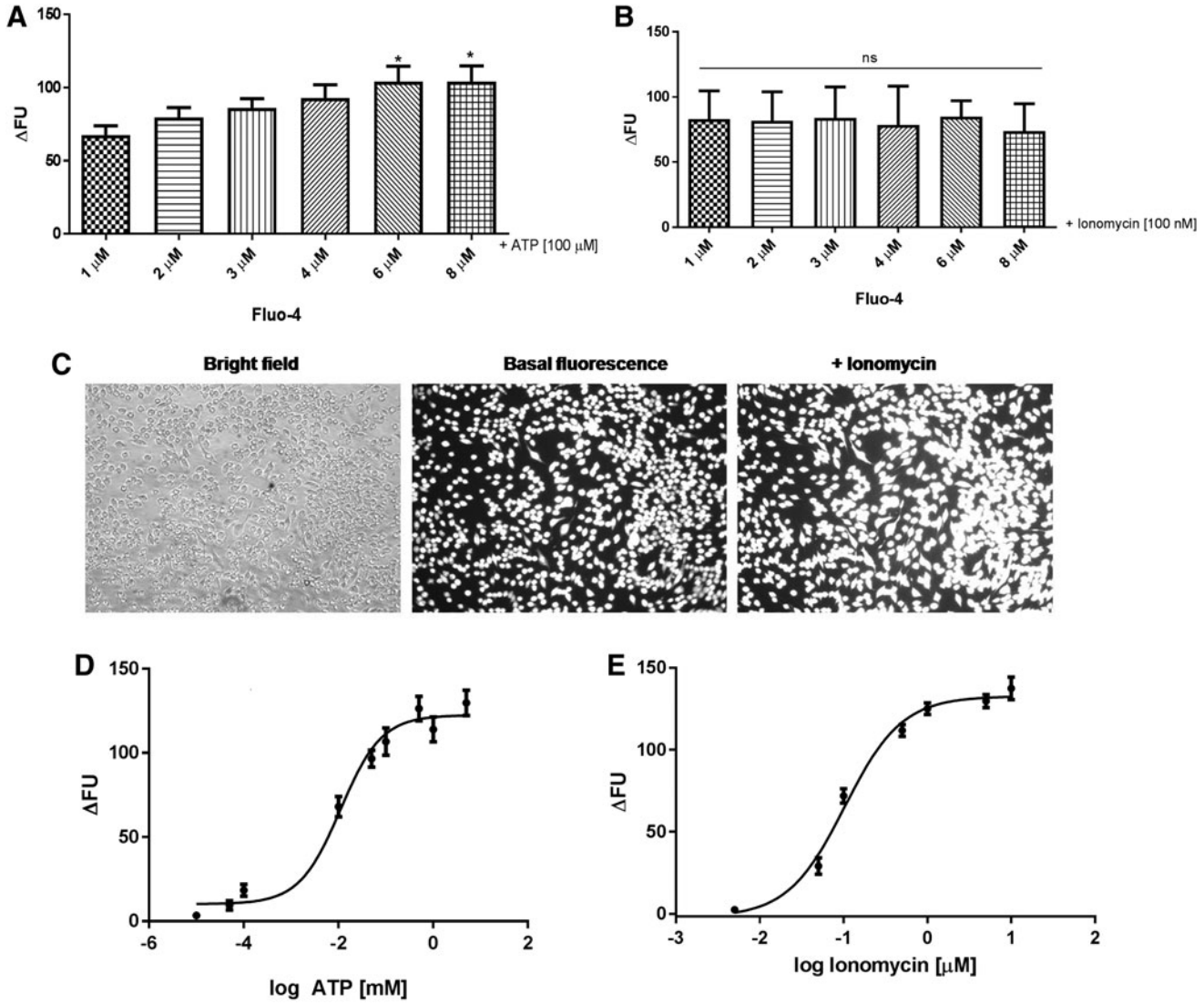

FIG. 1. Measurement of $[Ca^{2+}]_i$ after ATP and ionomycin stimulation in cells stained with Fluo-4. J774.G8 cells stained with different Fluo-4 concentrations were stimulated with [100 μM] ATP **(A)** and ionomycin [100 nM] **(B)** and were monitored for 90 sec on a FlexStation III equipment. Data are presented as Mean ± SDM of four independent experiments performed in triplicate. Data analyses were made using one-way ANOVA and Holm–Sidak post-test; $P < .05$. Asterisks indicates significant differences in relation to the 1 μM concentration. (ns) means not significant. Observation of J774.G8 cells in brightfield and fluorescence after loading with Fluo-4 and stimulated with ionomycin during 1 minute (objective 20×) **(C)**. Representative images from three independent experiments performed in triplicate. ATP **(D)** and ionomycin **(E)** curves were obtained from J774.G8 cells stained with 2 μM of Fluo-4. Data are presented as Mean ± SEM of three independent experiments performed in triplicate.

associated with calcium, as shown in Figure 2C. Cells stimulated with ionomycin also increased their fluorescence (Fig. 2D). No decline in intracellular levels during the selected time frame for this experiment, that is, 90 sec, was observed. Therefore, these experiments validated our calcium measurement protocol using the FlexStation III equipment.

Characterization of P2 receptors in J774.G8 cells

Before beginning the screening experiments, we attempted to identify which P2 receptors (P2R) were expressed in this cell line to guide our assay, avoiding bias, since some P2R subtypes were activated by the same agonists. First, the RT-PCR technique was performed, aiming to verify which P2R are expressed in J774.G8 cells. As observed in Figure 3A and B, cDNA fragments corresponded to P2X1 (775 bp), P2X7 (186 bp), P2Y1 (683 bp), P2Y2 (850 bp), P2Y4 (544 bp), P2Y6 (480 bp), P2Y11 (274 bp), and P2Y12 (360 bp).

All these receptors, except P2Y12R, are associated with intracellular calcium increase signaling pathways through opening of a nonselective cation ion channel (P2X) or PLCβ/IP_3 system activation (P2Y). Thus, we further investigated the response of P2R subtypes expressed in J774.G8 cells using selective agonists. As shown in Figure 3C, this

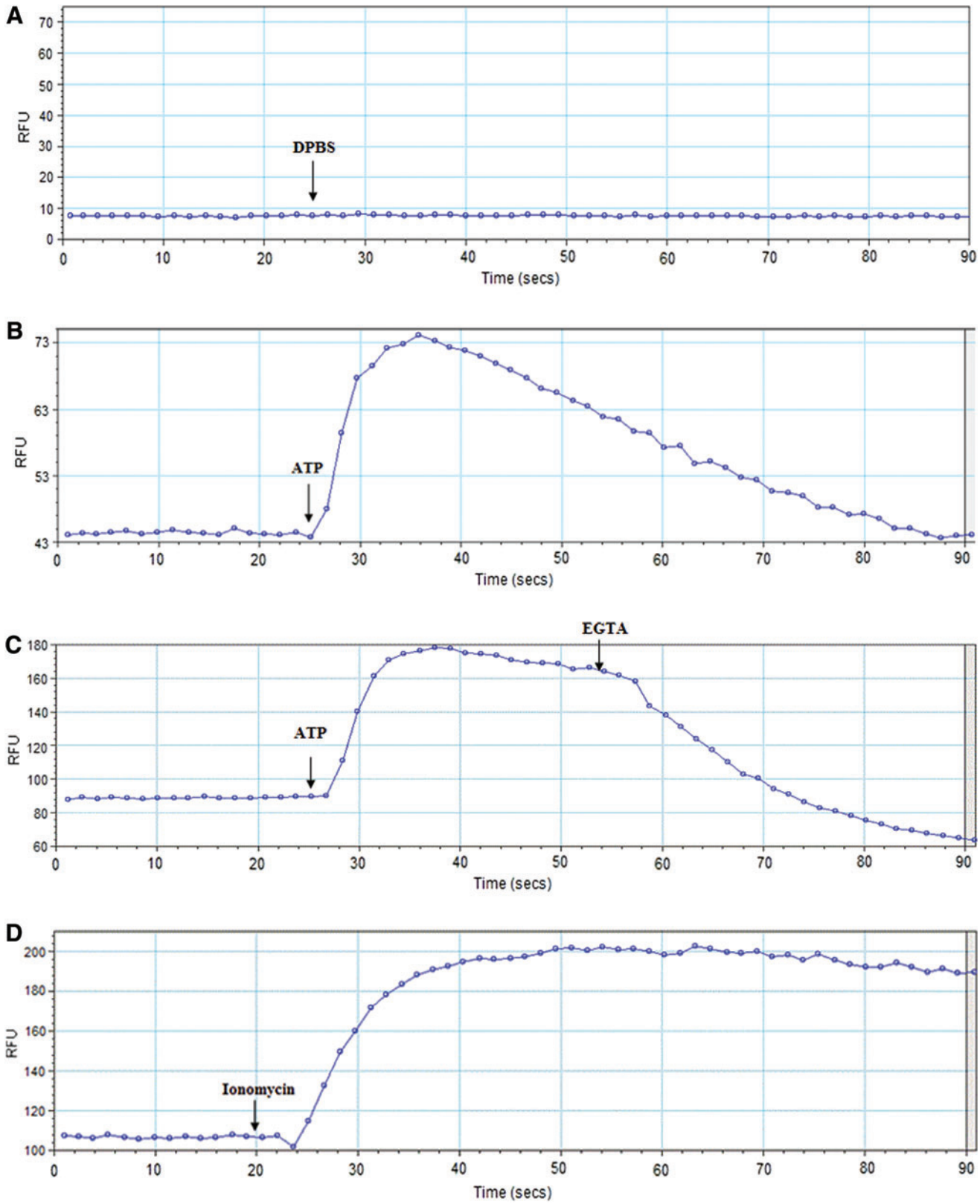

FIG. 2. Original records of calcium signals obtained on a FlexStation III. Original records of calcium signals obtained on a FlexStation III equipment after addition of 50 μL of DPBS **(A)**, ATP [100 μM] **(B, C)** at 25 sec, or Ionomycin [100 μM] **(D)** at 20 sec and EGTA [20 mM] (54 sec) **(C)** (indicated by the *arrows*) in J774.G8 cells stained with 2 μM of Fluo-4. Representative images from three independent experiments performed in triplicate. Color images available online at www.liebertpub.com/jmf

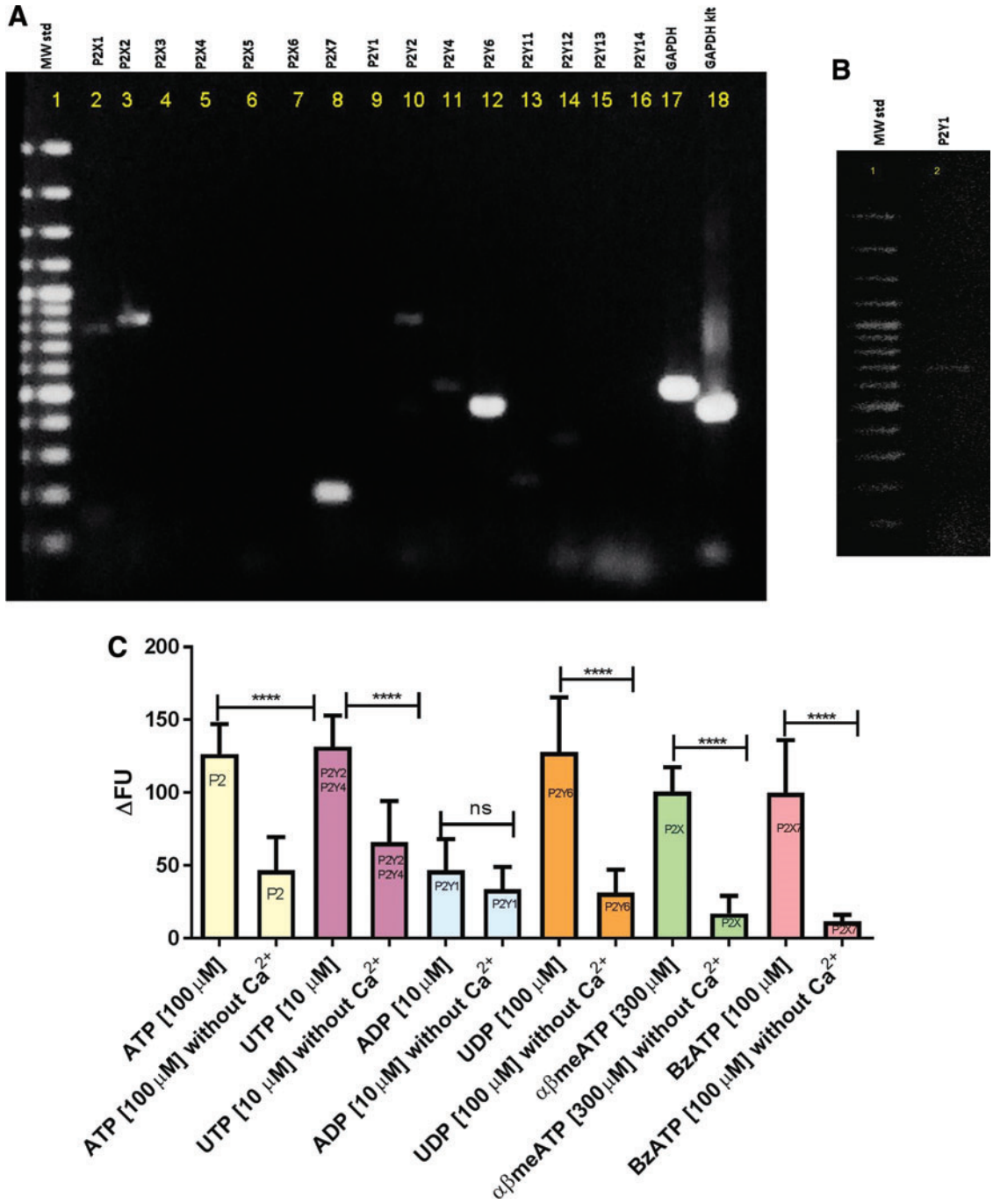

FIG. 3. P2 receptors subtypes expressed in J774.G8 cell line. In **(A)** and **(B)**, the columns correspond to cDNA fragments of subtypes described above them. MW std means molecular weight standard. Samples were run on 2% agarose gels and stained with ethidium bromide. Representative images from two experiments. In **(C)**, increase of intracellular calcium induced by agonists of P2 receptors. Cells stained with 2 μM of Fluo-4 were stimulated with ATP [100 μM], UTP [10 μM], ADP [10 μM], UDP [100 μM], $\alpha\beta$meATP [300 μM], and BzATP [100 μM], and were monitored for 90 sec. Data are presented as Mean ± SDM of three independent experiments performed in triplicate. Data analyses were made using one-way ANOVA and Holm–Sidak post-test; $P < .05$. Asterisks indicates significant differences in relation to the respective group without Ca^{2+}. (ns) means not significant. The receptors named inside bars correspond to subtypes activated by agonist used. Color images available online at www.liebertpub.com/jmf

cell line expresses P2X and P2Y receptors, such as P2Y2 and P2Y4, activated by UTP, P2Y1, and P2Y6, which were activated by ADP and UDP, respectively. Cells also express P2X7 that promoted calcium response after stimulation with BzATP (P2X7 selective agonist). ATP and $\alpha\beta$meATP, nonselective agonists, were used for observing general P2 and P2X receptor calcium responses, respectively. These results confirm the expression of P2 receptors observed in RT-PCR, including UTP-activated P2Y receptors, that is, P2Y2 and P2Y4, which are our target subtypes.

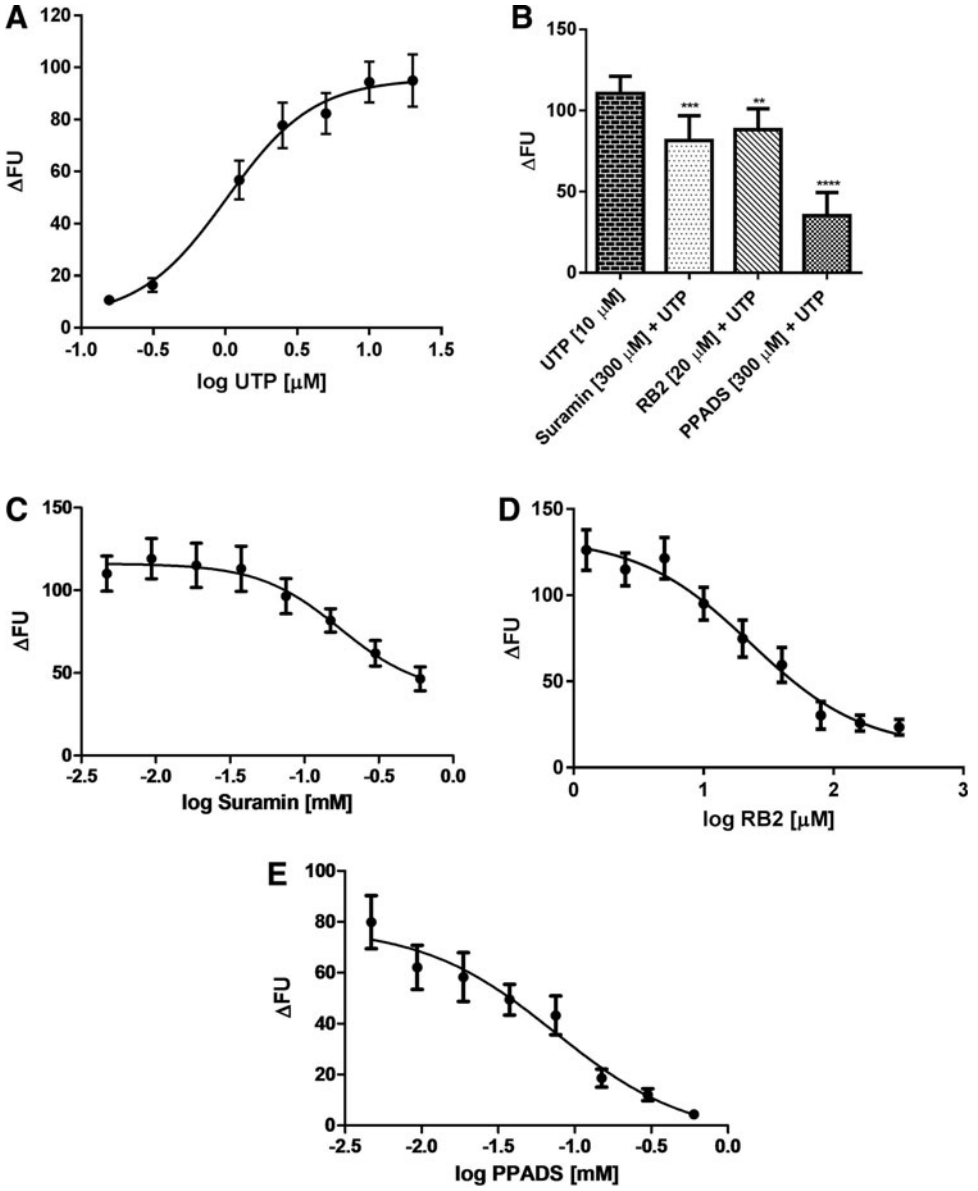

FIG. 4. Calcium mobilization induced by UTP can be partially inhibited by P2Y2 and P2Y4 nonselective antagonists. UTP concentration–response curve was obtained from J774.G8 cells stained with 2 μM of Fluo-4. Data are presented as Mean ± SEM of three independent experiments performed in triplicate **(A)**. J774.G8 cells stained with 2 μM of Fluo-4 were pretreated with Suramin [300 μM], RB2 [20 μM], and PPADS [300 μM] for 30 min. Cells were then stimulated with UTP [10 μM] and monitored for 90 sec. Data are presented as Mean ± SDM of three independent experiments performed in triplicate. Data analyses were made using one-way ANOVA and Holm–Sidak post-test; $P < .05$. Asterisks indicates significant differences in relation to UTP **(B)**. J774.G8 cells were pretreated with increasing concentrations of antagonists Suramin **(C)**, RB2 **(D)**, and PPADS **(E)** during 30 min, then were stimulated with UTP [10 μM]. Data are presented as Mean ± SEM of four independent experiments performed in triplicate.

Interestingly, we observed that all agonists, with the exception of ADP, presented lower responses without extracellular calcium, possibly indicating the participation of store-operated calcium channels. In the case of ATP and $\alpha\beta$meATP, this response could be regarding ionotropic channels.

Characterization of UTP-induced calcium responses

Since our cells express UTP-activated receptors, that is, P2Y2 and P2Y4, they were stimulated with increasing concentrations of this agonist, to observe UTP response profile (Fig. 4A). The determined EC_{50} was of 1.021 μM. Next, we obtained the P2Y2 and P2Y4 antagonist profiles by performing an antagonism assay using Reactive-Blue 2 (RB2), Suramin, and PPADS.[2] As shown in Figure 4B, RB2, Suramin, and PPADS partly inhibited UTP-induced intracellular calcium mobilization. To observe the antagonistic profile of these inhibitors of P2Y2 and P2Y4 receptors, we pretreated cells with different Suramin, RB2, and PPADS concentrations and obtained IC_{50} of 173.3, 22.98, and 74.24 μM, respectively (Fig. 4C–E). We also observed that Suramin and RB2 partially block intracellular UTP-induced calcium mobilization.

After characterization of UTP calcium responses and P2Y2 and P2Y4 antagonist inhibition profile, we calculated the z′-factor of our assay. The z′-factor is a statistical parameter used to measure assay robustness. After applying the mathematical z′-factor equation, we obtained a value of 0.645. Therefore, the z′-factor of our assay, which is above 0.5, is within the optimal range ($z' \geq 0.5$). Taken together, these evaluated characteristics suggest that our protocol was appropriate for use.

Mini HTS application

Finally, we performed the screening of 100 natural product extracts, summarized in Table 2. Nine extracts

Table 2. Mini High-Throughput Screening Application with Extracts from Brazilian Plants: Inhibition Effect on Calcium Mobilization Induced by UTP through P2Y2 and P2Y4 Receptors

Sample	*ΔFU mean ± SDM*	*ΔFU of control (UTP) ± SDM*	*Sample*	*ΔFU mean ± SDM*	*ΔFU of control (UTP) ± SDM*
A2	84.00 ± 39.67	136.3 ± 22.41	F6	140.1 ± 15.32	131.1 ± 17.41
A3	96.11 ± 34.83	136.3 ± 22.41	F9	131.4 ± 19.00	131.1 ± 17.41
A4	93.64 ± 23.63	136.3 ± 22.41	G4	125.9 ± 22.93	131.1 ± 17.41
A5	121.50 ± 26.98	136.3 ± 22.41	G6	116.6 ± 34.57	131.1 ± 17.41
A6	114.70 ± 25.68	136.3 ± 22.41	H2	99.88 ± 26.96	131.1 ± 17.41
A7	118.20 ± 25.61	136.3 ± 22.41	H7	120.60 ± 26.54	131.1 ± 17.41
A8	114.60 ± 28.77	136.3 ± 22.41	H8	128.60 ± 30.45	131.1 ± 17.41
A9	103.80 ± 19.04	136.3 ± 22.41	H9	122.50 ± 24.30	131.1 ± 17.41
A10	102.00 ± 37.68	136.3 ± 22.41	H10	121.00 ± 22.07	131.1 ± 17.41
A11	90.91 ± 23.84	136.3 ± 22.41	H11	118.80 ± 25.45	131.1 ± 17.41
B2	103.20 ± 31.93	136.3 ± 22.41	JA2[a]	52.97 ± 29.48***	117.2 ± 21.81
B3	96.54 ± 30.47	136.3 ± 22.41	JA4	87.34 ± 17.22	117.2 ± 21.81
B4	98.59 ± 32.42	136.3 ± 22.41	JA5	69.73 ± 12.95**	117.2 ± 21.81
B5	104.40 ± 31.61	136.3 ± 22.41	JA8	88.61 ± 17.55	117.2 ± 21.81
B6	112.00 ± 32.86	136.3 ± 22.41	JA9	101.4 ± 16.28	117.2 ± 21.81
B7	101.30 ± 42.64	136.3 ± 22.41	JA10	106.8 ± 20.23	117.2 ± 21.81
B8	101.10 ± 45.63	136.3 ± 22.41	JA11	113.6 ± 21.24	117.2 ± 21.81
B9	79.80 ± 32.15	136.3 ± 22.41	JB2	68.70 ± 25.67**	117.2 ± 21.81
B10	83.52 ± 38.49	136.3 ± 22.41	JB3	103.3 ± 22.15	117.2 ± 21.81
B11	81.18 ± 39.85	136.3 ± 22.41	JB4	83.31 ± 15.39	117.2 ± 21.81
C2	97.98 ± 28.62	136.3 ± 22.41	JB5	95.08 ± 23.12	117.2 ± 21.81
C3	86.42 ± 33.81	136.3 ± 22.41	JB7	72.98 ± 27.89*	117.2 ± 21.81
C4	84.02 ± 32.69	136.3 ± 22.41	JB9	94.51 ± 15.21	117.2 ± 21.81
C5	89.70 ± 32.30	136.3 ± 22.41	JC4	80.44 ± 21.36	117.2 ± 21.81
C7	128.40 ± 35.47	136.3 ± 22.41	JC7	104.4 ± 23.61	117.2 ± 21.81
C8	114.90 ± 34.39	136.3 ± 22.41	P2B6	88.47 ± 7.95**	133.0 ± 24.81
C9	123.10 ± 23.62	136.3 ± 22.41	P5C1	90.14 ± 19.28*	133.0 ± 24.81
C10	126.30 ± 16.57	136.3 ± 22.41	P5F3	94.78 ± 26.75*	133.0 ± 24.81
C11	129.20 ± 16.79	136.3 ± 22.41	RA2	47.18 ± 72.71	99.71 ± 62.55
D2	130.90 ± 16.78	136.3 ± 22.41	RA3[b]	11.15 ± 10.11**	99.71 ± 62.55
D3	121.60 ± 21.87	136.3 ± 22.41	RA4	126.00 ± 41.12	99.71 ± 62.55
D4	128.00 ± 20.63	136.3 ± 22.41	RA5	121.10 ± 47.06	99.71 ± 62.55
D5	119.90 ± 26.90	136.3 ± 22.41	RA6	92.82 ± 38.43	99.71 ± 62.55
D6	101.00 ± 36.11	136.3 ± 22.41	RA7	64.31 ± 19.61	99.71 ± 62.55
D7	98.50 ± 21.90	136.3 ± 22.41	RA8	101.40 ± 34.38	99.71 ± 62.55
D8	111.70 ± 28.66	136.3 ± 22.41	RA9	105.90 ± 26.27	99.71 ± 62.55
D9	123.80 ± 11.76	136.3 ± 22.41	RA10	100.80 ± 39.60	99.71 ± 62.55
D10	109.60 ± 21.55	136.3 ± 22.41	RA11	117.30 ± 30.47	99.71 ± 62.55
D11	127.00 ± 36.18	136.3 ± 22.41	RB2	51.19 ± 27.55	99.71 ± 62.55
E2	117.40 ± 20.61	142.0 ± 18.04	RB3[c]	19.96 ± 31.03**	99.71 ± 62.55
E3	103.20 ± 18.34	142.0 ± 18.04	RB4	106.90 ± 32.81	99.71 ± 62.55
E4	102.30 ± 47.03	142.0 ± 18.04	RB5	85.30 ± 15.44	99.71 ± 62.55
E5	100.10 ± 36.62	142.0 ± 18.04	RB6	110.50 ± 37.21	99.71 ± 62.55
E6	95.00 ± 36.32	142.0 ± 18.04	RB7	110.80 ± 36.12	99.71 ± 62.55
E7	91.00 ± 41.39	142.0 ± 18.04	RB8	97.02 ± 29.97	99.71 ± 62.55
E8	115.30 ± 41.97	142.0 ± 18.04	RB9	114.70 ± 37.33	99.71 ± 62.55
E9	108.00 ± 45.52	142.0 ± 18.04	RB10	91.47 ± 35.80	99.71 ± 62.55
E10	129.80 ± 45.93	142.0 ± 18.04	RB11	99.84 ± 51.68	99.71 ± 62.55
E11	91.27 ± 30.64	142.0 ± 18.04	RC2	60.97 ± 23.75	99.71 ± 62.55
F2	132.1 ± 23.55	131.1 ± 17.41	RC3	59.46 ± 21.70	99.71 ± 62.55

J774.G8 cells labeled with 2 μM Fluo-4 were pretreated with extracts [50 μg/mL] for 30 min. Then, cells were stimulated with UTP [10 μM] and were monitored for 90 sec. Mean ± SDM of three independent experiments. Data analyses were made using one-way ANOVA and Tukey post-test; $P < .05$.

Asterisks Indicates significant differences in relation to UTP.

[a]*Joannesia princeps* Vell. stem extract.

[b]*Peixotoa* A. Juss. flower extract.

[c]*Peixotoa* A. Juss. leaf extract.

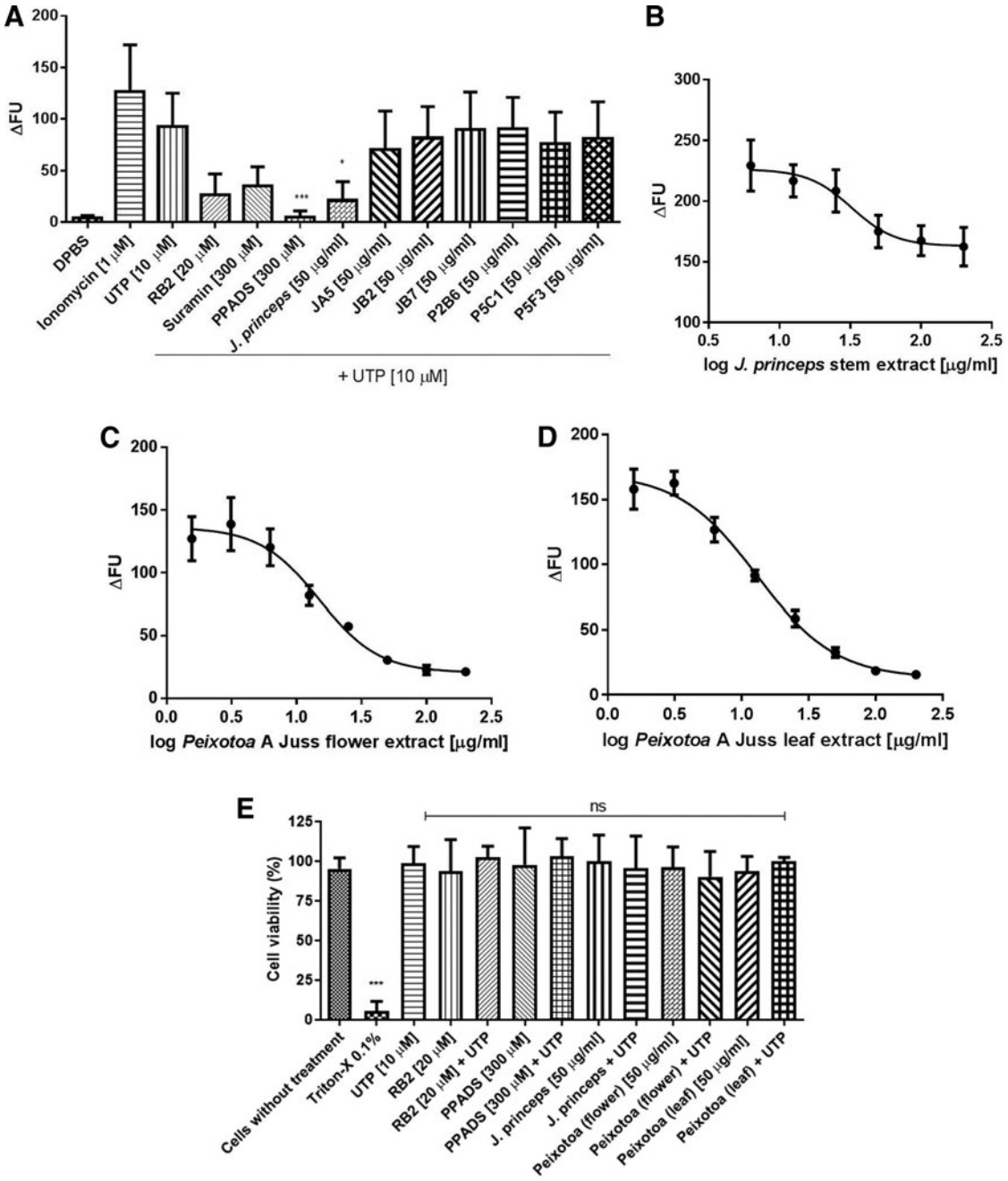

FIG. 5. *J. princeps* and *Peixotoa* A. Juss extracts partially inhibited calcium responses induced by UTP. Peritoneal macrophages stained with 2 μM of Fluo-4 were pretreated with RB2 [20 μM], Suramin [300 μM], PPADS [300 μM], and extracts [50 μg/mL] during 30 min. Then, cells were stimulated with UTP [10 μM] and monitored for 90 sec. Data are presented as Mean ± SDM of three independent experiments performed in triplicate. Data analyses were made using one-way ANOVA and Holm-Sidak post-test; $P < .05$. (*) indicates significant differences in relation to UTP **(A)**. J774.G8 cells were pretreated with increasing concentrations of candidate extracts: *J. princeps* **(B)**, *Peixotoa* A. Juss (flower) **(C)** and *Peixotoa* A. Juss (leaf) **(D)** during 30 min, then were stimulated with UTP [10 μM]. Data are presented as Mean ± SEM of four **(B)** and three **(C, D)** independent experiments performed in triplicate. J774.G8 cells were pretreated with RB2 [20 μM], PPADS [300 μM], and *J. princeps* and *Peixotoa* A. Juss extracts [50 μg/mL] in the presence or absence of UTP [10 μM] during 1 h. Then, cell viability was assessed by MTT technique **(E)**. Data are presented as Mean ± SDM of three independent experiments performed in triplicate. Data analyses were made using one-way ANOVA and Holm-Sidak post-test; $P < .05$. Asterisks indicates significant differences in relation to UTP. ns, not significant.

partially inhibited calcium mobilization induced by UTP, namely: JA2 (*Joannesia princeps* Vell. - stem), JA5, JB2, JB7, P2B6, P5C1, P5F3, RA3 (*Peixotoa* A. Juss - flower), and RB3 (*Peixotoa* A. Juss - leaf). The same experiment was repeated using peritoneal macrophages from mice, a primary cell that expresses P2R (Fig. 5A). When using these cells, we observed that only *J. princeps* Vell. extract was able to significantly inhibit UTP-calcium responses. The mean of *J. princeps* Vell in peritoneal macrophages was less than in J774.G8 cells (Table 2) (21.07 ± 18.24 compared with 52.97 ± 29.48), which could reflect differences between cell lines and primary cells. *Peixotoa* A. Juss extracts (RA3 and RB3) were not been tested in peritoneal macrophages due to sample scarcity. Subsequently, cells were treated with increasing concentrations of *J. princeps* Vell. and *Peixotoa* A. Juss (flower and leaf) extracts, and the obtained IC_{50} were equal to 32.32, 14.99, and 12.98 μg/mL, respectively (Fig. 5B–D). Aiming to verify if calcium response inhibition was the result of possible extract cytotoxicity, viability tests were performed using the MTT technique. As observed in Figure 5E, only leaf extract caused a slight decrease in cell viability, but although nonsignificant. Taken together, we characterized *J. princeps* Vell. and *Peixotoa* A. Juss extracts as potential antagonist candidates for UTP-stimulated receptors.

DISCUSSION

P2Y2 and P2Y4 are purinergic receptors physiologically activated by UTP, which promote an increase of intracellular calcium through PLCβ/IP_3 when activated.[2] They are expressed in many cell types, such as epithelial cells and leukocytes, and even in organs, such as the brain, heart, kidneys, liver, spleen, and muscle,[3] where they play important roles. Likewise, these P2Y receptors are associated with certain diseases, such as cystic fibrosis, dry eye disease, Alzheimer, and cancer.[2,4,5] Thus, pharmaceutical companies have been developing drugs that act on these P2Y receptors, especially P2Y2, to treat these related diseases.[37–39] However, the lack of selective ligands that act on P2Y2 and P2Y4 receptors compromises the discovery of new functions associated with these receptors, which can be solved by the search for new antagonist compounds.[17] Therefore, the aim of this study was to improve a methodology for the detection of intracellular calcium mobilization to discover new molecules with antagonistic activity on UTP-activated P2Y receptors. For this reason, we established a protocol to evaluate intracellular calcium mobilization.

First, the calcium indicator concentration was optimized. Fluo-4 is widely applied in HTS assays of metabotropic receptors that induce an increase of intracellular calcium.[32] We chose 2 μM as the final concentration, due to the apparent homogenous cellular loading in cells and the fact that no significant differences were observed compared with other concentrations. In addition, it is important to use the lowest concentration that results in an adequate signal, thereby reducing reagent consumption and expenses during screening.[31] Gee *et al.* also demonstrated that the use of the lowest concentrations of calcium indicators can reduce the buffer effect on calcium, as well as minimize the levels of toxic products, such as formaldehyde and acetic acid, which are produced through the hydrolysis of acetoxymethyl esters.[40] Furthermore, using this concentration, we demonstrated that cells were able to react to different stimuli, which increase intracellular calcium levels in a concentration-dependent manner, such as P2R agonists, including ATP and UTP. The EC_{50} found for ATP (11 μM) is slightly higher than that reported by Li *et al.* when they performed calcium experiments using the FlexStation III (1.1 μM and 58 nM for HEK293 and CHO-K1 cells, respectively).[32] This could be a sum of responses from various ATP-activated P2R, such as P2Y2, P2Y11, P2X1, P2X2, and P2X7 subtypes. The latter could have displaced the EC_{50}, since its activation occurs at concentrations higher than 100 μM. Meanwhile, the EC_{50} found for ionomycin (103 nM) was lower than that reported by Valentin *et al.* (2011), that is, 1.5 μM when they performed a calcium assay using a microplate reader with human endothelial cells from the umbilical vein (HUVEC).[41]

We also observed some expected calcium signal characteristics, such as the uniformity of the basal signal, a signal peak after the ATP stimulus, indicating an increasing of $[Ca^{2+}]_i$ and its return to basal levels after a response time, which, in calcium events, usually occurs in seconds. In addition, a decrease of calcium responses in the presence of EGTA, a calcium chelating agent, was also observed. A similar response profile was obtained through calcium imaging (data not shown). Ionomycin stimulation also induced an increase in intracellular calcium levels, although with no return to basal levels during the experiment period (90 sec). However, a longer measurement time concerning the ionomycin response could be required to observe this return. However, increasing the well-measurement time could lead to a decrease in the number of samples to assess in an established period of time. Therefore, we opted to maintain the set time at 90 sec.

Moreover, we calculated the z′-factor, a measure for assay robustness, obtaining a value of 0.645, indicating that our protocol was adequate for use. Ito *et al.* performed an HTS campaign to identify novel P2Y6R antagonists using P2Y6-1321N1 cells, and obtained a z′-factor of 0.80,[28] whereas Valentin *et al.* (2011) used primary cells (HUVEC) to perform a HTS based on calcium measurement and obtained a z′-factor of 0.60.[41] These findings suggest that the use of transfected cells could improve the performance of the assay. However, the use of transfected cells can add costs to the screening. Thus, we suggest that it be applied in a second research moment, to verify hit responses.

J774.G8 murine macrophage cell line expresses P2Y activated by UTP, targets of the screening assay proposed herein. Using the RT-PCR technique, we detected cDNA for P2X1, P2X2, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, and P2Y12 subtypes. These P2R subtypes have been described in macrophages in many studies applying molecular and pharmacological methods.[42–45] Furthermore, all are associated with intracellular calcium increase signaling pathways

through the opening of a nonselective cation ion channel (P2X) or PLCβ/IP_3 (P2Y), with the exception of P2Y12. Therefore, we characterized these subtypes using the following agonists: ATP, a nonselective P2 receptor agonist; UTP, a P2Y2 and P2Y4 receptor agonist; ADP for the P2Y1 receptor; UDP for the P2Y6 receptor; BzATP, a selective P2X7 agonist; and $\alpha\beta$meATP for P2X receptors. These data showed the functional expression of these receptors. Calcium responses decreased in the absence of extracellular calcium, suggesting the participation of store-operated calcium channels and ionotropic receptors in the amplification of intracellular calcium mobilization.[46]

We also obtained the profiles of P2Y2 and P2Y4 agonist and antagonists. J774.G8 cells were stimulated with increasing concentrations of UTP and the EC_{50} obtained was 1.021 μM. It was similar to that cited by King *et al.*: 1.1 μM for P2Y2 and 0.20 μM for P2Y4 receptors.[47] In relation to the antagonists, we observed that Suramin and RB2 partially block intracellular UTP-induced calcium mobilization, whereas PPADS was more effective. King *et al.* cited that PPADS (100 μM) and RB2 (100 μM) inhibited approximately 30% of P2Y4R, whereas Suramin is ineffective.[47,48] von Kügelgen cited that P2Y2 is blocked by Suramin and RB2, but not by PPADS.[48] We also hypothesize that the participation of store-operated calcium channels could have influenced their inhibition at some level.

It is worth mentioning that our study is one of the pioneers in the search for novel antagonists using natural products, which present significant diversity concerning number of species and chemical structures, both acquired over millions of years of evolution. Thus, we tested 100 extracts from a natural library at a cutoff concentration established at 50 μg/mL. First, nine extracts demonstrated partial inhibition of UTP-induced calcium responses in J774.G8 cells. After a repetition of this experiment with a primary cell, that is, peritoneal macrophages, which express P2Y2 and P2Y4 receptors,[44] we found that only *J. princeps* Vell. extract inhibited this activity. Two other extracts (*Peixotoa* A. Juss leaf and flower) were not tested in these cells due to sample scarcity. However, aiming at observing the occurrence of this inhibition at higher doses, we treated J774.G8 cells with increasing concentrations of the assessed extracts, and observed that calcium responses were inhibited in a concentration-dependent manner, suggesting potential ability to block UTP-activated P2Y receptors. We then asked if inhibition could be due to cell toxicity. To answer this question, we performed a cell viability assay applying the MTT technique and observed no cytotoxicity.

J. princeps Vell. is a tree approximately 20 m in height belonging to the Euphorbiaceae family. In Brazil, it is found in the Atlantic Rainforest and Caatinga biomes, and can also be found in some regions of Africa and Asia. It is popularly known as ''cotieira'' in Brazil, with economic value in landscaping, wood extraction, and medicinal applications.[49,50] The only part of the plant used for medicinal purposes is its seed. The seed oil is used as a laxative, whereas its extract displays antihelminthic activity.[50] Recently, Donato-Trancoso *et al.* demonstrated that *J. princeps* Vell. seed oil can improve cutaneous wound closure in an experimental mice model.[51] Sousa *et al.* cite that the use of *J. princeps* Vell. in folk medicine is indicated to treat menstrual and digestive disorders, pernicious fever, microbial diseases, syphilis, scrofulous, and swelling.[50] In relation to chemical profile, Achenbach and Benirschke, in their work using *J. princeps* Vell. root bark, stem bark, and leaves, demonstrated that this species presents compounds belonging to the following classes: sesquiterpenes, bis-sesquiterpenes, diterpenes, triterpenes, and steroids.[52] Waibel et al. demonstrated that the methanol extract of the seeds presents secondary metabolites, such as lignans, neolignans, and sesquineolignans.[53] Recently, Camero *et al.* isolated 28 secondary metabolites from *J. princeps* Vell. leaves for the first time, including gallic acid derivatives, gallotannins, flavonoids, α-ionones, glycosylated monoterpenes, lignan, and other phenolic derivatives.[54] However, scarce reports in the literature are available concerning *J. princeps* Vell. stem phytochemistry, which was used in this work.

Peixotoa A. Juss is a bush belonging to the botanical family Malpighiaceae, which, in Brazil is represented by 32 genera and 300 species. The *Peixotoa* A. Juss genus comprises shrubs, subshrubs, and creepers, which grow on rocky and earthy substrates. It is a native plant presenting widespread distribution in Brazil.[55] Only one species belonging to this botanical genus presents folk medicinal use reported in the literature. The roots of *Peixotoa cordistipula* A Juss, popularly known as ''joão-da-costa'' are used to treat spine disorders in the west-central region of Brazil.[56] The scarcity of information about this botanical genus is also reflected in the phytochemistry field, since no studies are available in the literature concerning the characterization of its metabolites. Some information about the chemical profile of other Malpighiaceae family members is, however, available. For example, De Frias and collaborators evaluated the chemical profile of leaves of *Banisteriopsis anisandra* A. Juss., belonging to the *Banisteriopsis* genus, and found alkaloids, anthraquinones, flavonoids, and tannins.[57] Andrade *et al.* analyzed the chemical profile of another Malpighiaceae family member, *Byrsonima crassifolia*, which belongs to the *Byrsonima* genus, and found that barks produce the following secondary metabolites: flavonoids, anthraquinones, reducing sugars, phenolic compounds, tannins, and cardiotonic glycosides.[58] Therefore, based on the presence of metabolites in these two genera, it is possible that *Peixotoa A. Juss* may also synthesize some of these substances.

Despite this information, scarce studies demonstrating the potential medicinal use of other plant parts, such as leaf, stem, root, and flower, are available. In the present study, we demonstrate the potential antagonistic activity of a stem extract from *J. princeps* Vell. and flower and leaf extracts from *Peixotoa* A. Juss on P2Y2 and P2Y4 receptors, inhibiting UTP-induced calcium responses in a dose–concentration manner without causing cytotoxic effects. However, it is important to understand and characterize the active molecules from these extracts responsible for the observed inhibitory effect, which will be carried out in further studies by our group.

In conclusion, our results point to the discovery of potential antagonists for P2Y2 and P2Y4 receptors from Brazilian botanical species *J. princeps* Vell. and *Peixotoa* A. Juss. This discovery may significantly contribute to purinergic pharmacology and the application of P2 receptor antagonists in clinical therapy.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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- **Situação:** Submetido para a Revista Phytotherapy (FI 3,349)

- **Descrição:** Nesse artigo foi caracterizada a atividade antagonista do extrato de galhos de *Joannesia princeps* Vell. através da técnica de mensuração de cálcio intracelular. Foi demonstrado que o extrato inibiu a resposta de cálcio proveniente da ativação dos receptores P2Y2 e P2Y4, apesar de o mesmo ter sido capaz de diminuir parcialmente a mobilização de cálcio induzida pelo UDP. Observamos também que a atividade inibitória não foi causada por um efeito *quenching*, e parece não ter sido afetada por canais de cálcio operados em estoque. Por fim, demonstramos que ambos os extratos, metanólico e etanólico, de *J. princeps* Vell. apresentaram efeitos similares sobre a inibição da mobilização de cálcio, bem como não afetaram a viabilidade das células após 24h de tratamento. Esses dados sugerindo a existência de uma possível seletividade entre os receptores P2 e podem nortear o desenvolvimento de novos antagonistas para os receptores P2Y2 e P2Y4. Esse trabalho atende aos objetivos específicos 2 e 3 da tese.

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The antagonistic effect of *Joannesia princeps* Vell. stem extract on UTP-activated receptors

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Abstract

P2Y2 and P2Y4 are purinergic receptors physiologically activated by UTP. They are expressed in organs such as lung, heart, skeletal muscle, spleen, and kidneys, as well as in immune system cells. Once activated, they promote calcium mobilization and act on ion flux regulation. These receptors are associated with several diseases, such as inflammation and cancer, making them important therapeutic targets. The scarcity of selective antagonists for these receptors limits their application in clinical therapy, encouraging the search for new molecules presenting antagonistic activity. In the present study, we characterized the antagonistic activity of the *Joannesia princeps* Vell. stem extract. Using calcium measurement technique, we demonstrated that the extract inhibits calcium response for P2Y2 and P2Y4, but also P2Y6 receptor suggesting the existence of selectivity among P2 receptors. We also observed that inhibitory activity was not due to the quenching effect, and was not affected by stock-operated calcium channels. Finally, we demonstrated that both methanolic and ethanolic *J. princeps* Vell. extracts are able to reduce UTP-induced calcium responses and do not cause cytotoxicity. Our data suggest that *J. princeps* Vell stem extracts act as an UTP-activated receptors receptor antagonist.

Key words: P2Y2, P2Y4, UTP, antagonist, calcium, natural products

1 Introduction

Purinergic receptors are plasma membrane receptors activated by extracellular nucleotides. They are classified into two families: P1 and P2 receptors (P1R and P2R), which are activated by adenosine and its derivated-nucleotides, respectively. P2R is also subdivided into two classes: ionotropic P2X and metabotropic P2Y (Abbracchio & Burnstock, 1994).

P2Y receptors comprise seven transmembrane domains (TM1-7), in addition to an intracellular C-terminal and extracellular N-terminal. Currently, eight P2Y receptors subtypes are described. P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 are coupled to Gq protein, although the latter can also couple to the Gs protein, while PY12-P2Y14 are coupled to the Gi protein. These receptors can be activated by different extracellular nucleotides, including ADP, ATP, UDP, UDP-glucose and UTP, which will be addressed in this study (Jacobson et al., 2012).

P2Y2 is a receptor physiologically activated by UTP and ATP and has aroused much therapeutic interest, first, due to its expression in a variety of biological tissues, including the lung, heart, skeletal muscle, spleen and kidneys, as well as immune system cells, such as lymphocytes and macrophages (von Kügelgen, 2006; Von Kügelgen & Hoffmann, 2016). Second, this receptor plays important roles related to the ion flow in epithelial cells, which can contribute significantly to the treatment of cystic fibrosis and dry eye disease (Jacobson & Boeynaems, 2010). In view of this, an ophthalmic solution that mimics UTP has been developed and is able to attenuate the dry eye disease symptoms. This drug, called Diquafosol, is marketed only in Japan, South Korea, Thailand and Vietnam (Santen, 2017). In addition, several studies have indicated that P2Y2 is involved in the development of certain diseases, such as neuropathic pain (Li et al., 2014; Magni et al., 2015), cancer (Chadet et al., 2014; Choi et al., 2013; Jin et al., 2014; Li et al., 2013; Xie et al., 2014) and inflammation (Ayata et al., 2012; Degagné

et al., 2009; Eun et al., 2014; Inoue et al., 2008; Vanderstocken et al., 2010; Zhang et al., 2010), thus, constituting an adequate therapeutic target.

P2Y4 is another UTP-activated receptor. It is expressed in the gut, brain, lung, heart, among other cell types (Rafehi & Müller, 2018). Because it is structurally and pharmacologically similar to the P2Y2 receptor and sometimes expressed in the same cell types, the characterization of P2Y4 physiological effects has become a significant challenge (Rafehi & Müller, 2018; von Kügelgen, 2006). However, P2Y4 is known to also act on ion regulation in the intestine and cochlea, besides promoting the inhibition of spontaneous longitudinal ileum muscle contractile activity (Ghanem et al., 2005; Kim et al., 2010; Zizzo et al., 2012).

The scarcity of selective antagonists for these receptors limits their application in clinical therapy, encouraging the search for new molecules with antagonistic activity. Natural products are noteworthy in the scenario for the search of new drugs, due to their enormous structural and chemical diversity, acquired over thousands of years of evolution. These characteristics inspire new discoveries to this day, in the chemistry, biology, and medicine fields, making them an important source for the discovery of novel drugs. In addition, natural products and their derivatives present higher hit rates during the high throughput screening (HTS) process than compounds obtained from synthetic libraries (Chen et al., 2017).

In a previous study, we demonstrated for the first time the potential antagonistic activity of the crude extracts of *Joannesia princeps* Vell. stems after a mini-screening campaign to discover new P2Y2 and P2Y4 receptor antagonists. *J. princeps* Vell. extracts were able to inhibit concentration-dependent UTP-induced intracellular calcium mobilization without promoting cytotoxicity (Ferreira et al., 2018). *J. princeps* Vell. is a botanical species belonging to the Euphorbiaceae family, popularly known as "cotieira". This species is found in Brazil and in some African and Asian continent regions. Some literature reports have indicated that this species presents laxative, anti-parasitic and antimicrobicide activities (Donato-Trancoso et al., 2014; Sousa et al., 2007). In this present study, we demonstrate that calcium inhibition is selective for UTP-activated receptors, and was not caused by quenching effects or cell death.

2 Material and Methods

2.1 Reagents

ADP, ATP, UDP, UTP, PPADS, RB2, Suramin, Triton-X, DMEM and RPMI culture mediums, NaCl, KCl, Na_2HPO_4, KH_2PO_4, $MgCl_2$, $CaCl_2$, Hepes, Glucose, DMSO, MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium, Probenecid and Ionomycin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). 2-thio-UTP and MRS 4062 were purchased from Tocris (Bristol, UK). Fetal bovine serum was obtained from Gibco (Oklahoma, USA). Fluo-4 AM was obtained from Life Technologies (California, USA). YO-PRO-1 was obtained from Molecular Probes (Oregon, USA).

2.2 Extracts

J. princeps Vell. stems were collected at Linhares, in the state of Espírito Santo, Brazil. The samples were crushed and extracted with a 1:1 mixture of dichloromethane-methanol. The crude extract was deposited under voucher number EX294 at the Natural Product Chemistry Laboratory Bioprospecting Platform. Other *J. princeps* Vell. stem and leaf samples were obtained from Maricá, in the state of Rio de Janeiro, Brazil. The plant was oven dried (40°C) for one weekin an air circulation oven and reduced to small fragments. The dried plant materials were submitted to a static extraction with ethanol for about seven days and dried under reduced pressure. They were resuspended in a Dulbecco's PBS solution containing 0.5% DMSO for the experiments. All collections of botanical materials were authorized by the Chico Mendes Institute for Biodiversity Conservation, an environmental agency of the Brazilian government.

2.3 Cell culture

J774.G8 cells were cultivated with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere. The cellular medium was changed twice a week and cell density was adjusted to $2x10^6$ cells per 150 cm^2 cell culture flask (Corning, New York, USA). Cell viability was measured by trypan blue exclusion and experiments were conducted only when viability was above 90%.

2.4 Calcium assays

J774.G8 cells were plated on a 96-well black-wall and clear-bottom plate (Corning, New York, USA), at a concentration of $2x10^5$ cells/well. They were maintained in culture with DMEM supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24h. Then, the medium was replaced by 100 μl of Dulbecco's PBS (DPBS) (136 mM NaCl, 2.68 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5.55 mM Glucose and 2.5 mM Probenecid – pH 7.4) containing [2 μM] Fluo-4 AM and cells were incubated for 60 minutes at 37ºC in a 5% CO_2 atmosphere. Subsequently, the wells were washed three times with 200 μl DPBS and each well received a final volume of 150 μl DPBS. Afterwards, cells were treated with PPADS [300 μM], Suramin [300 μM], Reactive Blue-2 (RB2) [20 μM] and *J. princeps* Vell. extracts [50 μg/mL] for 30 minutes. Intracellular calcium mobilization was determined using a FlexStation III

(Molecular Devices, California, USA) in real time during 90 seconds, at 485 nm and 525 nm wavelengths for excitation and emission, respectively. The agonists were added at 20 seconds and readings were performed every 1.52 seconds, totaling 60 readings/well. Calcium mobilization was quantified as ΔFU (the difference between the fluorescence peak and basal fluorescence) (Hansen & Bräuner-Osborne, 2009).

2.5 Dye uptake assay

J774.G8 cells were plated on a 96-well black plate (Corning, New York, USA), at a concentration of 2x10^5 cells/well. They were maintained in culture with DMEM supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24h. Then, the medium was replaced by an extracellular saline solution (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4). Subsequently, cells were treated with Brilliant Blue G (BBG) [200 nM] and the *J. princeps* Vell. extract [50 μg/mL] for 30 minutes. Then, cells were stimulated with ATP [5 mM] for 15 minutes, in order to allow the opening of the membrane pore associated with the P2X7 receptor. Next, the cationic dye YO-PRO-1 was added to each well at a concentration of 2 μM, and after 3 minutes, fluorescence signals were determined using a FlexStation III equipment (Molecular Devices, California, USA) at 488 nm and 509 nm for excitation and emission, respectively. Cells treated with Triton-X [0.1%] were considered the maximum dye uptake control.

2.6 Animals

Animal experiments followed all animal experimentation ethical principles as determined by the Brazilian College of Animal Experimentation. All experiments were approved by the Ethics Committee on the use of Animals from the Oswaldo Cruz, Institute under identification code L-037/2017 (approved on November 24, 2017). Healthy male Swiss mice weighing between 30 and 40g were obtained from the Oswaldo Cruz Central Bioterium. The animals were housed under a 12h/12h light and dark cycle conditions with free access to food and water.

2.7 Peritoneal macrophage isolation

Animals were euthanized by CO_2 asphyxiation. Subsequently, 10 mL of cold PBS were injected into the peritoneal cavity. Peritoneal cells were collected and macrophages were isolated by centrifugation at 1,500 RPM for 10 minutes. Cell viability was analyzed by the trypan blue exclusion assay. Cells were plated on a 96-well black-wall and clear-bottom plate at a density of 4x10^5 cells/well and maintained in culture with RPMI supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 1h, to allow a pre-adhesion. Then, each well was washed with 200 μl of RPMI medium and peritoneal macrophages were maintained in culture with RPMI supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24h. Calcium assays were performed as previously described.

2.8 Cell viability measurement

J774.G8 cells were plated on a 96-well plate (Corning, New York, USA) at a concentration of 2x10^5 cells/well. They were maintained in culture with DMEM without phenol red supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24h. Next, cells were treated with extracts [50 μg/mL] and the RB2 [20 μM] antagonist for 1 or 24h. Untreated cells were considered as a positive viability control, while cells treated with Triton-X [0.1%] were considered as the negative control. After the treatments, the medium was replaced by 200 μL of DMEM without phenol red containing an MTT solution [100 μg/well]. The plate was incubated for 3h at 37°C in a 5% CO_2 atmosphere, and was then centrifuged at 1,500 RPM for 1 minute. Supernatants were collected and formazan crystals were dissolved in 100 μl of DMSO. Well absorbance was determined measured using a Spectramax 190 equipment (Molecular Devices, California, USA) at 570 nm.

2.9 High Performance Liquid Chromatography (HPLC) analysis

The qualitative analyses were carried out using an HPLC system consisting of a Shimadzu Liquid Chromatograph, LC-20ADXR pump equipped with a quaternary gradient valve, Shimadzu SPD-M20A diode array detector (DAD), Shimadzu SIL-30AC autosampler, CTO-20AC oven, DGU-20A5R degasser and a Shimadzu CBM 20-A communications bus module.

Methanol was added to the sample until reaching 20 mg/mL. The sample was then vortexed and sonicated for 10 minutes Subsequently, the sample was membrane filtered PVDF 0.22 μm and 20 µL were injected.

The *Joannesia princeps* chemical profile fingerprint was obtained using a Supelcosil C-18 column (5μm particle size, 250 mm X 4,6mm internal diameter) and oven temperature of 30^o C. Solvent system conditions were trifluoroacetic acid pH 2.5 and acetonitrile (95:5 v/v to 95:5 v/v, 90 minutes) with a mobile flow rate of 1.0mL/min with HPLC effluent monitoring at 254-365 nm by a DAD.

2.10 Data analysis

Each sample was analyzed in triplicate and all experiments were performed on at least three independent days. All data are presented as means $\pm$ standard deviation (SD). The D' Agostino and Pearson normality test was used in order to test for a Gaussian distribution. If data followed a Gaussian distribution, an appropriate parametric test was applied and, if not, an appropriate non-parametric test was used. Tests are specified in figure legends. P values of 0.05

or less were considered significant. Graphs and statistical analyses were performed on the GraphPad Prism version 7 (GraphPad Software, San Diego, California, USA).

3 Results

3.1 Selectivity of *J. princeps* Vell. extract regarding P2Y2 and P2Y4 receptors

In a previous study, we identified the potential antagonistic activity of a *J. princeps* Vell. extract, which inhibited UTP-induced calcium mobilization in the J774.G8 cell line and in peritoneal macrophages (Ferreira et al., 2018). These two cell types express UTP- activated P2Y2 and P2Y4. In the present study, our first aim was to verify if this extract could inhibit other P2 receptors. In order to verify whether the *J. princeps* extract is able to inhibit P2 receptors in a promiscuous manner, we stimulated J774.G8 cells with two non-selective agonists, ATP and its derivative, BzATP. As in Figure 1A, the extract was unable to inhibit intracellular calcium mobilization induced by these two agents. On the other hand, PPADS, a non-selective P2 receptor antagonist, significantly inhibited the calcium mobilization induced by these two agents, whereas RB2, a non-selective antagonist, reduced the calcium increase induced by BzATP only partially.

We then stimulated the pretreated cells with the *J. princeps* Vell. extract with ADP, in order to verify if it would be able to inhibit the calcium mobilization induced by the P2Y1 receptor. As shown in Figure 1B, the extract was unable to inhibit ADP-induced calcium mobilization, as well as RB2. Only the PPADS antagonist promoted a significant reduction in calcium levels.

We also verified whether *J. princeps* Vell. extract would be able to inhibit the P2Y6 receptor, by stimulating cells pretreated with this extract using UDP. As shown in Figure 1C, both the extract and RB2 partially reduced intracellular calcium levels after the UDP stimulus.

Finally, we tested whether the *J. princeps* Vell. extract would be able to inhibit the calcium mobilization induced by selective P2Y2 and P2Y4 receptor agonists, i.e., 2-thio-UTP and MRS 4062, respectively. As indicated in Figures 1D and 1E, the extract reduced intracellular calcium levels produced by these two agonists by half. The RB2 antagonist, however, reduced only the calcium levels induced by MRS 4062.

Since the cell line used in the study is well recognized regarding P2X7 receptor expression, we performed the final test to verify whether the *J. princeps* Vell. extract could influence the activity of this receptor. With this objective, we applied the cellular permeabilization technique, which is based on dye incorporation, such as YO-PRO-1, ethidium bromide, propidium iodide, among others, from the membrane pore formed in the plasma membrane of ATP-activated cells at millimolar levels (Alves et al., 2014; Di Virgilio et al., 2018). As indicated in Figure 1F, the extract was unable to inhibit the dye uptake induced by ATP. However, BBG, a P2X7 antagonist, partially reduced YO-PRO-1 uptake by the membrane pore.

Thus, these experiments demonstrate that the extract can only inhibit the calcium response from the two UTP- and UDP-activated receptors at a lower level, suggesting the existence of certain selectivity among P2 receptors.

3.2 Inhibition of *J. princeps* Vell. extract is not due to the quenching effect nor to stock-operated calcium channels

Although the *J. princeps* Vell. extract displayed inhibition of calcium responses from P2Y2 and P2Y4 receptors; we speculated whether this inhibition could be due to the sequestration of Fluo-4 fluorescence by molecules present in the extract. Thus, to verify if the decrease of the calcium levels was due to a reduction in the dye fluorescence, cells pretreated with the extract were stimulated with the calcium ionophore, ionomycin. As shown displayed in Figure 2A, cell extract treatment did not alter ionomycin-induced calcium fluorescence levels.

Another important issue raised was the possible influence of stock-operated calcium channels (SOC) on the extract inhibition response. As noted in Figure 2B, the extract significantly inhibited UTP-induced calcium mobilization. Surprisingly, the absence of calcium in the extracellular medium drastically decreased intracellular calcium levels promoted by UTP. In this situation, the SOC response appears to have been eliminated and both the extract and RB2 did not promote any basal UTP response inhibition.

Thus, we conclude that the calcium inhibition response is not due to sequestration of the calcium indicator fluorescence, nor is it affected by stock-operated calcium channels, suggesting the presence of a molecule present in the *J. princeps* Vell. extract responsible for this activity.

3.3 Effect of the ethanolic *J. princeps* Vell. extract on UTP-induced calcium responses

The extract tested up until now presents favorable characteristics for therapeutic applications. This extract was prepared using a methanol and dichloromethane mixture. However, this extraction is not usually the most convenient for herbal medicine approval. Thus, we speculated whether the ethanolic *J. princeps* Vell. extract would still present this same response profile concerning the P2Y2 and P2Y4 receptors. As shown in Figures 3A and 3B, the extract was able to significantly inhibit UTP-induced calcium mobilization in J774.G8 cells and peritoneal macrophages, as well as the RB2 and Suramin antagonists. We further tested whether another part of the *J. princeps* Vell. plant, leaves, would also exhibit this inhibitory activity. However, as indicated in Figure 3C, although the leaf sample decreased part

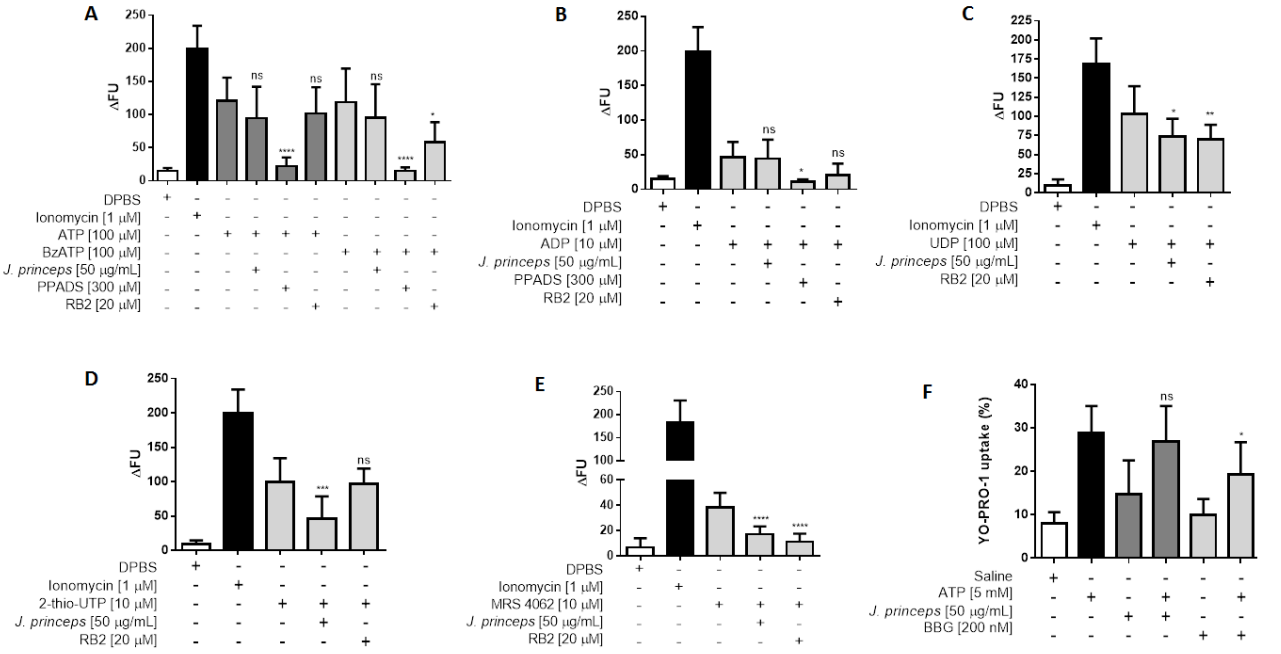

FIGURE 1: **The *J. princeps* Vell. extract only inhibits calcium mobilization induced by P2Y2 and P2Y4 agonists.** J774.G8 cells stained with 2 μM of Fluo-4 were pretreated with *J. princeps* Vell. stem extract [50 μg/mL], RB2 [20 μM] and PPADS [300 μM] for 30 minutes and then stimulated with agonists. Cells were stimulated with ATP [100 μM] or BzATP [100 μM] and monitored for 90 seconds. Data are presented as the means $\pm$ SD of three independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidak post-test; $p<0.05$. Asterisks indicate significant differences in relation to ATP or BzATP controls. (ns) non-significant means (A). Cells were stimulated with ADP [10 μM] and monitored for 90 seconds. Data are presented as the means $\pm$ SD of three independent experiments performed in triplicate. The data analysis was performed by a Kruskal-Wallis and Dunn's post-test; $p<0.05$. Asterisks indicate a significant difference in relation to ADP and (ns) non-significant means (B). Cells were stimulated with UDP [100 μM] and monitored for 90 seconds. Data are presented as the means $\pm$ SD of four independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidak post-test;$p<0.05$. Asterisks indicate a significant difference in relation to UDP (C). Cells were stimulated with 2-thio-UTP [10 μM] and monitored for 90 seconds. Data are presented as the means $\pm$ SD of three independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidakpost-test; $p<0.05$. Asterisks indicate a significant difference in relation to 2-thio-UTP and (ns) non-significant means (D). Cells were stimulated with MRS 4062 [10 μM] and monitored for 90 seconds. Data are presented as the means $\pm$ SD of three independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidak post-test;$p<0.05$. Asterisks indicate a significant difference in relation to MRS 4062(E). J774.G8 cells were pretreated with *J. princeps* Vell. stem extract [50 µg/mL] and BBG [200 nM] during 30 minutes. Then, cells were stimulated with ATP [5 mM] for a further 15 minutes and the YO-PRO-1 dye [2 µM] was added to the wells and fluorescence of cells was measured. Data are presented as the means $\pm$ SD of three independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidak post-test; $p<0.05$. Data were normalized as a percentage in function of the mean of units of arbitrary fluorescence (RFU) obtained by Triton-X, the maximum fluorescence control. Asterisks indicate a significant difference in relation to ATP and (ns) non-signficant means (F).

of the UTP-induced calcium response, this reduction was not statistically significant.

Therefore, we conclude that both *J. princeps* Vell. extracts, methanolic and ethanolic are able to reduce the calcium response from P2Y2 and P2Y4 receptor the activation.

3.4 *J. princeps* Vell. extract did not present cytotoxicity

A final criterion for the analysis of the *J. princeps* Vell. extract inhibition was to test whether this activity could be due to cell death. In the case of cell death, cell fluorescence would tend to decrease, causing an artifact in the measurement of calcium levels. In order to test this hypothesis, we treated the cells with the *J. princeps* Vell. methanolic and ethanolic extracts for 1h, an estimated period for the duration of the calcium recordings. As indicated in Figures 4A and 4B, both extracts did not cause cell death compared to untreated cell control. Finally, we also tested the cell viability profile after 24 hours of treatment with the extracts.

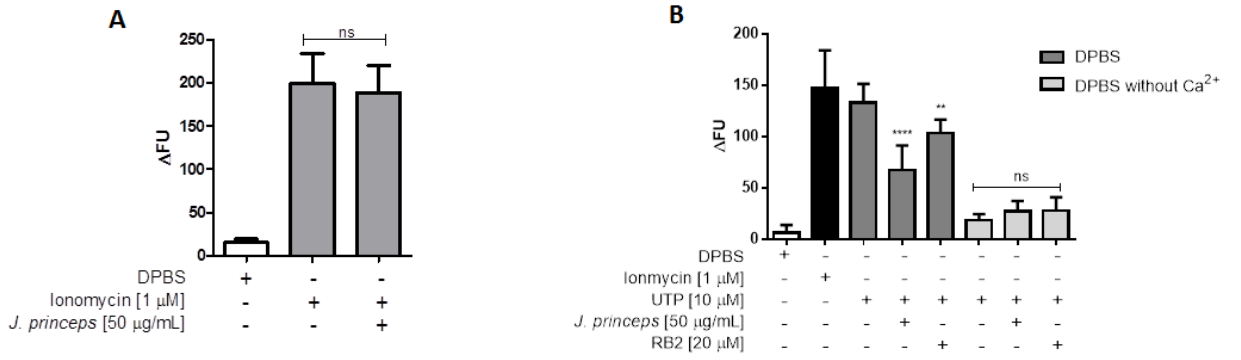

FIGURE 2: **The *J. princeps* Vell. extract did not present the quenching effect and did not inhibit stock-operated calcium channels.** J774.G8 cells stained with 2 μM of Fluo-4 were pretreated with *J. princeps* Vell. stem extract [50 μg/mL] for 30 minutes. Cells were then stimulated with Ionomycin [1 μM] and monitored for 90 seconds. Data are presented as the means $\pm$ SD of three independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidak post-test; $p<0.05$.(ns) non-significant means (A). Cells loaded with Fluo-4 and bathed in DPBS with or without calcium were pretreated with *J. princeps* Vell. stem extract [50 μg/mL] for 30 minutes, and stimulated with UTP [10 μM], were then monitored for 90 seconds. Data are presented as the means $\pm$ SD of three independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidak post-test; $p<0.05$. Asterisks indicate a significant difference in relation to UTP and (ns) non-significant means (B).

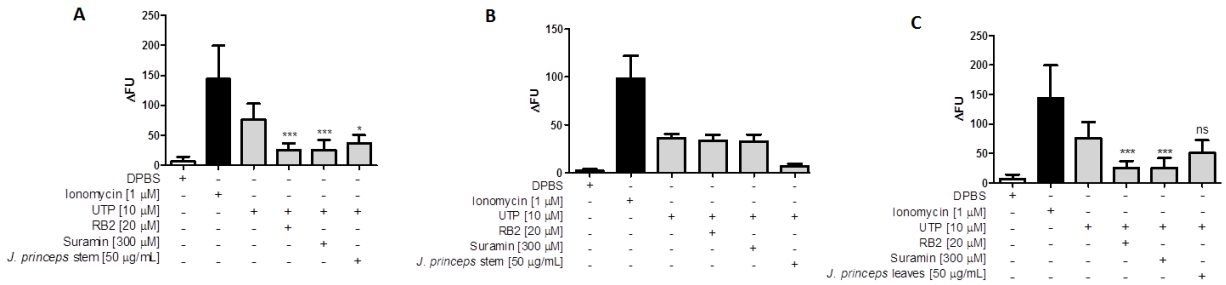

FIGURE 3: **Effects of *J. princeps* Vell. ethanolic extracts on UTP-induced calcium responses**. J774.G8 cells stained with 2 μM of Fluo-4 were pretreated with *J. princeps* Vell. stem extract [50 μg/mL], RB2 [20 μM], and Suramin [300 μM] for 30 minutes. Cells were then stimulated with UTP [10 μM] and monitored for 90 seconds. Data are presented as the means $\pm$ SD of four independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidak post-test; $p<0.05$. Asterisks indicate a significant difference in relation to UTP (A). Peritoneal macrophages loaded with Fluo-4 were pretreated with *J. princeps* Vell. stem extract [50 μg/mL], RB2 [20 μM], and Suramin [300 μM] for 30minutes, and stimulated with UTP [10 μM], were then monitored for 90 seconds. Data are presented as the means $\pm$ SD of one independent experiment performed in triplicate (B). J774.G8 cells stained with 2 μM of Fluo-4 were pretreated with *J. princeps* Vell. leave extract [50 μg/mL], RB2 [20 μM], and Suramin [300 μM] for 30 minutes. Cells were then stimulated with UTP [10 μM] and monitored for 90 seconds. Data are presented as the means $\pm$ SD of four independent experiments performed in triplicate. The data analysis was performed by a one-way ANOVA and Holm-Sidak post-test; $p<0.05$. Asterisks indicate a significant difference in relation to UTP and (ns) non-significant means (C).

As shown in Figures 4C and 4D, none of the extracts reduced cell viability, similarly to untreated cell controls and the RB2 antagonist.

Therefore, we discarded the hypothesis that extract inhibition was due to cell death and concluded that they do not affect cell viability after 24h of treatment at the tested concentration.

3.5 Chemical J. princeps profile determined by HPLC

Both *J. princeps* methanolic and ethanolic extracts were submitted to the HPLC technique for chemical profile determinations as shown in Figures 5A and 5B, the methanolic and ethanolic extracts presented between 31-45 compounds at 254 and 365 nm, even though the method was not efficient regarding substance separation. The peak times of both samples was observed at approximately at 15 minutes.

4 Discussion

In a previous study, we described the potential antagonistic activity of *J. princeps* Vell. extract on UTP-activated receptors for the first time after holding a mini high throughput screening campaign. We observed that the extract inhibited calcium responses in a dependent-concentration manner and found an IC_{50} of 32.32 μg/mL (Ferreira et al., 2018). However, some questions remained open for the conclusion that the extract could be considered a P2Y2 and P2Y4 receptor antagonist.

Herein, we first performed a pharmacological characterization of the *J. princeps* Vell. extract on P2 receptors associated with calcium signaling, i.e., P2X and P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 using physiological or selective agonists (Ralevic & Burnstock, 1998). To stimulate P2X receptors, we used ATP and BzATP, but did not observe any inhibitory response. ATP is a non-selective agonist for P2X receptors and is also capable of activating the P2Y2 and P2Y11 receptors. The EC_{50} range for the activation of these receptors ranges from 0.1 to 10 μM, with the exception of P2X7, whose activation occurs at concentrations over 100 μM (Ralevic & Burnstock, 1998). However, the J774.G8 line expresses several of these receptors (Coutinho-Silva et al., 2005; Ferreira et al., 2018), and the calcium response observed after ATP stimulation may be a kind of summation of the activation of these receptors simultaneously. Even if it were able to inhibit only P2Y2, by adding the response of the other receptors, it would become imperceptible. In an attempt to select a small portion of this response for the P2X receptors, an ATP derivative was used. BzATP functions as a potent agonist for P2X7 as well as a total agonist for P2X4 and a partial agonist for P2X1, P2X2, P2X3 and P2X5 (Coddou et al., 2011). Despite this, the response profile was similar to that observed for ATP.

Another technique used to verify if the extract is able to exert antagonistic activity on P2X7, which is widely expressed in this cell line, was cellular permeabilization, which consists of cell stimulation at high ATP concentrations (above 1 millimolar) in order to observe the formation of a membrane pore. This membrane pore allows the passage of molecules up to 900 Da, including ions (Na^+, Ca^{2+}, K^+), water molecules, ATP and dyes such as propidium iodide (668 Da), ethidium bromide (394 Da), and YO-PRO-1 (629 Da), which are widely used for the study of P2X7 activity (Alves et al., 2014; Di Virgilio et al., 2018). However, no inhibitory response of the extract to the P2X7 receptor was observed.

We also used ADP and UDP, physiological P2Y1 and P2Y6 receptor agonists, respectively, and observe a slight inhibition response of P2Y6. Interestingly, UDP is a product of UTP degradation by ectonucleotidases, and, by displaying a structure similar to UTP, could be easily targeted for inhibition by the extract. However, some studies have recently demonstrated that UTP binding to P2Y2 and P2Y4 receptors do not result in conformational modifications, whereas UDP binding to P2Y6 receptor results in conformational modifications at the quaternary level of the protein (D'Ambrosi et al., 2007; Kotevic et al., 2005). As we still could rule out the possibility of the extract functioning as a negative allosteric modulator, these conformational modifications may continue to occur even, although at a lower level, but sufficient for the P2Y6 receptor to remain active.

Since UTP is able to activate both P2Y2 and P2Y4 subtypes, we use selective agonists for these receptors in order to try to identify whether the extract is able to inhibit a single specific subtype or both subtypes (Jacobson et al., 2012). However, the data suggest that the extract can inhibit the response of both P2Y2 and P2Y4 receptors. Although they are considered selective agonists, it is known that, depending on the concentration, they may present a certain loss of specificity. According to the manufacturer, both agonists, 2-thio-UTP (P2Y2) and MRS 4062 (P2Y4), are capable of activating other P2Y receptors in the submicromolar range. With all these challenges, the experiment that can be performed in attempting to conclude this pharmacological characterization is the use of cells transfected with a single P2Y receptor subtype that is activated by UTP at a time.

Another important point questioned was whether the inhibition caused by the *J. princeps* Vell. extract could be due to a quenching effect. It is known that some plant substances, such as quercetin or vitexin, are able to fluoresce or display a quenching effect, both of which can result in artifacts in experiments using fluorescence (Zou et al., 2002). In this sense, we performed a test using ionomycin, a calcium ionophore that displays the ability to increase the intensity of calcium-related fluorescence. With this, we ob-

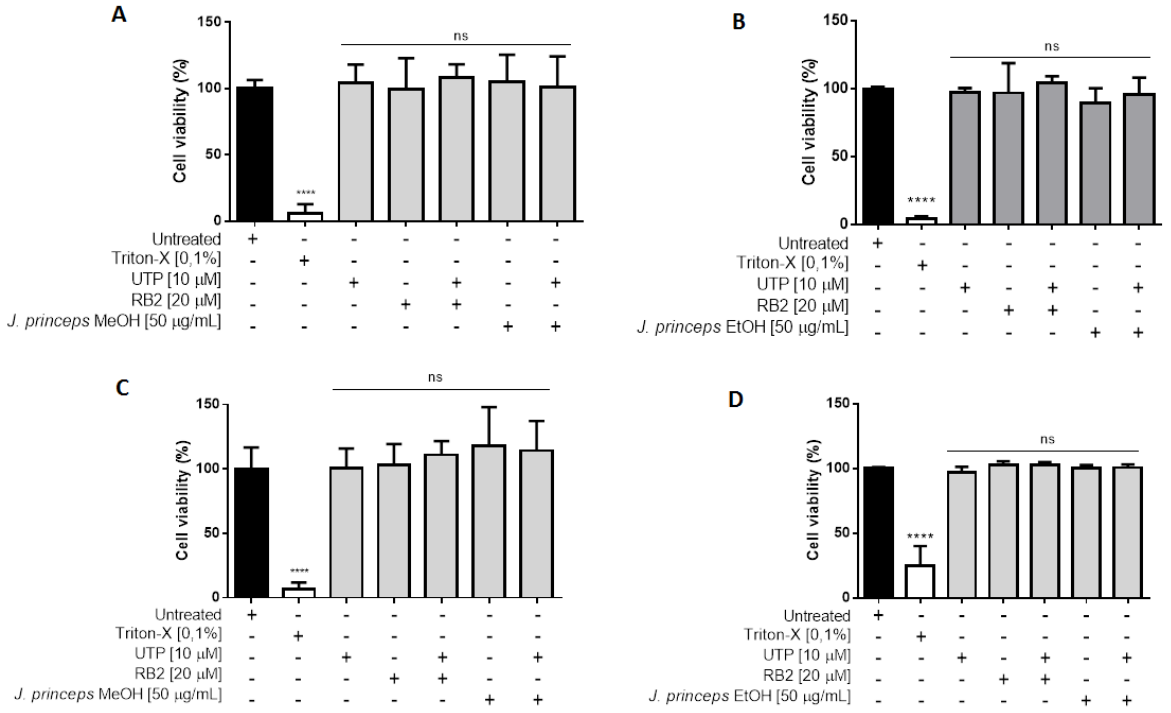

FIGURE 4: ***J. princeps* Vell. cytotoxicity evaluation.** J774.G8 cells were pretreated with *J. princeps* Vell. methanolic (MeOH) or ethanolic (EtOH) extracts [50 μg/mL] and RB2 [20 μM] in the presence or absence of UTP [10 μM] during 1h (A and B) or 24h (C and D). Then, cell viability was assessed by MTT technique. Data are presented as the means $\pm$ SD of three independent experiments performed in triplicate. Data were normalized as a percentage in function of the mean of absorbance obtained by untreated cells, the cellular viability control. The data analysis was performed by a one-way ANOVA and Holm-Sidakpost-test; $p<0.05$. Asterisks indicate a significant difference in relation to UTP and (ns) non-significant means.

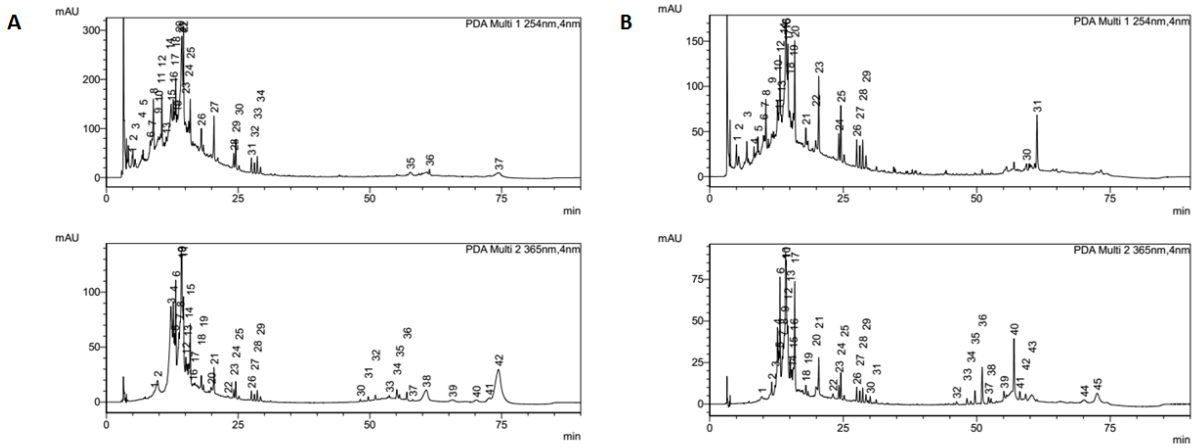

FIGURE 5: ***J. princeps* Vell. chemical profiles.** HPLC chromatograms of the methanol (A) and ethanol (B)extracts from *J. princeps* Vell. stems.

served that the presence of the extract did not result in any changes in the ionomycin response profile, thus ruling out the possibility of a quenching effect.

We also speculated whether the opening of stock-operated calcium channels (SOC) could influence the extract response, i.e., the opening of these channels could be raising intracellular calcium levels (Azimi et al., 2014). This increase in calcium levels could make it difficult to observe calcium mobilization via the phospholipase C/IP_3 pathway. Thus, by removing the extracellular calcium from the buffer solution, we expected to continue to observe inhibition of the UTP response. Surprisingly, calcium mobilization reduced drastically and the extract response was similar to baseline UTP. This led us to question two facts. The first, whether it would be possible that the extract could inhibit the SOC response rather than the receptors, and the second whether, in fact, the extract inhibits SOC, would it not inhibit, even partially, the calcium mobilization induced by ATP, ADP, BzATP and UDP? Another hypothesis is that the absence of extracellular calcium could generate experimental artifacts or unclarified phenomena that could affect the observed response.

Another concern was with regard to the applicability of the extract as a possible phytotherapic. The first sample used in the present study was extracted with a mixture of methanol and dichloromethane. However, according to Food and Drug Administration (FDA) regulations, for a botanical drug to be approved as a phytotherapic it must be extracted in either an aqueous solution or ethanol (Center for Drug Evaluation and Research, 2016). Thus, we performed a new extraction in ethanol and similar to what was observed for the methanolic extract, this new ethanolic extract was also able to inhibit the UTP response in J774.G8 cells and peritoneal macrophages. It is noteworthy that this inhibition was not caused by cell death and that both extracts did not present cytotoxicity within 24 hours of treatment.

J. princeps Vell. is a botanical species found in Brazil and in some African and Asian regions. It belongs to the Euphorbiaceae family and each individual measures around 20 m in height. In Brazil, it is popularly known as "cotieira" and its seeds present laxative and healing properties, as well as anti-parasitic and anti-microbicide activities (Donato-Trancoso et al., 2014; Sousa et al., 2007). *J. princeps* Vell. presents compounds belonging to the sesquiterpene, bis-sesquiterpene, diterpene, triterpene and steroid classes (Achenbach & Benirschke, 1997). However, the few studies that describe the phytochemistry and medicinal properties of *J. princeps* Vell. focus specifically on seeds and leaves. In this sense, the study of the chemical composition and medicinal application of stem extracts is still scarce, and our group is a pioneer in this scenario. However, the characterization of the active molecule, responsible for the antagonistic activity, is indispensable and is the next aim of our research group.

The characterization of the active molecule responsible for the inhibition of P2Y2 and P2Y4 receptors will certainly contribute to the pharmacology of these receptors, which participate in the development of various inflammatory diseases (Ayata et al., 2012; Inoue et al., 2008; Vanderstocken et al., 2010), cancer (Chadet et al., 2014; Choi et al., 2013; Jin et al., 2014; Li et al., 2013; Qiu et al., 2018; Xie et al., 2014), and contribute to cardiac pathologies (Horckmans et al., 2015). Although P2Y2 possesses a single selective antagonist (AR-C118925), it has failed to advance to the clinical application due to certain undesired pharmacological properties, which include high polarity and low bioavailability through the oral route (Rafehi & Müller, 2018). The P2Y4 receptor, however, still lacks selective antagonists, which makes it difficult to characterize associated physiological functions. In this sense, the RB2 antagonist is often used to obtain references regarding the expected antagonistic effect. However, due to its ability to inhibit other P2 receptors, the results should be evaluated at with caution. In this scenario, the description of an antagonist that acts on these two receptors, even without selectivity, will certainly contribute to the development of several *in vitro* and *in vivo* studies, and may serve as basis for the synthesis and development of new selective antagonists for these receptors (Rafehi & Müller, 2018).

5 Conclusions

This study characterized the stem *J. princeps* Vell. extract as a potential antagonist for P2Y2 and P2Y4 receptors. Both methanolic and ethanolic extracts are capable of inhibiting calcium responses promoted by these receptors without causing cytotoxicity and quenching effects. The methanolic extract also inhibits calcium mobilization induced by P2Y6 receptor, but not P2X and P2Y1.

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Conflict of interest

The authors declare no conflicts of interest.

Abbracchio and Burnstock (1994); Achenbach and

Benirschke (1997); Alves et al. (2014); Ayata et al. (2012); Azimi et al. (2014); for Drug Evaluation and Research. (2016); Chadet et al. (2014); Chen et al. (2017); Choi et al. (2013); Coddou et al. (2011); Coutinho-Silva et al. (2005); D'Ambrosi et al. (2007); Degagné et al. (2009); Virgilio et al. (2018); Donato-Trancoso et al. (2014); Eun et al. (2014); Ferreira et al. (2018); Ghanem et al. (2005); Hansen and Bräuner-Osborne (2009); Horckmans et al. (2015); Inoue et al. (2008); Jacobson et al. (2012); Jacobson and Boeynaems (2010); Jin et al. (2014); Kim et al. (2010); Kotevic et al. (2005); Li et al. (2014, 2013); Magni et al. (2015); Qiu et al. (2018); Rafehi and Müller (2018); Ralevic and Burnstock (1998); Santen (2017); de Sousa et al. (2007); Vanderstocken et al. (2010); Kügelgen (2006); Kügelgen and Hoffmann (2016); Xie et al. (2014); Zhang et al. (2010); Zizzo et al. (2012); Zou et al. (2002)

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- **Título do artigo 3: <u>Discovery of antagonistic activity of CS-15 compound on P2X7 receptor</u>**

- **Referência bibliográfica: <u>Ferreira, NCS</u>**; Pontes, MC; Brito, LC; Figueiredo, MR; Calheiros, AS; Alves, LA; Soares-Bezerra, RJ. Discovery of antagonistic activity of CS-15 compound on P2X7 receptor.

- **Situação:** Submetido para a Revista Scientific Reports (FI 4,122)

- **Descrição:** Nesse artigo foi descrito um potencial antagonista para o receptor P2X7, o qual era o terceiro receptor purinérgico estudado na tese. Com o intuito de identificarmos novos antagonistas, aplicamos uma metodologia de permeabilização celular previamente padronizada pelo nosso grupo de pesquisa realizando algumas modificações, sendo a principal delas o uso do corante YO-PRO-1. Com isso, realizamos uma mini-campanha de *screening* e identificamos o efeito inibitório da molécula CS-15, um triterpeno isolado da planta *Clusia studartiana* C.M. Vieira & Gomes da Silva. Essa molécula foi capaz de inibir a captação do YO-PRO-1 em células das linhagens J774.G8 e U937, uma função relacionada com a ativação do P2X7, bem como apresentou um valor de IC_{50} na faixa de nanomolar. Além disso, a referida molécula não inibiu a mobilização de cálcio intracelular induzida pelo ATP e pelo UTP, assim como não reduziu a viabilidade celular após 24h de tratamento. Essa molécula ainda aliviou os sintomas da nocicepção em modelo experimental *in vivo*. Cabe ressaltar, que esse trabalho foi o primeiro a descrever a atividade antagonista de um triterpeno sobre o receptor P2X7, bem como o pioneiro no sentido de avaliar uma propriedade medicinal da referida espécie botânica. Esse trabalho atende aos objetivos específicos 1 a 5 da tese.

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Abstract	P2X7 is a purinergic receptor that is widely expressed in cells of the hematopoietic lineage and Central Nervous System. When activated, it promotes a cation efflux through the membrane and induces the reversible formation of a membrane pore, which allows the passage of molecules up to 900 Da. P2X7 prompts a series of proinflammatory responses, including inflammasome formation, activation of caspases, cytokines release and cell death. However, there are no medicines yet with action on P2X7, which stimulates the search for new molecules with antagonistic activity. Thus, the aim of this study was to identify new compounds with potential antagonistic activity on P2X7 receptor. In this work, we demonstrated the potential antagonistic activity of CS-15 compound, a triterpene isolated from botanical species Clusia studartiana C.M. Vieira & Gomes da Silva. CS-15 compound was able to inhibit approximately 50% of YO-PRO-1 uptake in J774.G8 and U937 cells, with an IC50 value of 610 nM. CS-15 did not inhibit calcium mobilization induced by ATP and UTP and did not reduce cell viability. This compound also alleviates symptoms of neuropathic pain in vivo. These data suggest the discovery of a new potential antagonist for P2X7 receptor with a natural origin.
Techniques	Life sciences techniques, Cellular imaging [Fluorescent dyes]; Life sciences techniques, Experimental organisms [Mouse]; Physical sciences techniques, Spectroscopy [NMR spectroscopy]; Physical sciences techniques, Spectroscopy [Mass spectrometry]; Life sciences techniques, Cell/tissue technologies [Apoptosis]; Life sciences techniques, High throughput screening [High-throughput screening assays];
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Discovery of antagonistic activity of CS-15 compound on P2X7 receptor

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ABSTRACT

P2X7 is a purinergic receptor that is widely expressed in cells of the hematopoietic lineage and Central Nervous System. When activated, it promotes a cation efflux through the membrane and induces the reversible formation of a membrane pore, which allows the passage of molecules up to 900 Da. P2X7 prompts a series of proinflammatory responses, including inflammasome formation, activation of caspases, cytokines release and cell death. However, there are no medicines yet with action on P2X7, which stimulates the search for new molecules with antagonistic activity. Thus, the aim of this study was to identify new compounds with potential antagonistic activity on P2X7 receptor. In this work, we demonstrated the potential antagonistic activity of CS-15 compound, a triterpene isolated from botanical species *Clusiastudartiana* C.M. Vieira & Gomes da Silva. CS-15 compound was able to inhibit approximately 50% of YO-PRO-1 uptake in J774.G8 and U937

cells, with an IC_{50} value of 610 nM. CS-15 did not inhibit calcium mobilization induced by ATP and UTP and did not reduce cell viability. This compound also alleviates symptoms of neuropathic pain *in vivo*. These data suggest the discovery of a new potential antagonist for P2X7 receptor with a natural origin.

Keywords: P2X7, antagonist, natural products

Introduction

Purinergic receptors are plasma membrane receptors, which are activated by extracellular purines and pyrimidines. In humans, these receptors are divided into two families: adenosine-activated P1 receptors and nucleotide-activated P2 receptors. P2 receptors are also grouped into two classes: G-coupled P2Y receptors and ionotropic P2X receptors[1]. Among P2X receptors, P2X7 stands out by its biophysical characteristics and physiological roles in inflammation, pain, and neurodegeneration.

P2X7 is expressed mainly in cells of the hematopoietic lineage, such as mast cells, erythrocytes, monocytes, macrophages, dendritic cells, and lymphocytes, as well as glial cells[2]. Once activated, this receptor promotes the flow of Ca^{2+}, $Na^{+,}$ and K^{+} cations through the cell membrane according to the electrochemical gradient. However, in cases of prolonged activation, it induces the reversible formation of a pore, which allows the passage of molecules up to 900 Da, such as dyes propidium iodide (668 Da), ethidium bromide (394 Da) and YO- PRO-1 (629 Da), which are widely used for the study of P2X7 activity[3].

In addition, activation of P2X7 prompts a series of proinflammatory responses, including inflammasome formation, activation of caspases, phospholipases, mitogen-activated protein kinases

(MAPKs), transcription of pro-inflammatory genes, cytokines release and cell death[4−8]. Due to these functions, several research groups have already observed the participation of P2X7 in the development of diseases such as inflammation[4], neurological and neurodegenerative diseases[9−13], cancer[14−18] and pain[19−23].

Although P2X7 has several selective antagonists characterized, only two of them have advanced to the clinical trials stage. AZD9056 and CE-224,535 were studied as anti-inflammatory agents for the treatment of rheumatoid arthritis, but both failed to demonstrate significant efficacy when compared to the drug available on the market[6]. Thus, this gap in therapy associated with the P2X7 receptor stimulates the research and development of drugs acting on this promising therapeutic target.

One important source for the discovery of new drugs is natural products since they have been used for many years in the treatment of various diseases that afflict humanity[24,25]. In addition, the natural products still present a great wealth of terms of biodiversity, as well as a variety of chemical structures with potential bioactivity[26]. Thus, more than 10 compounds from plants, animals, and microorganisms with activity on P2X7[24]. Among these compounds is amentoflavone, derived from the methanolic extract of *Rheedia longifolia* Planch & Triana, an antagonist of this receptor, which demonstrated analgesic and anti-inflammatory activities[27,28]. Emodin, an anthraquinone derived from rhubarb *Rheum officinale* Baill, also demonstrated the ability to inhibit membrane pore formation, intracellular calcium mobilization, ionic current, NLRP3 inflammasome activation and cell death promoted by P2X7[29−31].

Once these antagonists from natural products are also not applied in clinical therapy, the aim of this study was to identify and to characterize the antagonistic activity of a new compound

isolated from the botanical species *Clusiastudartiana* C.M.Vieira & Gomes da Silva. After running a mini-screening campaign, we identified a triterpene isolated from this Brazilian botanical species, which inhibited P2X7-associated membrane pore formation in a concentration-dependent manner and alleviated pain symptoms.

Results

Optimization of the cell permeabilization assay in J774.G8 cells and discovery of the inhibitory activity of CS-15 compound

In previous work, our research group standardized a methodology of high-throughput screening that allowed the identification of new antagonists for the P2X7 receptor[32]. This methodology was used in a screening campaign conducted by Soares Bezerra[33], in which approximately 1800 extracts were tested. However, the difference between that protocol for the current used in this study was the use of the YO-PRO-1 dye, which became increasingly popular in cell permeabilization assays. Thus, before carrying out the campaign, we sought to understand the profile of YO-PRO-1 uptake in J774.G8 cells.

In order to define the concentration of YO-PRO-1 that offered the best cost-benefit ratio, we tested different concentrations (62.5 nM to 8 μM) in three situations: i) untreated cells in order to observe the basal uptake of the dye; ii) cells treated with Triton-X [0.1%] to observe its maximum uptake; iii) cells stimulated with ATP [5 mM] in order to observe the dye uptake mechanism of YO-PRO-1 via membrane pore associated with P2X7 (data not shown). From this test, we observed that the equipment used to measure YO-PRO-1 fluorescence only distinguish the signal and basal responses from the concentration of 1 μM. Figures 1A and 1B demonstrate the difference between the basal and ATP responses using two different concentrations of YO-PRO-1 (1 and 2 μM). We

observed that there was a significant difference between the basal and ATP responses, and the signal-background ratio was 1.42 and 1.65 for the concentrations of 1 and 2 μM, respectively. Respecting the concentration in which we obtained the best signal-background ratio, we chose to use the concentration of 2 μM.

Using this concentration of YO-PRO-1, we obtained the P2X7 agonist and antagonist's profiles. J774.G8 cells were stimulated with increasing concentrations of ATP and the EC_{50} found was 2.84 mM, as shown in Figure 1C. To obtain an inhibition profile, we selected two P2X7 antagonists, one selective (A-740003) and another non-selective (Brilliant Blue G - BBG). Cells were treated with increasing concentrations of these two antagonists and then stimulated with ATP. Thus, we obtained an IC_{50} of 511 nM for A-740003 (Figure 1D) and 106 nM for BBG (Figure 1E). In addition, we used the formula for calculating z', a statistical parameter that measures how robust the test is, and we obtained a value of 0.59. Since the value found was greater than 0.5, this means that the conditions of our trial are optimal for the execution of the screening.

Finally, we conducted our mini-screening campaign and tested 134 extracts and substances isolated from natural products. Among these samples, the CS-15 compound was distinguished by its ability to reduce by almost half the uptake of YO-PRO-1 after stimulation with ATP.

CS-15 compound significantly inhibited dye uptake in murine and human cell lines, but not calcium mobilization

During the screening campaign, we observed that the CS-15 compound reduced 52% of the YO-PRO-1 uptake in J774.G8 cells stimulated with ATP, as shown in Figure 2A. We asked if this compound could inhibit this activity in lower concentrations. For this, we treated cells with increasing concentrations of the compound and then stimulated with ATP. As demonstrated in

Figure 2B, CS-15 compound inhibited the dye uptake in a concentration-dependent manner and presented an IC_{50} in the nanomolar range (610 nM). We also verified whether this inhibitory effect was restricted only to murine cells and therefore, we performed the same assay on U937 human cells. As shown in Figure 2C, the compound significantly inhibited the YO-PRO-1 uptake in these cells.

We also verified if the compound was able to inhibit the intracellular calcium mobilization induced by P2X7 activation. However, the compound did not exhibit such activity as demonstrated in Figure 2D. Since the cell line used expresses several P2X and P2Y receptors[34,35], this ATP-induced calcium response may not be considered specific for the P2X7 receptor. In an attempt to check if the compound could inhibit calcium responses promoted by P2Y2 and P2Y4 receptors, which were expressed in the J774.G8 cell line[,35], we used UTP as stimulus. As shown in Figure 2E, the CS-15 compound did not inhibit UTP-induced calcium mobilization. Therefore, the data suggest that the compound is able to inhibit the P2X7 receptor in mice and humans.

CS-15 compound did not cause cytotoxicity

Seeking for a possible therapeutic application, we evaluated whether treatment with the CS-15 compound is capable of affecting cell viability. For this reason, we treated the cells with the compound for 1, 6 and 24h. According to the results presented in Figures 3A-C, the compound did not reduce cell viability. These findings suggest that the compound at 1 μM concentration is safe for use.

CS-15 compound alleviates neuropathic pain *in vivo*

Since the activation of the P2X7 receptor is related to pain generation, we used an experi-

mental model of neuropathic and inflammatory pain evaluation. Thus, we observed that the CS-15 compound produced anti-nociceptive behavior associated to neuropathic pain (Figure 4A) and inflammatory pain (Figure 4B). This finding points to the discovery of possible analgesic property of the CS-15 compound.

Discussion

In this present work, we identified the CS-15 compound that presented potential antagonist activity on the P2X7 receptor, besides being able to relieve the symptoms of pain. This compound was discovered during a mini-screening campaign, in which about 134 extracts and isolated substances of plant origin were tested.

In a previously published study, our research group standardized a cellular permeabilization methodology to be applied in high-throughput screening campaigns aimed at identifying new antagonists for the P2X7 receptor [32]. In this protocol, the dye used was propidium iodide. In the present work, we reproduce the same protocol, except by the use of YO-PRO-1, which is being more popularized in dye uptake assays. This modification in the protocol allowed us to verify the reproducibility of the previously standardized methodology. For that, some observations were made. The first one was the choice of the dye concentration that would be used. In a test performed (data not shown), we tested a concentration range between 62.5 nM to 8 μM, but we observed that the equipment used only demonstrated sensitivity to measure differences in fluorescence relative to the dye uptake at concentrations higher than 1 μM. We also evaluated the signal-background ratio of two concentrations (1 and 2 μM), however, we obtained a higher ratio at the concentration of 2 μM, which is commonly used in several scientific studies[36−39]. The ratio obtained was similar to that found by Rat et al. (2017) for YO-PRO-1 [2 μM] in human retinal (ARPE-19) and

keratinocyte (HaCat) cell lines (1.7 and 1.45, respectively) in the presence of 1 and 10 mM ATP[36].

With the definition of the YO-PRO-1 concentration, we continue with our experiments in the spectrophotometer, evaluating the profiles of dye uptake in the presence of the agonist and antagonists. The EC_{50} detected for ATP was 2.18 mM, and thus was within the EC_{50} range for P2X7 (2 to 4 mM) described in the work of Coddou et al. (2011)[40]. However, in our previous work, we found an EC_{50} between 0.7114 and 0.7663 mM[32]. This difference could be due to variations in the sensitivity of the equipment or dye used. We cannot rule out that there may be differences in the J774.G8 subclones, which could for some reason be expressing the P2X7 receptor in lower density or a different splice variant, resistance in forming the membrane pore associated with that receptor[41]. Moreover, we calculated z'-factor, a measure of robustness of assay, and we found a value of 0.59, which indicates that our protocol was adequate for use. This value was similar to that found in our protocol with propidium iodide (0.635) but was smaller than that found using Lucifer yellow (0.876), indicating perhaps that there are variations in sensitivity between the dyes[32].

The IC_{50} detected for the BBG antagonist was 106 nM and also falls within the range of IC_{50} described in the literature (ranging from 15 to 250 nM)[40]. On the other hand, it was lower than that found in our protocol with propidium iodide, which was 1.3 to 2.6 μM[32]. We also tested the P2X7 selective antagonist, A-740003, and obtained an IC_{50} of 511 nM, which is also within the range described in the literature (ranging from 20 to 700 nM)[40].

After the characterization of YO-PRO-1 profile applied to cell permeability assays in J774.G8, we performed a mini-screening campaign and identified CS-15 compound as a promising hit. It blocked around 50% YO-PRO-1 uptake in J774.G8 and U937 cell lines. In addition, it was able to inhibit the uptake of YO-PRO-1 via a P2X7-associated pore in a concentration-dependent manner,

presenting an IC_{50} at submicromolar concentration. This effect is interesting because most hits have the desired response of 1 to 10 μM, and what the data suggest, it is active in the nanomolar range[42]. We further tested whether it could produce any toxicity to the cells, and it has been shown safe for use within 24 hours of treatment using the MTT technique.

We also tried to verify whether this compound would be able to inhibit ATP-induced calcium mobilization. In order to activate the P2X7 receptor, we need to use a high ATP concentration (100 μM); however, it was high sufficient to activate other P2 receptors. Since the J774.G8 cell expresses several P2 receptor subtypes including P2X and P2Y[34,35], we are unable to observe the expected inhibition using this model. An alternative to achieving a higher success rate in this experiment would be the use of cells transfected with P2X7 or the use of BzATP, a more potent but not selective agonist[40]. We further tested another nucleotide, UTP, in order to try to observe whether this inhibition extended to P2Y receptors, but the compound was not able to inhibit the calcium response. In any case, new assays with agonists of other P2 receptors must be made in order to confirm if there is selectivity for the P2X7 receptor.

Finally, we tested the effect of the compound in alleviating the pain sensation induced by intraplantar injection of formalin. The compound significantly reduced the sensation of pain originating from the activation of neuropathic and inflammatory pathways. This finding has already been observed in experiments with other P2X7 antagonists[20,28], and support the pharmacological potential of this compound.

CS-15 compound is a triterpene isolated from botanical species *Clusia studartiana* C.M. Vieira & Gomes da Silva, which belongs to Clusiaceae family. It is a tree of terrestrial habit, but occasionally it can appear hemiepiphyte form. This species is endemic in Brazil, although other

individuals of this family can be found in the tropics. This species is native to the Atlantic Forest in Southwest Brazil[43]. This species was discovered in the early 1990s and to date, there is no information in the literature about its medicinal properties, which makes our study a pioneer in this regard. Despite this, there is some information about the medicinal properties of the botanical family Clusiaceae. The latex present in the fruits of *Clusia* sp., for example, are popularly used in the treatment of rheumatic diseases and infant oral candidiasis, while the *C. grandiflora* seed contains compounds with antimicrobial activity[44−47].

We do not yet know whether these effects are dependent or independent of P2X7 receptor activation. For this, new experiments in which the analysis of cytokines such as IL-1β and other inflammatory mediators related to P2X7 need to be investigated *in vitro* and *invivo*. Besides that, experiments using hP2X7-transfected cells, whole cell patch-clamp, and selective agonists could significantly contribute to characterize the activity of CS-15 compound on P2X7. Despite these limitations, which are within the future perspectives of our group, this study demonstrated for the first time a medicinal effect of the *C. studartiana* compound on inflammation and pain possibly caused by the P2X7 receptor blockade.

Thus, in this work, we characterized and standardized a protocol for dye uptake assay in J774.G8 cells using YO-PRO-1 dye and applied it in a mini-screening campaign. We discovered the inhibitory activity of the CS-15 compound on pore formation associated with P2X7 activation. This compound also alleviates the symptoms of neuropathic pain and it did not cause cytotoxicity *in vitro*. These data suggest the discovery of a new potential antagonist for P2X7 receptor that has a natural origin.

Methods

Reagents

ATP, BBG, A-740003, Triton-X, DMEM and RPMI culture mediums, NaCl, KCl, $MgCl_2$, $CaCl_2$, Na_2HPO_4, KH_2PO_4, Glucose, Hepes, DMSO, MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) tetrazolium, Probenecid and Ionomycin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Fetal bovine serum was obtained from Gibco (Oklahoma, USA). Fluo-4 AM was obtained from Life Technologies (California, USA). YO-PRO-1 was obtained from Molecular Probes (Oregon, USA). Morphine was acquired from Cristália (São Paulo, Brazil) and Sodium diclofenac was obtained from Medley (São Paulo, Brazil).

Plant material

Aerial parts of *C. studartiana* C.M. Vieira & Gomes da Silva (Clusiaceae) were collected in the Ecological Reserve of Macaé de Cima, Atlantic Forest, Nova Friburgo, Rio de Janeiro, RJ. The plant material was identified by Dr. Marcos Nadruz and a voucher specimen was deposited at the Herbarium of the Botanical Garden of Rio de Janeiro under the number RB 336999. The species is authorized by the CGEN at the register number AB5D582.

Extraction, isolation, purification and methylation of 3-oxo-olean-12-en-28-oic-acid (CS-15 compound)

The collected material of *C. studartiana* was dried at 40°C in an oven with air circulation and reduced to small fragments. The extract was prepared by dynamic maceration at room temperature with hexane to exhaustion, and then, filtered and evaporated under reduced pressure. During the concentration of the extract, an amorphous solid was obtained and was chromatographed on silica

gel using solvent mixtures with increasing polarities (cyclopentane, cyclopentane/ethyl acetate, ethyl acetate, and methanol). The resulting fractions were analyzed by thin layer chromatography (CCD) and visualized with Godin's detection reagent. After successive recrystallizations, the procedure resulted in the isolation of a pentacyclic triterpene (3-oxo-olean-12-en-28-oic acid). The molecular formula of this acid was determined by direct insertion in a Bruker micrOTOF-QII mass spectrometer by precise mass and algorithmic analysis of its isotope standard (Bruker SmartFormula 3D True Isotopic Pattern). Methylation of this acid with diazomethane[48] yielded methyl 3-oxo-olean-12-en-28-oate (Figure 5), confirmed by GC-MS data (Figures 6A and 6B). The triterpenes were characterized by uni and bidimensional methods in ^{1}H (500 MHz) and ^{13}C NMR (125MHz) and confirmed by comparison with spectral literature data[49].

Methyl 3-oxo-olean-12-en-28-oate

^{13}C NMR, δ ppm (125 MHz; $CDCl_3$): 39.14 (C-1), 34.16 (C-2), 217,60 (C-3), 47,46 (C-4), 55,36 (C-5), 19,59 (C-6), 32,21 (C-7), 39,32 (C-8), 46,90 (C-9), 36,83 (C-10), 22,97 (C-11), 122,40 (C-12), 143,66 (C-13), 41,79 (C-14), 27,72 (C-15), 23,51 (C-16), 46,60 (C-17), 41,12 (C-18), 45,86 (C-19), 30,70 (C-20), 33,83 (C-21), 32,43 (C-22), 26,48 (C-23), 21,45 (C-24), 15,03 (C-25), 17,03 (C-26), 25,84 (C-27), 176,27 (C-28), 33,06 (C-29), 23,58 (C-30), 51,4 (OCH_3).

Cell culture

J774.G8 and U937 cells were cultivated with Dulbecco's Modified Eagle's Medium (DMEM) or RPPMI medium, respectively, supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere. The cellular medium was changed twice a week and the cells density were adjusted to $2x10^6$ cells per 150 cm^2 cell culture flask (Corning, New York, USA). Cell viability was measured by trypan blue exclusion and the experiments were conducted only when viability was above 90%.

Dye uptake assay

J774.G8 and U937 cells were plated in a 96-well black plate (Corning, New York, USA), at a concentration of 2x10^5 cells/well. They were maintained in culture with appropriated medium supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24h. Then, the medium was replaced by extracellular saline solution (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4). Subsequently, cells were treated with Brilliant Blue G (BBG) [1 μM] and CS-15 compound [1 μM] for 30 minutes. Then, cells were stimulated with ATP [5 mM] for 15 minutes, in order to allow the opening of the membrane pore associated with the P2X7 receptor. Next, the cationic dye YO-PRO-1 was added on each well at a concentration of 2 μM, and after 3 minutes, its fluorescence was measured using FlexStation III equipment (Molecular Devices, California, USA) at wavelengths of 488 nm and 509 nm for excitation and emission, respectively. Cells treated with Triton-X [0.1%] were considered as the control of maximum dye uptake.

Calcium assays

J774.G8 cells were plated in a 96-well black-wall and clear-bottom plate (Corning, New York, USA), at a concentration of 2x10^5 cells/well. They were maintained in culture with DMEM supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24h. Then, the medium was replaced by 100 μl of Dulbecco's PBS (DPBS) (136 mM NaCl, 2.68 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5.55 mM Glucose and 2.5 mM Probenecid – pH 7.4) containing [2 μM] Fluo-4 AM and cells were incubated for 60 minutes at 37ºC in a 5% CO_2 atmosphere. Next, the wells were washed three times with 200 μl of DPBS and then, each well received a final volume of 150 μl of DPBS. Subsequently, cells were

treated with BBG [1 μM], Reactive Blue-2 (RB2) [20 μM] and CS-15 compound [1 μM] for 30 minutes. The mobilization of intracellular calcium was measured in FlexStation III (Molecular Devices, California, USA) in real time during 90 seconds, at wavelengths of 485 nm and 525 nm for excitation and emission, respectively. The agonists (ATP and UTP) were added at the time of 20 seconds and the readings were conducted every 1.52 seconds, totaling a value of 60 readings/well. The calcium mobilization was quantified as ΔFU (the difference between fluorescence peak and basal fluorescence)[50].

Cell viability measurement

J774.G8 cells were plated in a 96-well plate (Corning, New York, USA) at a concentration of 2x10^5 cells/well. They were maintained in culture with DMEM without phenol red supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere for 24h. Next, cells were treated with BBG [1 μM] and CS-15 compound [1 μM] for 1, 6 or 24h. Untreated cells were considered our positive control of viability, while cells treated with Triton-X [0.1%] were considered our negative control. After the treatments, the medium was replaced by 200 μL of DMEM without phenol red containing MTT solution [100 μg/well]. The plate was incubated for 3h at 37°C in a 5% CO_2 atmosphere, and then, was centrifuged at 1500 RPM for 1 minute. Supernatants were collected and formazan crystals were dissolved in 100 μl of DMSO. The absorbance of the wells was measured in Spectramax 190 equipment (Molecular Devices, California, USA), using λ: 570 nm.

Animals

The experiments with animals follow ethical principles in animal experimentation of the Brazilian College of Animal Experimentation. They were approved by the Ethics Committee on

the use of Animals from Oswaldo Cruz Institute on November 24, 2017 (L-037/2017). Healthy male Swiss mice with a weight between 30 and 50g were obtained from Oswaldo Cruz Central Bioterium. The animals were housed under conditions of 12h/12h light and dark cycle with free access to food and water.

Formalin test

At the day of the test, animals were deprived of food and water for 1h before the experiment. Subsequently, the animals were separated by groups with six animals each: (1) vehicle group, which received 200 μL of saline; (2) positive control groups, which received anti-inflammatory or analgesic drugs: sodium diclofenac [50 mg/kg] and morphine [10 mg/kg], respectively; (3) P2X7 reference antagonist group, which received BBG [100 mg/kg]; and (4) test groups, which received CS-15 compound [100 or 200 µg/kg]. All drugs were orally administered with the exception of morphine, which was administered via intraperitoneally. After one hour of treatment (or 30 minutes, in the case of morphine-treated group), all animals received a formalin injection (the pain inductor agent) [2.5%] into the hind paw. The time that each animal spent licking the hind paw was counted at 0 to 5 minutes to analyze the activity of the compound on neuropathic pain, and later, between 15 to 30 minutes to evaluate inflammatory pain.

Data analysis

Each sample was measured in triplicate and all experiments were performed on at least three independent days. All data are presented as mean $\pm$ S.D. The D' Agostino and Pearson normality test was used in order to test if samples follow a Gaussian distribution. If data follow a Gaussian distribution, an appropriate parametric test was applied, if not, an appropriate non-parametric test was applied. Used tests were specified in figure legends. P values of 0.05 or less were considered

significant. Graphs and statistical analyses were performed by GraphPad Prism version 5 (GraphPad Software, San Diego, California, USA). We also assessed the quality of our dye uptake assay by calculating the z'-factor. z'-factor is a valuable tool to evaluate the robustness and suitability of HTS assays. z'-factor values above 0.5 mean that the assay is considered excellent and equal to 1, an ideal assay. However, values below 0.5, mean that the assay needs to be reformulated[51]. This parameter was calculated using the following equation:

$$z' = 1 - \frac{(3SD\ of\ positive\ control + 3SD\ of\ negative\ control)}{(mean\ of\ positive\ control - mean\ of\ negative\ control)}$$

where cells stimulated with ATP at [10 mM] were considered as a positive control, while cells without treatment were considered as a negative control.

The signal-background ratio between the positive and negative permeabilization controls represented by ATP and untreated cells, respectively, was obtained by the following calculation[51]:

$$Signal - background\ ratio = \frac{positive\ control\ mean\ (ATP)}{negative\ control\ mean\ (untreated\ cells)}$$

References

1–51

Authors' contributions

N.C.S.F., L.A.A. and R.J.S.B. designed experiments, N.C.S.F., M.C.P. and A.S.C. performed experiments, N.C.S.F., L.C.B and L.A.A. performed data analysis, M.R.F. and L.C.B performed the

extraction, isolation and purification of chemical compound, N.C.S.F., M.R.F., L.C.B and R.J.S.B. wrote the paper.

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Competing interests

The authors declare no conflicts of interest.

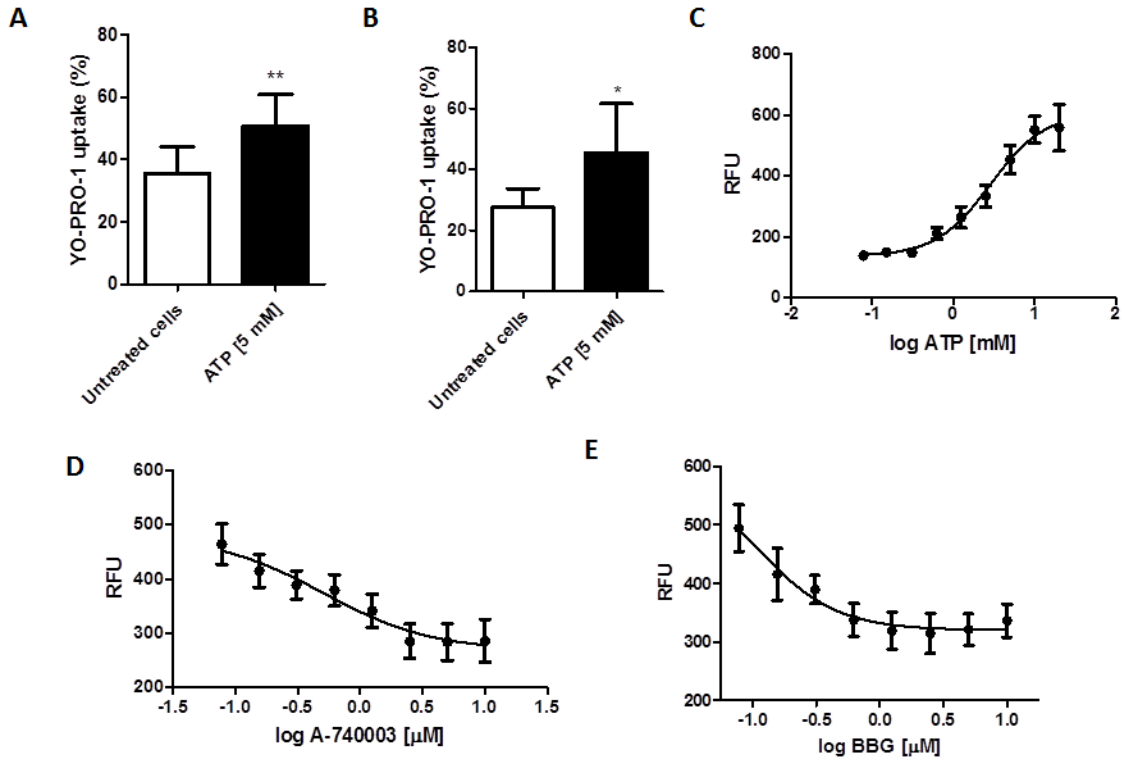

Figure 1: **Profile of YO-PRO-1 uptake assay via P2X7 pore with J774.G8 cells.** J774.G8 cells were stimulated with ATP [5 mM] for 15 minutes and then, received 1 μM (A) or 2 μM(B) of YO-PRO-1. Data are presented as mean $\pm$ SD of three independent experiments performed in triplicate. Data analysis were made using Paired t-Test; p= 0.0099 (A) and p=0.0132 (B). Data were normalized as a percentage in function of the mean of units of arbitrary fluorescence (RFU) obtained by Triton-X, the maximum fluorescence control. Asterisks indicate a significant difference between means. J774.G8 cells were stimulated with increasing concentrations of ATP [0.078 to 20 mM] for15 minutes and then, received YO-PRO-1 [2 μM] (C). J774.G8 cells were pretreated with increasing concentrations of A740003 [0.078 to 10 μM] (D) and BBG [0.078 to 10 μM] (E) for 30 minutes. Then, cells were stimulated with ATP [5 mM] for 15 minutes and received YO-PRO-1 [2 μM]. Data are presented as mean $\pm$SE of three independent experiments performed in triplicate.

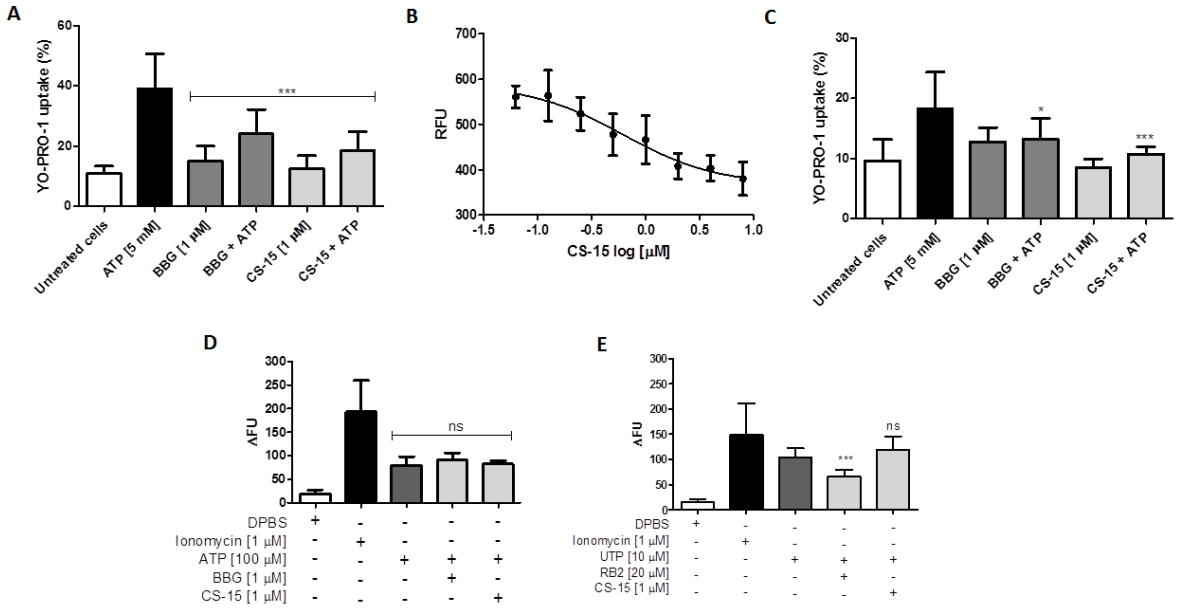

Figure 2: **Compound CS-15 inhibits P2X7 pore formation.** J774.G8 cells were pretreated with CS-15 compound [1 µM] and BBG [1 µM] during 30 minutes and were stimulated with ATP [5 mM] for further 15 minutes. Then, YO-PRO-1 dye [2 µM]was added to the wells and fluorescence of cells was measured. Data are presented as mean ± SD of three independent experiments performed in triplicate. Data analysis were made using one-way ANOVA and Tukey post-test; $p<0.0001$. Data were normalized as a percentage in function of the mean of units of arbitrary fluorescence (RFU) obtained by Triton-X, the maximum fluorescence control. Asterisks indicate a significant difference in relation to ATP (A). J774.G8 cells were pretreated with increasing concentrations of CS-15 compound [0.0625 to 8 μM]for 30 minutes. Then, cells were stimulated with ATP [5 mM] for 15 minutes and received YO-PRO-1 [2 μM]. Data are presented as mean ± SE of three independent experiments performed in triplicate(B). U937 cells were pretreated with CS-15 compound [1 µM] and BBG [1 µM] during 30 minutes and were stimulated with ATP [5 mM] for further 15 minutes. Then, YO-PRO-1 dye [2 µM] was added to the wells and fluorescence of cells was measured. Data are presented as mean ± SD of three independent experiments performed in triplicate. Data analysis were made using one-way ANOVA and Tukey post-test; $p<0.0001$. Data were normalized as a percentage in function of the mean of units of arbitrary fluorescence (RFU) obtained by Triton-X, the maximum fluorescence control. Asterisks indicate a significant difference in relation to ATP (C). J774.G8 cells stained with 2 μM of Fluo-4 were pretreated with CS-15 compound [1 μM], BBG [1 μM], RB2 [20 μM] for 30 minutes and then, were stimulated with ATP [100 μM] (D) or UTP [10μM] (E), and monitored for 90 seconds. Data are presented as mean ± SD of three independent experiments performed in triplicate. Data analysis were made using one-way ANOVA and Tukey post-test; $p<0.0001$. Asterisks indicate significant differences in relation to ATP or UTP controls. (ns) means not significant.

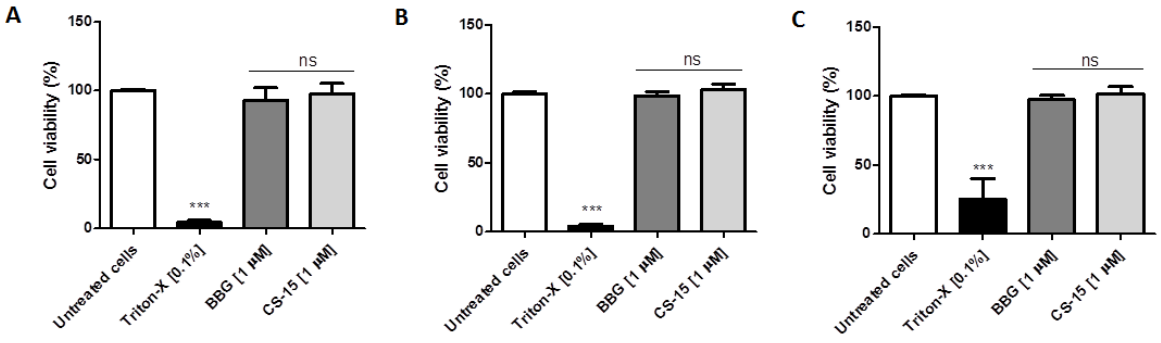

Figure 3: **CS-15 compound did not cause cytotoxicity.** J774.G8 cells were pretreated with CS-15 compound [1 μM] and BBG [1 μM] during 1h (A), 6h (B) or 24h (C). Then, cell viability was assessed by MTT technique. Data are presented as mean $\pm$ SD of three independent experiments performed in triplicate. Data were normalized as a percentage in function of the mean of absorbance obtained by untreated cells, the cellular viability control. Data analysis were made using one-way ANOVA and Tukey post-test; $p<0.0001$. Asterisks indicate a significant difference in relation to untreated cells and (ns) mean not significant.

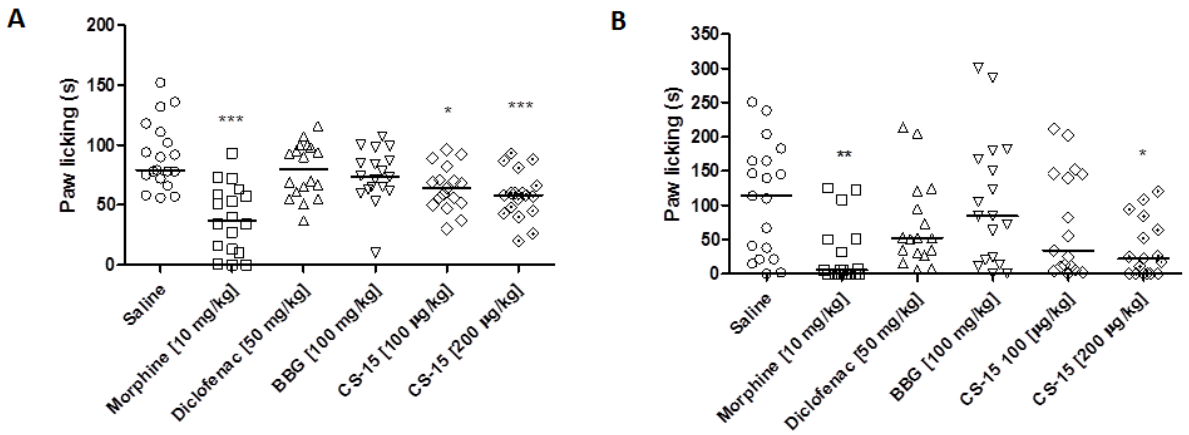

Figure 4: **CS-15 compound attenuated paw licking behavior.** Mice were treated with sodium diclofenac [50 mg/kg] or morphine [10 mg/kg] as positive controls, CS-15 compound [100 and 200 μg/kg] or BBG [100 mg/kg] as test treatments, or saline as vehicle control. After 1h, formalin [2.5%] was injected into the hind paw. The time spent at licking paw was counting in the first 5 minutes (neuropathic pain) (A) and then, between 15 and 30 minutes (inflammatory pain) (B). Data are presented as the median of three independent experiment (n= 17-19 animals per group). Data analysis were made using one-way ANOVA and Tukey post-test; $p< 0.0001$ in A and 0.002 in B. Asterisks indicate a significant difference in relation to saline control group.

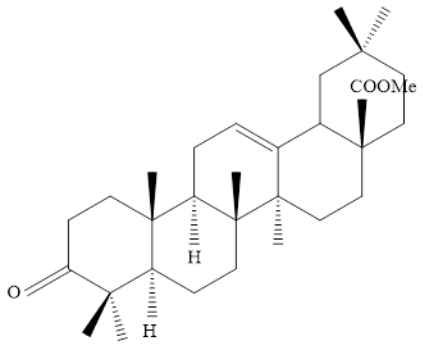

Figure 5: **Chemical structure of CS-15 compound.** CS-15 compound is named 3-oxo-olean-12-en-28-oicacid methyl ester**,** a triterpene isolated from *C. studartiana*.

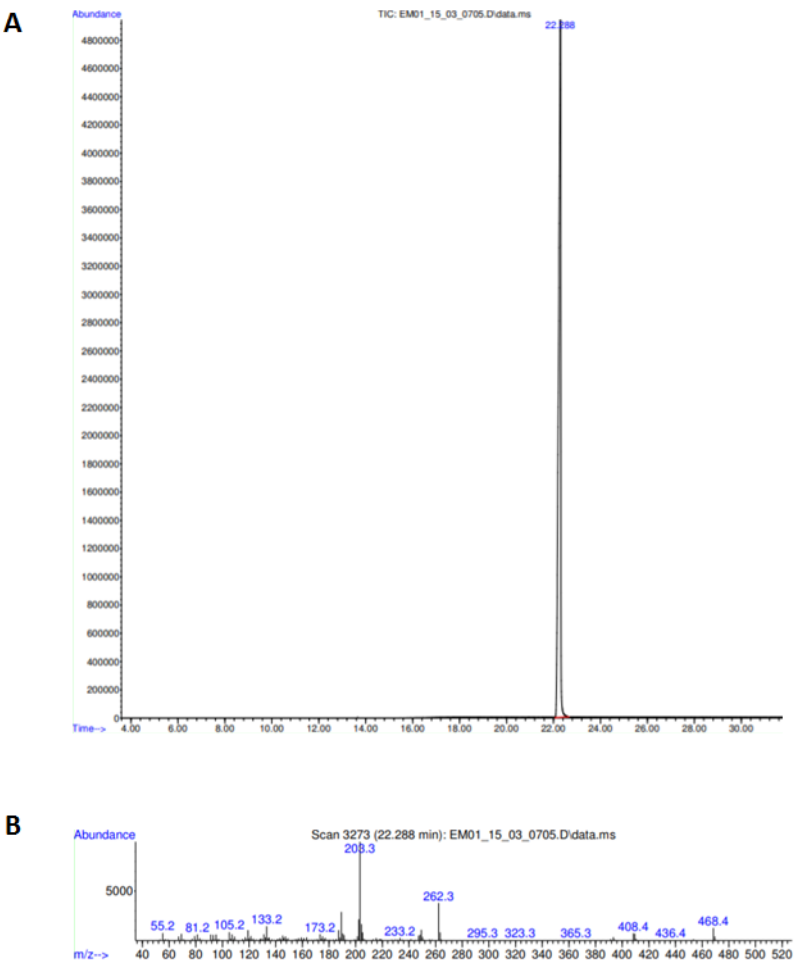

Figure 6: **Chemical spectrum of CS-15 compound from *C. studartiana*.** Gas chromatogram (A) and mass spectrum (B) of the compound methyl 3-oxo-olean-12-en-28-oate.

- **Título do artigo 4: <u>Potential therapeutic applications of P2 receptor antagonists: from bench to clinical trials</u>**

- **Referência bibliográfica: <u>Ferreira NCS</u>**; Alves LA; Soares-Bezerra RJ. Potential therapeutic applications of P2 receptors antagonists: from bench to clinical trials. *Curr Drug Targets*. 2019;20(9):1-18.

- **Situação:** Publicado na Revista *Current Drug Targets*.

- **Descrição:** Esse artigo de revisão foi redigido durante o meu doutorado e aborda o desenvolvimento de fármacos com atuação antagonista sobre diferentes subtipos de receptores P2 na terapia clínica. O trabalho discute os principais achados sobre o uso dos antagonistas em modelos de dor, neurodegeneração, câncer, inflamação e trombose em fase pré-clínica. Em seguida, aponta os resultados e os *status* dos antagonistas mais promissores que avançaram para as etapas 2, 3 e 4 da fase clínica, isto é, ligantes seletivos dos receptores P2X3, P2X7 e P2Y12. O artigo ainda apresenta uma pequena discussão sobre os antagonistas do receptor P2Y12, os quais são até o momento o único caso de sucesso da terapia purinérgica e encerra-se tecendo alguns comentários sobre os principais desafios para o desenvolvimento de fármacos nessa área de atuação.

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REVIEW ARTICLE

Potential Therapeutic Applications of P2 Receptor Antagonists: From Bench to Clinical Trials

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Abstract: ***Background*:** Extracellular purines and pyrimidines have important physiological functions in mammals. Purines and pyrimidines act on P1 and P2 purinergic receptors, which are widely expressed in the plasma membrane in various cell types. P2 receptors act as important therapeutic targets and are associated with several disorders, such as pain, neurodegeneration, cancer, inflammation, and thrombosis. However, the use of antagonists for P2 receptors in clinical therapy, with the exception of P2Y12, is a great challenge. Currently, many research groups and pharmaceutical companies are working on the development of specific antagonist molecules for each receptor subtype that could be used as new medicines to treat their respective disorders.

***Objective*:** The present review compiles some interesting findings on the application of P2 receptor antagonists in different *in vitro* and *in vivo* experimental models as well as the progress of advanced clinical trials with these compounds.

***Conclusion*:** Despite all of the exciting results obtained on the bench, few antagonists of P2 receptors advanced to the clinical trials, and once they reach this stage, the effectiveness of the therapy is not guaranteed, as in the example of P2X7 antagonists. Despite this, P2Y12 receptor antagonists have a history of success and have been used in therapy for at least two decades to prevent thrombosis in patients at risk for myocardial infarctions. This breakthrough is the motivation for scientists to develop new drugs with antagonistic activity for the other P2 receptors; thus, in a matter of years, we will have an evolution in the field of purinergic therapy.

Keywords: P2X receptors, P2Y receptors, antagonists, therapy, thrombosis, pain, inflammation, clinical trials.

1. INTRODUCTION

Purinergic receptors are a family of receptors expressed in the membrane surface of vertebrates, invertebrates, and protists [1, 2]. These receptors are activated by extracellular purines and pyrimidines, which initiate intracellular signaling that culminates in physiological responses, including inflammation [3], neurotransmission [4], platelet aggregation [5], and pain [6], among others.

In humans, 19 subtypes of purinergic receptors have been cloned since the 1990s. In 1994, a group of researchers proposed a classification for purinergic receptors into two classes according to their natural ligand and effector system. Thus, purinergic receptors were classified as either adenosine-activated P1 receptors (P1R) or nucleotide-activated P2 receptors (P2R) [7]. Recently, Thimm and collaborators (2013) identified a new family of adenine receptors in rodents that were named the P0 receptors. Naturally, the inclusion of P0 receptors in the traditional classification of purinergic receptors could occur in the future after the characterization of these receptors in humans [8].

P1 receptors are associated with the Gs/i protein and thus act on the protein-effector adenylyl cyclase to activate or inhibit the formation of cyclic adenosine 5'-monophosphate (cAMP), respectively. P1 receptors exhibit four subtypes: A1, A2A, A2B and A3, and their major physiological ligand is adenosine [7].

P2 receptors are divided into two major classes (P2X and P2Y) according to their pharmacological responses [7]. P2X receptors are ionotropic cation nonselective ion channels that allow for rapid Ca^{2+}, $Na^{+,}$ and K^{+} flux according to the electrochemical gradient of each ion. Currently, seven subtypes of these receptors are described: P2X1-P2X7, and adenosine 5'-triphosphate (ATP) is their physiological ligand. Among all P2X receptors, P2X7 has the unique ability to form membrane pores, *i.e.*, when activated by micromolar concentrations of ATP, P2X7 forms a membrane pore that allows for the passage of molecules up to 900 Da [9]. It

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has been described that other subtypes of P2X receptors, P2X2 and P2X4, also demonstrate the ability of membrane pore formation similar to P2X7 [10, 11]. Despite this, the origin of the membrane pore remains unclear. Some authors believe that the opening of the membrane pore is related to dilation of the ion channel, while others support the hypothesis that another membrane protein could function as a pore and could be activated by second messengers derived from activated-P2X receptors [12-14].

P2Y receptors have eight subtypes: P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11-P2Y14. These receptors are Gq/s/i protein-coupled, and their major effectors are the phospholipase C/inositol triphosphate system and adenylyl cyclase, which act in the regulation of intracellular calcium stores and cAMP formation, respectively. In humans, P2Y receptors are activated by different nucleotides, including ATP (P2Y2, P2Y11), adenosine 5'-diphosphate (ADP) (P2Y1, P2Y12, P2Y13), uridine 5'-triphosphate (UTP) (P2Y2, P2Y4), uridine 5'-diphosphate (UDP) (P2Y6, P2Y14) and UDP-glucose (P2Y14) [15, 16].

P2 receptors are widely expressed in many cell types and tissues in mammals, including hematopoietic-derived cells, platelets, skeletal muscle, glial cells, some neuronal cells, hepatocytes and some digestive tract cells [16, 17]. The wide expression of P2 receptors in numerous cell types, especially in humans, mice and rats, allowed the conduction of several *in vitro* and *in vivo* experiments aiming to discover and understand important physiological roles of P2 receptors. These findings also demonstrated the participation of P2 receptors in the development of various pathologies and noted that the use of antagonists could relieve the symptoms of these diseases.

2. ON THE BENCH: PROMISING EFFECTS OF P2 RECEPTOR ANTAGONISTS IN DIFFERENT EXPERIMENTAL MODELS OF DISEASES

There are many selective and nonselective antagonists described for P2 receptors, although the establishment of their use in a therapeutic context is still very difficult. Several groups have demonstrated that the use of antagonists for P2 could attenuate the effects of some disorders associated with P2R activation, such as pain, neurodegeneration, and inflammation. Here, we briefly discuss some findings on the use of P2R antagonists in pain, neurodegeneration, cancer, inflammation, and thrombosis.

2.1. Pain

The participation of P2 receptors in pain is well-investigated through the use of several animal models. In terms of P2X subtypes, the participation of P2X3, P2X4, and P2X7 in pain development has been demonstrated.

P2X3 expression is upregulated in the dorsal ganglion neurons of animals with bone cancer and is associated with cancer-induced pain behavior [18] and visceral pain transduction from the ureter, bladder, gut, and uterus [19]. In animal models of visceral inflammation pain, the use of A317491 (a P2X3-selective antagonist), pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and Suramin (P2R-nonselective antagonist) attenuated pain sensation [20-23].

Meanwhile, P2X4 is upregulated in microglia after nerve injury, and it was suggested that P2X4 could be implicated in neuropathic pain [24, 25]. This upregulation in microglia after nerve injury is dependent on lyn tyrosine kinase activity, which is a member of the src kinase family (SFK) [26]. Tsuda *et al.* (2009) demonstrated that $P2X4^{-/-}$ mice exhibited a reduction in pain responses in models of chronic pain [27].

P2X7 is associated with inflammatory hyperalgesia since its activation stimulates the release of inflammatory mediators, such as IL-1β, TNF-α, IL-6 and cytokine-induced neutrophil chemoattractant-1 (CINC-1). It has already been demonstrated that selective antagonists of P2X7, A438079, AACBA, and A740003 could attenuate hyperalgesia and allodynia in animal models of pain [28-32]. Recently, Munoz *et al.* (2017) demonstrated that P2X7-induced nociception could be related to the production of reactive oxygen species (ROS) since the administration of 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP) in mice induced ROS production in the spinal cord and nociceptive behavior, which were attenuated with the pretreatment of ROS scavengers [33].

It has already been observed that all P2Y subtypes, with the exception of P2Y4, partially contribute to pain development. MRS2179, a selective antagonist for P2Y1, relieves nociception transmission in cancer-induced bone pain and thrombus-induced thermal hyperalgesia in rats [34, 35]. P2Y2 plays a role in trigeminal neuropathic pain, and the use of antisense oligonucleotides, Suramin and ARC-118925 (a selective P2Y2 antagonists) promoted an anti-allodynic effect [36, 37].

It has been observed that nerve injury in animals upregulates the expression of P2Y6 and P2Y11 receptors and that tactile allodynia could be inhibited by P2Y6 and P2Y11 antagonists MRS2578 and NF340, respectively [38]. MRS2578 and MRS2211 (P2Y6 and P2Y13 antagonists, respectively) and P2Y14 anti-sense nucleic acids also attenuated neuropathic pain in an animal model of nerve injury [39]. Barragán-Iglesias and collaborators (2015) also observed an increase of 0.5% in formalin-induced flinching behavior after treatment with P2Y1, P2Y6 and P2Y11 agonists; on the other hand, selective antagonists of these receptors significantly reduced inflammatory pain [40]. Recently, Huang *et al.* (2018) demonstrated P2Y6 expression in the spinal cord of rats and that treatment with UDP increased mechanical allodynia and thermal hyperalgesia in a model of chronic constriction injury of the sciatic nerve, whereas MRS2578 alleviated these symptoms [41].

P2Y12 antagonists also demonstrated analgesic effects. In animal models of neuropathic and inflammatory pain, and acute thermal nociception, P2Y12 antagonists attenuated hyperalgesia and the expression of pro-inflammatory cytokines [42]. Recently, Jia *et al.* (2018) showed that nanoparticle-encapsulated curcumin, a metabolite with antagonistic activity against P2Y12, reduced P2Y12 expression, the release of pro-inflammatory cytokines, and thermal hyperalgesia in type 2 diabetic rats [43].

Therefore, these findings demonstrate that P2 receptor antagonists are able to attenuate different pain mechanisms, including inflammatory and neuropathic pain. Thus, P2 re-

ceptor antagonists could contribute to the development of a new class of analgesic agents. Although promising, the results should be evaluated with caution because some of the antagonists used in the studies present some problems. PPADS and Suramin are nonselective antagonists, and the latter is also an inhibitor of G-protein receptors. MRS2578, a P2Y6 selective antagonist, exhibited reactivity and hydrophobicity limitations. A-317491 also demonstrated low bioavailability. BzATP, a selective P2X7 agonist, has more affinity for the P2X1 receptor [44]. Curcumin is a molecule from the plant *Curcuma longa* that has several molecular targets, including those that are antioxidants and involved in anti-inflammatory activities [45].

2.2. Neurodegenerative Diseases

In animal models of neurodegenerative diseases, it was observed that P2X7 was upregulated in some cells in the brain and contributed to the aggravation of neurodegenerative diseases. In fact, P2X7 is the most studied subtype in neurodegenerative diseases. Little is known about the contribution of the other P2 receptors. As will be discussed below, several works demonstrated that its antagonists could alleviate the symptoms of different types of neurodegenerative diseases.

P2X7 antagonists, A438079 and JNJ-47965567, alleviated seizures in epileptic mice. A438079 also had a neuroprotector role in the animal model of kainic acid-induced seizures by decreasing seizure severity and neuronal death in the hippocampus [46, 47]. P2X7 antagonists also ameliorated neuroinflammation, neuronal damage, and glial activation thereby alleviating seizure severity [48].

P2X7 expression is upregulated in lateral amyotrophic sclerosis, and activation of the P2X7 receptor is associated with motor neuronal death, which was inhibited by Brilliant Blue G (BBG), oxidized ATP (oATP) and KN-62. Treatment with these antagonists also decreased the expression of microgliosis and inflammatory markers, such as NF-κB, NADPH oxidase 2 and IL-1β, and improved motor performance in mice [49-51].

BBG treatment blocked preterm birth in an animal model of intrauterine inflammation induced by lipopolysaccharide (LPS) and ameliorated fetal cortical injury, increased the number of dendrites and preserved the architectural morphology of cortical neurons [52]. In an experimental model of Parkinson disease, BBG ameliorated rotational behavior, improved memory, increased dopamine levels in the striatum and mesencephalon, and attenuated gliosis [53].

In Alzheimer's disease, P2X7 stimulates amyloid precursor protein (APP) release and decreases α-secretase activity, which cleaves APP, increasing amyloid plaque accumulation. P2X7 also produces reactive oxygen species, which leads to oxidative stress in Alzheimer's disease. P2X7 antagonists inhibit APP release and enhance APP processing *via* α-secretase, thereby preventing amyloid plaque accumulation. P2X7 knockdown also stimulated microglial phagocytosis of amyloid β (Aβ). Additionally, BBG prevented cognitive deficits and improved learning and memory [54-60].

Delekate *et al.* (2014) observed the participation of the P2Y1 receptor in an animal model of Alzheimer's disease. They showed that astrocytes near Aβ were hyperactive and that astrocyte activity diminished in the presence of P2Y1 antagonists [61].

These observations illustrate the potential use of antagonists to alleviate symptoms of neurodegenerative diseases to help improve patients' quality of life. However, the major antagonist used in the experiments, BBG, is not selective for P2X7. Thus, since BBG inhibits the P2X4 receptor and voltage-gated sodium channels, it would be important to verify whether these other two molecular targets are actually unaffected in these experimental models [62]. In 2003, Beigi *et al.* [63] also described that oATP could have other molecular targets in addition to P2X7.

2.3. Cancer

The role of ATP in cancer is contradictory. ATP could activate P2 receptors to induce cellular proliferation or apoptosis. Sometimes, only one subtype of P2R could play this dual role. Pellegatti *et al.* (2008) established a protocol to quantify ATP concentration in the tumor *in vivo*, and found that the ATP concentration is above 100 μM [64]. This level of extracellular ATP is sufficient to activate all P2Rs, including P2X7, which is activated at concentrations above 100 μM.

In neuroblastoma and glioma, P2X7 is involved in cellular proliferation, which was blocked with intravenous administrations of BBG [65]. Activation of P2X7 by extracellular ATP also stimulates cellular proliferation, migration, and invasion in pancreatic ductal adenocarcinoma cells and in some cell lines of prostate, lung, melanoma and breast cancer. These activities could be prevented by treatment with receptor antagonists [66-70].

Amoroso *et al.* (2016) observed the dual role of P2X7 in malignant pleural mesothelioma cell lines (MMP89, IST-MES2, and MSTO-211H). They observed that P2X7 antagonists (AZ10606120 and oATP), as well as BzATP (P2X7 selective agonist), inhibited cell proliferation. However, this inhibitory effect was caused by cell death. Indeed, treatment with AZ10606120 in animals inoculated with tumor cells reduced the tumor volume after two weeks [71]. Similarly, systemic administration of ATPγS, a nonhydrolyzable ATP analog, reduced the tumor growth of MDA-MB-231 and Py8119 mammary cells *in vivo*, possibly by acting on P2X receptors [72]. BBG and P2X7 shRNA also increased tumor growth and angiogenesis in glioma C6 cells [73].

Extracellular ATP also activates P2Y2, which is associated with cellular proliferation, migration, invasion, and metastasis in some cell lines from prostate and breast cancer, as well as human hepatocellular carcinoma and human cancerous pancreatic duct epithelial cells [74-78].

P2Y6 can contribute to the development of colorectal tumors since $P2Y6^{-/-}$ animals presented a lower number of cancer cells and tumors with small volumes [79].

Recently, Khalid *et al.* (2017) demonstrated that ATP and NF546 (a selective agonist of P2Y11) induced migration of the human hepatocellular carcinoma lineages Huh-7 and HepG2. Migration of these cell lines, as well as calcium mobilization induced by ATP, were inhibited by NF340, a P2Y11 antagonist [80].

P2Y12 also plays a role in cancer. Treatment with ticagrelor and P2Y12 knockdown reduced the growth of ovarian cancer tumors [81]. Treatment with ticagrelor at clinical doses (10 mg/kg) also reduced metastasis of melanoma and breast cancer cells, and improved the survival of the animals [82]. Sarangi *et al.* (2013) demonstrated that treatment with cisplatin induced P2Y12 expression in the 4T1 breast cancer cell line and that the antagonist of this receptor (2-MeS-AMP) could improve cisplatin-induced cytotoxicity [83].

Collectively, these findings contributed to the potential application of P2 antagonists as chemotherapeutic or adjuvant therapies.

2.4. Inflammation

Extracellular purines and pyrimidines may act as danger signals and contribute significantly to inflammation by activating P2 receptors.

P2X4 is associated with the release of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, and IL-17, and contributes to neuroinflammation and rheumatoid arthritis, which were attenuated by treatment with nonselective antagonists (PPADS and TNP-ATP) and P2X4 anti-sense oligonucleotide [84-86]. Activation of P2X4 induces prostaglandin E2 (PGE_2) release from macrophages, and $P2X4^{-/-}$ mice show lower levels of the inflammatory mediators after the challenge with carrageenan and formalin [87]. In an allergic asthma model in which animals were challenged with ovalbumin, Zech *et al.* (2016) demonstrated an increase in P2X4 expression, the number of lymphocytes and eosinophils in bronchoalveolar lavage fluid **(**BALF), and pro-inflammatory cytokines, and a reduction of Th2 priming capacity. All of these features were alleviated with the P2X4 antagonist 5-BDBD or in knockout models. The researchers also observed an increase in P2X4 expression in individuals with asthma compared to expression in healthy individuals [88]. Patients with asthma also express functional P2X1 receptors in eosinophils. However, P2X1 current responses and cell adhesion were reduced in individuals with asthma compared to healthy individuals [89]. Alberto *et al.* (2016) also demonstrated the participation of different P2 receptors in eosinophil migration to the inflamed site that could be blocked with Suramin, a nonselective P2 antagonist [90].

P2X7 may induce activation of the NLRP3 inflammasome, resulting in IL-1β release and pro-inflammatory signaling. The P2X7 antagonist BBG suppressed inflammasome formation and IL-1β release, leading to a decrease in nephritis severity [91] and cisplatin-induced nephrotoxicity [91, 92]. BBG and P2X7 knockdown also alleviated lung inflammation and collagen deposition in mice exposed to silica [93]. KN-62 and Suramin attenuated pulmonary inflammation, emphysema formation and fibrosis [94, 95].

The P2X7 antagonists BBG, A740003 and A438079 also prevented colitis in rats and inhibited neutrophil infiltration in mouse ears [96-98]. A438079 decreased necrosis, inflammatory infiltration, collagen accumulation, and pro-inflammatory cytokines in an experimental model of liver injury and fibrosis [99]. P2X7 is also expressed in inflamed synovial tissue, and the use of the antagonists AZD9056, A740003 and AACBA alleviates joint inflammation in animal models [31, 100, 101].

A P2X7 antagonist permeable to the blood-brain barrier, JNJ-47965567, was developed to reach targets in the central nervous system. JNJ-47965567 blocked IL-1β release and attenuated amphetamine-induced hyperactivity and neuropathic pain in animal models [102]. Recently, some nanobodies against mouse P2X7 were developed. The systemic administration of these nanobodies could attenuate P2X7 activation in T cells and macrophages to ameliorate experimental glomerulonephritis and contact dermatitis. The human P2X7 nanobody, Dano1, was able to reduce the level of IL-1β release from primary human monocytes primed with LPS and stimulated with ATP [103].

P2Y1 expressed on platelets is responsible for leukocyte recruitment and platelet-leukocyte complex formation *in vivo* during allergic inflammation *via* the RhoA signaling pathway. This action could be reversed by treatment with the P2Y1 antagonists MRS2179 and MRS2500 [104]. Amison *et al.* (2017) also demonstrated that P2Y1 and P2Y14 antagonists (MRS2500 and PPTN, respectively) induce leukocyte chemotaxis to the lungs after an LPS challenge [105].

P2Y2, activated by extracellular ATP and UTP, participates in inflammation. P2Y2 is involved in cyclooxygenase-2 (COX-2) expression and PGE_2 release in intestinal cells and medullary collecting duct cells, thereby contributing to postobstructive uropathy bowel inflammation and diuresis, respectively [106, 107]. P2Y2 is also associated with eosinophil and neutrophil accumulation in lung inflammation as well as neutrophil infiltrates and cell death in the liver [108-110]. P2Y2 receptors induce ROS production in endothelial cells and THP-1 migration after UTP or oxidized low-density lipoprotein (oxLDL) stimulation [111]. Eun *et al.* (2014) demonstrated that treatment with LPS induces P2Y2 expression in macrophages. Macrophages primed with LPS and further stimulated with ATP or UTP also induce the production of several pro-inflammatory mediators, such as high mobility group box 1 (HMGB1) protein, COX-2, PGE_2, inducible nitric oxide synthase (iNOS) and nitric oxide (NO) [112]. Recently, Paniagua-Herranz *et al.* (2017) demonstrated that activation of the EP3 receptor by PGE_2 could inhibit calcium responses, cell migration, and ERK/Akt phosphorylation induced by UTP in cerebellar astrocytes [113].

P2Y6 activated by extracellular UDP stimulates CXCL8 expression and its release by intestinal epithelial cells [114]. MRS2693, a selective P2Y6 agonist, induces pro-inflammatory cytokine production (IL-6 and IL-8) and the activation of NF-κB, p38 and mitogen-activated protein kinase (MAPK) in a cell line from human bronchial epithelium [115]. In an asthma animal model, treatment with MRS2578, a selective P2Y6 antagonist, decreased asthma features such as eosinophilia in bronchoalveolar lavage, airway remodeling, cytokine production, bronchial hyperresponsivity and airway inflammation [116]. MRS2578 also decreases pro-inflammatory cytokine production and the number of neutrophil extracellular traps (NETs) induced by monosodium urate (MSU) crystals [117, 118]. The P2Y6 agonists UDP and 3P-UDP also induce cytokine production in THP-1 cells, mouse peritoneal macrophages, and human primary monocyte-derived macrophages. In this same work, the authors induced atherosclerosis in mice through a Western diet and after some weeks, observed that $P2Y6^{-/-}$ mice

presented a significant reduction in the plaque area on the aortic arch [119].

The P2Y12 receptor also plays a role in inflammation. In a model of intra-abdominal sepsis and acute lung injury, animals treated with clopidogrel and animals that were P2Y12-null presented with reduced levels of circulating white blood cells, platelet activation, cytokine release, and lung injury compared with the control animals [120]. Satonaka *et al.* (2015) also observed P2Y12 participation in vascular wall inflammation. They observed that treatment with ADP increased the expression and release of monocyte chemoattractant protein-1 (MCP-1), which is involved in monocyte migration to the vascular wall, in a time-dependent manner, and these features were inhibited by R-138727 (the active metabolite of prasugrel) and P2Y12-siRNA [121].

The P2Y13-selective antagonist MRS2211 and P2Y13-knockout significantly suppressed IL-6 production in Kupffer cells, alleviating liver inflammation [122].

Recently, PPTN was identified as an antagonist for P2Y14. PPTN inhibited neutrophil chemotaxis induced by UDP-glucose [123]. In collecting duct intercalated cells, P2Y14 activated by extracellular UDP-glucose induces pro-inflammatory chemokine expression and MAPK activation to mediate kidney inflammation; this effect was attenuated by PPTN [124].

Collectively, these findings endorse the role of P2R in inflammation, pointing to the possible use of P2R antagonists as anti-inflammatory agents to attenuate inflammatory events associated with the activation of these receptors.

2.5. Thrombosis

P2 antagonists can also be applied in thrombosis prevention. Thrombosis is generally associated with the P2Y12 subtype, but there is increasing evidence of a significant contribution from other subtypes, such as P2X1 and P2Y1, in thrombosis prevention.

Animals that are $P2X1^{-/-}$ presented with smaller thrombus formations compared to wild-type animals, and $P2X1^{-/-}$ animals survived longer during the time interval studied than the control group [125].

P2Y1 and P2Y12 are expressed in platelets, and the inhibition of P2Y1 and P2Y12 is associated with a decline in platelet aggregation, thus preventing thrombosis. The P2Y1 antagonists MRS2179 and MRS2500 prevented platelet aggregation and thrombus formation in humans and animal models [126-131]. Using MRS2500 in combination with clopidogrel increased the anti-thrombotic effect compared to using these compounds alone [130]. It is important to note that new drugs have been developed with simultaneous action on P2Y1 and P2Y12 due to their expression in platelets, activation by extracellular ADP and contribution in platelet aggregation [132].

3. P2 ANTAGONISTS IN CLINICAL TRIALS

3.1. Antagonists for P2X-related Diseases

Based on the results observed *in vitro* and in animal models of different diseases, some P2 antagonists have advanced to the clinical trial level. Based on the website of clinical trials (clinicaltrials.gov) from the US National Institutes of Health, we found approximately 15 studies in clinical phases involving P2X receptors worldwide. Among these results, only 5 studies were in phase 2 or later, and the results are shown in Table **1**.

The P2X3 receptor, a P2X subtype, is represented in three records of clinical trials in phase 2 or later. AF-219, a P2X3 selective antagonist, was studied for the first time in 2011 for the treatment of chronic cough by the Afferent Pharmaceuticals Company. P2X3 is expressed in the vagal nerve, and the hypothesis is that P2X3 could mediate cough reflex sensibilization. In the P2X3 study, 24 patients received treatment with AF-219 for 2 months, and 75% of the patients had decreased cough frequency. However, 6 patients presented adverse effects, such as taste disturbance, and abandoned the treatment. Some related adverse effects were dysgeusia (88%), hypogeusia (54%), nausea (38%) and oropharyngeal pain (21%), among others [133]. In 2015, AF-219 was studied in patients with idiopathic pulmonary fibrosis to assess its tolerability in relation to blood pressure since P2X3 is expressed in the carotid chemosensory afferent nerve and could influence sympathetic activity. However, before and after AF-219 use, the patients did not present a difference in systolic (means: 122 *vs* 122, respectively) and diastolic (means: 77 *vs* 76, respectively) blood pressure. In the same year, Afferent Pharmaceuticals began to study the application of AF-219 in patients with idiopathic pulmonary fibrosis with a persistent cough, but the obtained results were not disclosed. Interestingly, AF-219 was studied in 74 women with interstitial cystitis and/or bladder pain syndrome; AF-219 was administered for 4 weeks, and after this period, compared to the placebo, AF-219 reduced daily painful bladder or interstitial cystitis symptoms (13.3 *vs* 15.3, respectively) and the genitourinary pain index (20.8 *vs* 25.5, respectively). However, the authors did not mention if these effects were mediated by P2X3. The major related adverse events were dysgeusia (72.22%), headache (13.89%), ageusia (8.33%) and hypogeusia (8.33%); however, there were no related serious adverse effects [150].

Another P2X subtype that presents antagonists in clinical studies is P2X7. Based on the results observed in animal models of rheumatoid arthritis, many investigators studied the application of P2X7 antagonists in this disease. Using the parameters of the American College of Rheumatology of 20%, 50% and 70% improvement responses (ACR20, ACR50, and ACR70, respectively), the authors observed that treatment with AZD9056 did not show any significant benefit in patients with rheumatoid arthritis compared to the placebo, with patients in both groups continuing treatment with methotrexate. Among the 64 patients treated with AZD9056 at the maximal dose (400 mg), only 5 patients fell within the ACR70 criteria, compared to 3 of the 65 patients from the placebo group who fell inside this analyzed parameter. The major adverse effects were nausea, upper abdominal pain, diarrhea, and headache. The AZD9056 compound was also studied in patients presenting with acute Crohn's disease at moderate and severe levels. Patients mentioned that the AZD9056 compound alleviated their pain sensation and increased their well-being. However, the levels of biomarkers, such as reactive C protein, in the serum and fecal calprotectin did not decrease [134, 151].

Table 1. P2 antagonists in clinical trials at phase 2 or later.

P2	Antagonist(s)	Disease(s)	Aim	Number of Patients	Phase	Start Year	Sponsor and NCT Number	Status	The Major Result(s)
P2X3	AF-219	Chronic cough	Evaluate the effects of AF-219 on chronic cough treatment	n= 34; randomized and double-blind	2	2011	Afferent Pharmaceuticals, United Kingdom - NCT01432730	Completed	AF-219 reduced cough frequency [133].
P2X3	AF-219	Idiopathic pulmonary fibrosis (IPF)	Evaluate AF-219 tolerability in patients with IPF	n= 6	2	2015	Afferent Pharmaceuticals United States - NCT02477709	Completed	AF-219 did not alter blood pressure.*
P2X3	AF-219	Idiopathic pulmonary fibrosis in patients with a persistent cough	Evaluate changes in cough frequency	n= (51); randomized and double-blind	2	2015	Afferent Pharmaceuticals, United States - NCT02502097	Completed	ND
P2X7	AZD9056	Rheumatoid arthritis (RA)	Confirm the efficacy of treatment with the drug in RA patients	n= 385; randomized and double-blind	2	2007	AstraZeneca, United States - NCT00520572	Completed	AZD9056 did not have efficacy compared to placebo [134].
P2X7	CE-224,535	Rheumatoid arthritis	Improve signals and symptoms of RA in patients	n= 100; randomized and double-blind	2 and 3	2008	Pfizer, United States - NCT00628095	Completed	It did not have efficacy compared to placebo [135].
P2Y12	Clopidogrel and prasugrel	Coronary artery disease (CAD) and diabetes mellitus	Optimize antiplatelet therapy in patients with diabetes mellitus	n= 35; randomized and double-blinded	2	2008	Eli Lilly and Company, United States - NCT00642174	Completed	Prasugrel treatment was superior to clopidogrel treatment [136].
P2Y12	Ticagrelor and prasugrel	Acute coronary syndrome (ACS)	Evaluate the effects of aspirin and P2Y12 antagonists on platelet reactivity	n= (29); randomized and double-blinded	4	2015	Sheba Medical Center, Israel - NCT02049762	Completed	ND
P2Y12	Clopidogrel, ticagrelor, and prasugrel	ACS and coronary angioplasty	Compare the impact of conventional therapy to genotype-guided therapy with P2Y12 antagonists	n= (4000); randomized	4	2012	Italian Society of Invasive Cardiology, Italy - NCT01477775	Unknown	ND
P2Y12	Clopidogrel and ticagrelor	ACS, stents, and atherosclerosis	Compare the efficacy of mono- and dual-therapy with antiplatelets	n= (1520); randomized	4	2017	Samsung Medical Center, Korea - NCT03119012	In progress (recruiting)	ND

(Table 1) contd….

P2	Antagonist(s)	Disease(s)	Aim	Number of Patients	Phase	Start Year	Sponsor and NCT Number	Status	The Major Result(s)
P2Y12	Clopidogrel and ticagrelor	ACS	Compare the impact of conventional therapy to genotype-guided therapy with P2Y12 antagonists	n= 132; randomized	4	2013	The University of Hong Kong, China – NCT01994941	Completed	Of the 65 patients who were genotyped, 40 patients switch medication from clopidogrel to ticagrelor due to loss-of-function of the CYP2C19 allele [137].
P2Y12	Ticagrelor and prasugrel	ACS	Compare platelet response between ticagrelor and prasugrel	n= (50); randomized	4	2013	Federal University of São Paulo, Brazil – NCT02215993	Completed	ND
P2Y12	Prasugrel	Sepsis	Investigate possible attenuation effects of thienopyridines on coagulation	n= 20; randomized and double-blind	4	2009	Medical University of Vienna, Austria – NCT01099566	Completed	Prasugrel inhibited platelet aggregation, but its effects were partially antagonized by von Willebrand factor [138].
P2Y12	Clopidogrel and ticagrelor	Inflammation and thrombosis	Investigate possible attenuation effects of thienopyridines on cellular infiltration	n= (50); randomized	4	2015	First Affiliated Hospital of Harbin Medical University, China - NCT02639143	Unknown	ND
P2Y12	Clopidogrel, ticagrelor, and prasugrel	Dental diseases and bleeding	Evaluate bleeding rate after dental procedures	n= (428); randomized, double-blinded	4	2017	Chiang Mai University, Thailand – NCT03103685	In progress (not yet recruiting)	ND
P2Y12	Ticagrelor and prasugrel	Angina and myocardial infarction (MI)	Evaluate impact of P2Y12 antagonists in ACS patients	n= (2520); randomized	4	2015	University of Padova, Italy - NCT02618837	In progress (recruiting)	ND
P2Y12	Clopidogrel, ticagrelor, and prasugrel	Coronary artery disease	Evaluate the impact of cigarette smoking in platelet reactivity	n= 205; nonrandomized	4	2014	University of Roma La Sapienza, Italy - NCT02026713	Unknown	Cigarette smoking weakly influences the antiplatelet effects of P2Y12 inhibitors [139].
P2Y12	Clopidogrel and ticagrelor	CAD, myocardial ischemia and infarction, stent thrombosis and cardiovascular diseases	Compare the response between ticagrelor and clopidogrel in PCI	n= (1900); randomized	3	2017	Assistance Publique – Hôpitaux de Paris, France – NCT02617290	In progress (recruiting)	ND
P2Y12	PRT060128 (elinogrel) and clopidogrel	Percutaneous coronary intervention (PCI)	Compare the response between elinogrel and clopidogrel in PCI	n= 652; randomized and double-blind	2	2008	Portola Pharmaceuticals, United States – NCT00751231	Completed	Treatment with elinogrel increased the amount of bleeding and adverse effects compared to treatment with clopidogrel [136].

(Table 1) contd….

P2	Antagonist(s)	Disease(s)	Aim	Number of Patients	Phase	Start Year	Sponsor and NCT Number	Status	The Major Result(s)
P2Y12	Ticagrelor	ACS	Observe if ticagrelor could induce NO formation	n= (74); nonrandomized	4	2015	Jennifer Rossington, United Kingdom - NCT02169596	Completed	ND
P2Y12	Clopidogrel	CAD	Evaluate the possible interaction between clopidogrel and statins	n= (150); randomized and double-blind	4	2015	University of Roma La Sapienza, Italy - NCT02030054	Unknown	ND
P2Y12	Clopidogrel	CAD	Evaluate the possible interaction between clopidogrel, omeprazole, and pantoprazole	n= (150); randomized and double-blind	4	2014	University of Roma La Sapienza, Italy - NCT02028234	Unknown	ND
P2Y12	Clopidogrel, prasugrel, and ticagrelor	ACS and PCI	Compare the impact of conventional therapy to genotype-guided therapy with P2Y12 antagonists	n= (120); randomized, double-blinded	2 and 3	2014	Ottawa Heart Institute Research Corporation, Canada – NCT02044146	In progress (active, not recruiting)	ND
P2Y12	Clopidogrel and ticagrelor	ST elevation myocardial infarction (STEMI) and fibrinolysis	Compare the effects of ticagrelor and clopidogrel on patients with STEMI	n= (56); randomized and single-blind	4	2013	University of Patras, Greece - NCT01950416	Completed	ND
P2Y12	Clopidogrel, ticagrelor, and prasugrel	Myocardial infarction (MI)	Evaluate the possible interaction between clopidogrel and morphine	n= 36; randomized and double-blind	4	2011	Medical University of Vienna, Austria – NCT01369186	Completed	Morphine diminishes the C_{max} values of the active metabolites of prasugrel and clopidogrel [140, 141].
P2Y12	Clopidogrel	CAD, MI, stent thrombosis, ACS and heart diseases	Establish a cut-off level for platelet inhibition in patients with or without previous stent occlusion	n= (450); nonrandomized	2	2009	Uppsala University, Sweden – NCT00914368	Completed	ND
P2Y12	Clopidogrel, ticagrelor, and prasugrel	Stable angina	Implementation of genotype-guided therapy with P2Y12 antagonists	n= (150); randomized	3	2013	Medical University of Warsaw, Poland – NCT01930773	In progress (recruiting)	ND
P2Y12	Clopidogrel, ticagrelor, and prasugrel	Heart attack	Compare the effects of P2Y12 antagonists in patients with primary PCI	n= (90); nonrandomized	4	2015	The Royal Wolverhampton Hospitals, United Kingdom - NCT02376283	Unknown	ND

(Table 1) contd….

P2	Antagonist(s)	Disease(s)	Aim	Number of Patients	Phase	Start Year	Sponsor and NCT Number	Status	The Major Result(s)
P2Y12	Ticagrelor and prasugrel	MI	Evaluate the addition of vorapaxar in dual-antiplatelet therapy	n= (126); randomized	4	2016	University of Florida, United States – NCT02545933	In progress (recruiting)	ND
P2Y12	Clopidogrel	Pancreatic cancer	Evaluate the possible interaction between clopidogrel and chemotherapeutic agents	n= (39); randomized and double-blind	3	2016	Assistance Publique – Hôpitaux de Paris, France – NCT02404363	In progress (active, not recruiting)	ND
P2Y12	PRT060128 (elinogrel)	MI	Evaluate the safety and efficacy of elinogrel before PCI in dual-antiplatelet therapy	n= 70; randomized and double-blind	2	2007	Portola Pharmaceuticals, United States – NCT00546260	Terminated by administrative reasons	Treatment of patients with elinogrel is well-tolerated [142].
P2Y12	Clopidogrel, ticagrelor, and prasugrel	ACS	Evaluate the safety and efficacy of a ½ dose of P2Y12 inhibitors in patients with ACS	n= (100); randomized	3	2016	Dong-A University, Korea – NCT02944123	In progress (recruiting)	ND
P2Y12	Clopidogrel, ticagrelor, and prasugrel	ACS, unstable angina and MI	Compare the effects of P2Y12 antagonists in elderly patients (≥ 70 years)	n= (1000); randomized	4	2013	St. Antonius Hospital, The Netherlands - NCT02317198	In progress (recruiting)	ND
P2Y12	Cangrelor and ticagrelor	STEMI	Compare the effects of cangrelor and ticagrelor in patients with STEMI	n= (30); randomized and single-blind	4	2017	Attikon Hospital, Greece - NCT02943369	Completed	ND
P2Y12	Prasugrel	Chronic asthma	Evaluate prasugrel treatment in bronchial hyperreactivity	n= (26); randomized and single-blind	4	2011	University of Milan, Italy – NCT01305369	Completed	ND
P2Y12	Prasugrel	ACS	Investigate the effects of prasugrel pretreatment in MI	n= 4033; randomized and double-blind	3	2009	Eli Lilly and Company, United States – NCT01015287	Completed	Prasugrel did not reduce ischemia, but led to an increased rate of bleeding complications [143-145].
P2Y12	Clopidogrel, ticagrelor, and prasugrel	Cardiovascular diseases	Evaluate if ticagrelor improves endothelial function	n= (54); randomized	4	2015	Erasmus Medical Center, The Netherlands - NCT02587260	Completed	Ticagrelor did not improve endothelial functions in stabilized patients who suffered an ACS [146].

(Table 1) contd….

P2	Antagonist(s)	Disease(s)	Aim	Number of Patients	Phase	Start Year	Sponsor and NCT Number	Status	The Major Result(s)
P2Y12	Ticagrelor	ACS	Compare the antiplatelet effects between ticagrelor and tirofiban	n= 95; randomized	3	2012	Pusan National University Yangsan Hospital, Korea - NCT01660373	Unknown	Tirofiban demonstrated a greater antiplatelet effect than ticagrelor [147].
P2Y12	Prasugrel	MI	Compare the antiplatelet effects between ticagrelor and tirofiban	n= 100; randomized and single-blind	3	2010	Università degli Studi di Ferrara, Italy - NCT01336348	Completed	Tirofiban demonstrated a greater antiplatelet effect than prasugrel [148].
P2Y12	Clopidogrel	Coronary artery stenosis and platelet aggregation	Compare the antiplatelet effects of clopidogrel plus cilostazol	n= (80); randomized	3	2009	Gyeongsang National University Hospital, Korea - NCT00891670	Unknown	ND
P2Y12	Ticagrelor and prasugrel	CAD	Compare the pharmacodynamic effects of ticagrelor substituted with prasugrel	n= 110; randomized	4	2012	Daiichi Sankyo, United States - NCT01587651	Completed	Prasugrel increased the platelet reactivity index and presented a similar profile to that of ticagrelor on inhibition percentage of platelet reactivity.*
P2Y12	Clopidogrel and prasugrel	CAD	Compare the effectiveness of clopidogrel and prasugrel in reducing the number of cardiovascular adverse effects in patients with CAD	n= 423; randomized and double-blind	2	2009	Eli Lilly and Company, United States - NCT00910299	Terminated due to the low rate of primary endpoint events experienced	Clopidogrel substitution with prasugrel offered effective platelet inhibition, but slightly increased the risk of bleeding [149].
P2Y12	Ticagrelor	Peripheral artery disease	Compare ticagrelor and aspirin effects on walking	n= 40; randomized, double-blinded	2	2014	AstraZeneca, United States - NCT02227368	Terminated due to slow recruitment	There was a 0.1 log of difference between ticagrelor and aspirin treatment in seconds at peak walking time in 26 weeks.*
P2Y12	Clopidogrel, ticagrelor and prasugrel	MI and STEMI	Implementation of genotype-guided therapy with P2Y12 antagonists	n= (2700); randomized	4	2011	Vera HM Deneer, The Netherlands - NCT01761786	In progress (active, not recruiting)	ND
P2Y12	Clopidogrel and ticagrelor	CAD	Compare ticagrelor and clopidogrel effects in patients with CAD	n= (28); randomized	4	2012	Rapid City Regional Hospital, United States - NCT01706510	Completed	ND

ND: not disclosed; ACS: acute coronary syndrome; CAD: coronary artery disease; C_{max}: maximal plasma concentrations; IPF: idiopathic pulmonary fibrosis; MI: myocardial infarction; NO: nitric oxide; RA: rheumatoid arthritis; STEMI: ST elevation myocardial infarction.

The number of estimated patients in the study is provided between parentheses due to a lack of results being disclosed. NCT: ClinicalTrials.gov Identifier. *Information about the results are available at the site (clinicaltrials.gov)

CE-224,535, another compound with antagonistic activity on P2X7, was studied in patients with rheumatoid arthritis treated with methotrexate. However, CE-224,535 did not demonstrate efficacy compared with the placebo, since 34% *versus* 36.2% of the patients treated with CE-224,535 and the placebo, respectively, fell inside the ACR20 criteria after 12 weeks. Approximately 62.2% of the patients treated with this P2X7 antagonist presented adverse effects compared to 55.3% of the patients in the placebo group. The major adverse effects were nausea and diarrhea. Moreover, 9.4% of the patients treated with CE-224,535 discontinued treatment compared to 6.4% of the patients in the placebo group [135]. A summary of these results is described in Table **1**.

3.2. Antagonists for P2Y-related Diseases

The website of clinical trials (clinicaltrials.gov) has more than 300 records on clinical studies related to P2Y receptors, of which 206 studies were in phase 2 or later and involved only P2Y12 receptor antagonists. Of these 206 studies, 61 studies were active, 119 studies were finalized, and 26 study statuses were unknown. The majority of the studies compared P2Y12 antagonists (clopidogrel, prasugrel, ticagrelor, cangrelor, elinogrel, and ACT-246475) in pharmacodynamic parameters, efficacy and number of adverse effects in the aspect of cardiovascular diseases; some of these studies are listed in Table **1**.

Among the studies with disclosed results, some studies reported the effects of prasugrel in patients. One study with 110 participants aimed to observe the effects of substituting ticagrelor with prasugrel and showed that prasugrel-treated groups presented an increase in the platelet reactivity index (PRU) compared to the ticagrelor-treated groups. However, both ticagrelor and prasugrel maintained the same P2Y12 inhibition profile and adverse effects at similar rates. Trenk *et al.* (2012) compared the efficacy between prasugrel and clopidogrel in patients with high platelet reactivity on clopidogrel after coronary stent implantations and demonstrated the superiority of prasugrel in reducing the PRU [149]. In 212 patients treated with prasugrel, the PRU fell from 245 to 80, while in the clopidogrel-treated group (211 patients), this index fell from 249 to 241. However, after 90 days from the beginning of treatments, the PRU means did not differ between groups. Therefore, a possible substitution of clopidogrel for prasugrel offers effective platelet inhibition but slightly increases the risk of bleeding (1.4% observed in the prasugrel group compared to 0.5% in the clopidogrel group) in a period of 6 months. Moreover, adverse effects were observed in 52.38% of patients treated with prasugrel compared to 34.29% of patients treated with clopidogrel [149].

In another study that compared elinogrel and clopidogrel in a sample size of 652 patients with nonurgent percutaneous coronary intervention (PCI), the obtained results demonstrated the safety and tolerability of both drugs. However, patients treated with elinogrel at a lower dose (100 mg oral) presented an increase in bleeding in 120 days (10.9%) compared to patients treated with clopidogrel (6.7%). In addition, elinogrel-treated patients presented more adverse effects than clopidogrel-treated patients that include respiratory disorders (24.4% *versus* 9.6%, respectively) and skin and subcutaneous tissue disorders (8.5% *versus* 6.3%, respectively) [136].

Furthermore, Kim *et al.* (2016) compared the actions of prasugrel and tirofiban, an inhibitor of glycoprotein IIb/IIIa that is used in antiplatelet therapy. The researchers observed that treatment with tirofiban increase its inhibition of platelet aggregation from 83 to 92.9% compared to treatment with ticagrelor (from 56.2 to 91.3%) in a period between 2h and 24 h, after ADP stimulus. Similarly, after thrombin receptor activating peptide (TRAP) stimulation, tirofiban increased its inhibition of platelet aggregation from 61.3 to 76.5% compared to the increase from 21.2 to 58.3% of ticagrelor. However, the high on-treatment platelet reactivity did not differ between the two groups [147]. Nevertheless, Valgimigli *et al.* (2012) demonstrated that concomitant administration of tirofiban and prasugrel in patients inhibited almost 100% of the platelet aggregation induced by ADP and TRAP [148].

Other records from P2Y12 clinical trials available at the site (clinicaltrials.gov) aim to implement a personalized choice of platelet P2Y12R inhibitor according to the patient's genotype. The hypothesis is that CYP2C19, an important enzyme for thienopyridine metabolism, can present some loss-of-function polymorphisms that have an association with clopidogrel resistance and ischemic events. Thus, through genotyping, it would be possible to guide the treatment of patients according to two groups: group one presents a high risk of resistance due to polymorphisms, and group two presents a lower risk. Patients who fit the first group may be treated with more potent drugs such as ticagrelor and prasugrel, while other patients may be treated with clopidogrel. This strategy aims to reduce the risk of heart attacks, deaths from cardiovascular causes and the risk of complications from bleeding. Recently, Tam *et al.* (2017) conducted a study on the personalized choice of P2Y12 antagonist based on genotypes in 65 individuals from China. They found that 40 patients presented loss-of-function on the CYP2C19 allele, and treatment with clopidogrel was substituted with ticagrelor, which was effective in reducing platelet reactivity [137]. These results could also be an example of a pharmacogenomics application.

Finally, other studies have been dedicated to verifying the possible pharmacological interaction effects of P2Y12R inhibitors. One study of 250 patients with coronary artery disease (CAD) demonstrated that cigarette smoking weakly influences the antiplatelet effects of P2Y12R antagonists [139]. Another study showed the interaction between morphine and antiplatelets. The researchers observed that morphine diminishes the maximal plasma concentrations (C_{max}) of active metabolites from prasugrel and clopidogrel in healthy volunteers. In addition, morphine also delayed the absorption and effects of clopidogrel [140, 141]. There are still other studies that evaluate possible pharmacological interactions between P2Y12 inhibitors and drugs such as omeprazole, pantoprazole (proton pump inhibitors), vorapaxar (protease-activated receptor inhibitor), and rosuvastatin and atorvastatin (statins), along with chemotherapeutics, but the results have not been disclosed.

Through the presented general scenario, we observed that only P2Y12 receptor antagonists are applied in clinical therapy. P2X3 and P2X7 receptor antagonists are closer to reaching this stage in the future compared to other P2X receptors, as studies on P2X3 and P2X7 receptor antagonists

are in the clinical study phase. Despite this, there is still much room for the development of drugs that act on other P2 receptors.

4. FIRST CASE OF SUCCESS: CLINICAL APPLICATION OF P2Y12 RECEPTOR ANTAGONISTS IN THROMBOSIS PREVENTION

P2Y12 is a unique subtype of purinergic receptors that has selective compounds used as drugs in clinical therapy to prevent thrombosis since it decreases platelet aggregation. Clopidogrel was the first P2Y12 antagonist used in therapy and has been used since the 1990s. Clopidogrel is a prodrug that requires cytochrome P450 metabolism to generate the active metabolite, a derivative of reactive thiol that has antiaggregant and anti-thrombotic properties. Clopidogrel is an irreversible antagonist that is frequently administered with aspirin in patients at risk of ischemic events such as stroke, myocardial infarction and peripheral arterial disease [152]. A study with 12,562 patients demonstrated that 9.3% of patients who used clopidogrel plus aspirin died from cardiovascular causes compared to 11.4% of patients who used aspirin alone [153].

Prasugrel is another prodrug with antagonistic activity on P2Y12 that is similar in structure to clopidogrel. Prasugrel decreases the rates of ischemic events and death from cardiovascular causes when compared to clopidogrel. In a total of 13,608 patients, 9.9% of patients who used prasugrel died due to cardiovascular causes compared to 12.1% of patients who used clopidogrel. However, prasugrel use increased the risk of bleeding in 2.4% of patients compared to clopidogrel use (1.8%) [154, 155].

Ticagrelor is a reversible and selective P2Y12 antagonist. Ticagrelor inhibits platelet aggregation and the amplification of platelet activation but presents more side effects than clopidogrel [156]. A study of 18,624 patients demonstrated that there was a 90.2% reduction in deaths from cardiovascular events associated with ticagrelor compared to clopidogrel, *i.e.*, 9.8% of patients who received ticagrelor died compared to 11.7% of patients who received clopidogrel [157].

Elinogrel and cangrelor are also P2Y12 antagonists. Elinogrel is a potent, reversible and competitive antagonist with demonstrated activity in patients nonresponsive to clopidogrel in clinical studies. Although elinogrel is available in both oral and intravenous forms, it is not yet approved by FDA for therapeutic purpose [158, 159]. Cangrelor is an approved antagonist available in intravenous form. Cangrelor decreases the rate of ischemic events compared to clopidogrel without increasing the risk of bleeding and the need for blood transfusions [160].

Fig. (**1**) presents a summary of the major selective antagonists of P2 receptors used in *in vitro* and *in vivo* studies. The antagonists that have advanced into the clinical trial phase, as well as those who have entered therapy, are all listed.

5. THERAPEUTIC IMPLEMENTATION OF DRUGS ACTING ON P2 RECEPTORS: A CHALLENGE FOR THE FUTURE

Currently, there have been many studies conducted to discover new P2R antagonist molecules that could be used in therapy and implemented for "purinergic therapy", as could

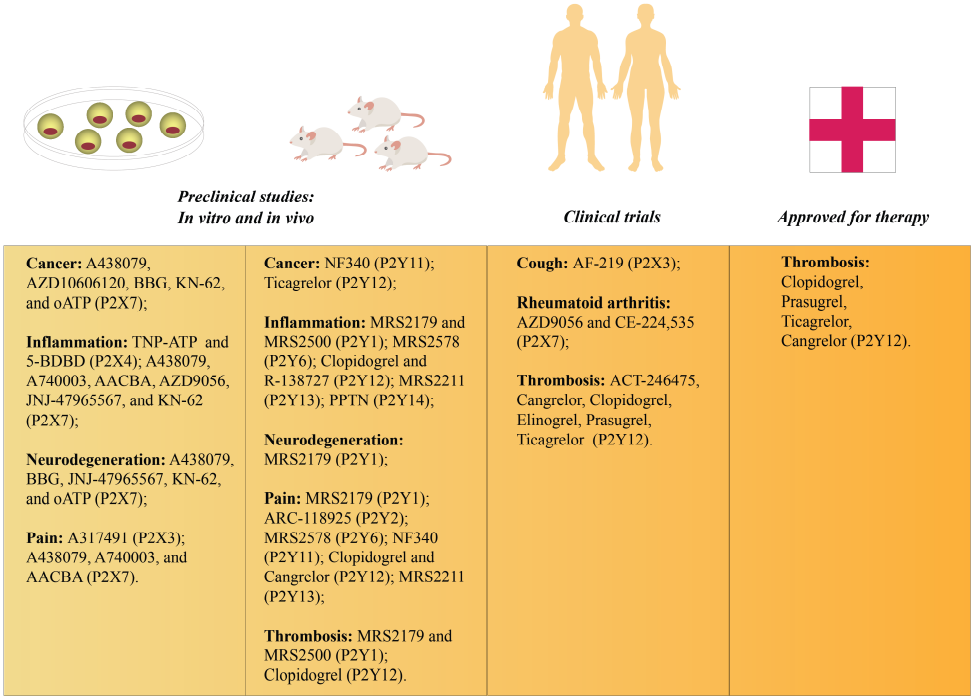

Fig. (1). Advancement of selective antagonist for implementation test in therapy. In the first part of the figure, the main P2 receptor antagonists tested in cells and animal models of cancer, inflammation, neurodegeneration, pain, and thrombosis associating the subtypes P2X3, P2X4, P2Y1, P2Y2, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 are listed. Among all of the antagonists used, only nine advanced to the clinical trial stage (phase 2 and onwards). Currently, only 4 antagonists (selective for the P2Y12 receptor) are approved for use in the prevention of thrombosis in patients at risk for infarction and are commonly used together with aspirin.

be seen in this work. Many research groups worldwide are studying new clinical therapies focusing on diseases related to P2R. Studies relating P2 antagonist applications to HIV have become very promising despite toxic effects and no curative efficacy related to the actual medicines [161]. As discussed above, the correlation between the purinergic system and pain mechanisms is well-established as well as the generation of potential therapeutic approaches [162], which could also be seen in the cancer research context seeking anti-tumor growth therapy [163]. The most important feature in "purinergic therapy" research is the correlation with the immune system, which could be useful for immunotherapeutic treatments for ischemia, organ transplantation, autoimmunity, and cancer [164].

Unfortunately, despite the therapeutic potential of P2 receptors, only the P2Y12 subtype has antagonists that have been applied in therapy. Other antagonists have been explored by pharmaceutical companies to treat diseases related to P2X receptors. Antagonists of the P2X7 receptor, for example, have been studied in the clinical phase to treat inflammatory diseases, such as rheumatoid arthritis, but have failed to demonstrate efficacy [134, 135, 151]. These failures could be related to the polymorphisms of P2X7 since this receptor is highly polymorphic [165]. Sorge *et al.* (2012) demonstrated that a single-nucleotide polymorphism (SNP) with a substitution of a proline to leucine at amino acid 451 (P451L) in the cytosolic domain of P2X7 is associated with less allodynia in mice. The researchers also observed that a missense SNP rs7958311 reduced the amount of chronic pain in patients suffering from osteoarthritis [166]. There is more evidence of these polymorphism effects on P2X7 due to the role of this receptor in tuberculosis. Extracellular ATP that acts on P2X7 is important in the killing of *Mycobacterium tuberculosis* but depends on a functional receptor. However, a variant of P2X7 with a 1513A>C loss-of-function polymorphism increases tuberculosis susceptibility [165]. Thus, a lack of therapy remains.

The absence of selective antagonists for the P2X5 and P2Y4 receptors impairs the characterization of physiological roles associated with these receptors. In this scenario, the search for new molecules with antagonistic activity on these purinergic receptors is very important to achieve therapeutic applications of drugs that act on these molecular targets.

Two important sources to discover new molecules with antagonistic activity on P2 receptors are natural products and synthetic compound libraries. However, these sources require a great demand for testing, the first because of biological richness and the latter due to a large number of compounds deposited. Thus, it is necessary to increase the number of available techniques that are able to solve the screening problem related to evaluating these samples. Therefore, high-throughput screening techniques may facilitate the discovery of new molecules with selective actions for P2 receptors subtypes since they allow for quick and efficient analyses of samples [167-169].

CONCLUSION

P2 purinergic receptors play roles in many biological systems due to their wide expression in mammals. Many therapies have been studied, and molecules with action against P2R have been discovered to seek new possibilities of treatments. Despite all the exciting results obtained on the bench, few antagonists of P2 receptors advanced to the clinical trials; even when an antagonist reaches this stage, the effectiveness of the therapy is not guaranteed, as in the example of P2X7 antagonists. Thus, the scarcity of ligands (agonists and antagonists) that could be useful in humans prevents the clinical applicability of purinergic therapy. Therefore, the search for new compounds with activity for P2 receptors has been growing, but only the P2Y12 subtype has antagonists with applications as medicines in humans. P2Y12 receptor antagonists have a history of success and have been used in therapy for at least two decades to prevent thrombosis in patients at risk for myocardial infarctions. This success story should be the motivation for scientists to develop new drugs with antagonistic activity for the other P2 receptors; thus, in a matter of years, we will have an evolution in the field of purinergic therapy.

LIST OF ABBREVIATIONS

Aβ	=	Amyloid β
ACR20	=	American College of Rheumatology of 20% improvement response
ACR50	=	American College of Rheumatology of 50% improvement response
ACR70	=	American College of Rheumatology of 70% improvement response
ADP	=	Adenosine 5'-diphosphate
APP	=	Amyloid precursor protein
ATP	=	Adenosine 5'-triphosphate
BALF	=	Bronchoalveolar lavage fluid
BBG	=	Brilliant Blue G
CAD	=	Coronary artery disease
cAMP	=	Cyclic adenosine 5'-monophosphate
CINC-1	=	Cytokine-induced neutrophil chemoattractant-1
C_{max}	=	Maximal plasma concentration
COX-2	=	Cyclooxygenase-2
HMGB1	=	High mobility group box 1
IPF	=	Idiopathic pulmonary fibrosis
iNOS	=	Inducible nitric oxide synthase
LPS	=	Lipopolysaccharide
MAPK	=	Mitogen-activated protein kinase
MCP-1	=	Monocyte chemoattractant protein-1
MI	=	Myocardial infarction
MSU	=	Monosodium urate
NETs	=	Neutrophil extracellular traps
NO	=	Nitric oxide
oATP	=	Oxidized adenosine 5'-triphosphate
oxLDL	=	Oxidized low-density lipoprotein
P1R	=	P1 receptors
P2R	=	P2 receptors
PCI	=	Percutaneous coronary intervention
PGE_2	=	Prostaglandin E_2
PPADS	=	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PRU	=	Platelet reactivity index
RA	=	Rheumatoid arthritis
ROS	=	Reactive oxygen species

STEMI = ST elevation myocardial infarction
TRAP = Thrombin receptor activating peptide
UDP = Uridine 5'-diphosphate
UTP = Uridine 5'-triphosphate

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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IV. DISCUSSÃO

No presente trabalho, cujo objetivo foi identificar novos antagonistas para receptores P2, nos detivemos em estudar três subtipos desses receptores. São eles: os subtipos ativados por UTP (P2Y2 e P2Y4) e o receptor ionotrópico P2X7. Eles foram escolhidos devido à carência de antagonistas aplicáveis na clínica, bem como a sua importante contribuição para o desenvolvimento de doenças inflamatórias, câncer e dor. Nos dois primeiros artigos foram apresentados os resultados referentes ao estudo dos receptores P2Y2 e P2Y4, enquanto que o terceiro artigo apresenta os resultados oriundos da pesquisa com o receptor P2X7. Já o quarto artigo apresenta uma compilação entre os principais resultados descritos na literatura conduzidos *in vitro*, *in vivo* e até mesmo em seres humanos, reforçando a importância da pesquisa por novas moléculas com atividade antagonista sobre os receptores P2. Portanto, no tópico de discussão, será realizada uma abordagem crítica sobre os resultados referentes ao estudo com o extrato *Joannesia princeps* Vell., o qual apresentou potencial atividade antagonista sobre os receptores P2Y2 e P2Y4. Em seguida, concluiremos com a discussão dos resultados referentes a molécula CS-15 que foi capaz de inibir significativamente algumas funções relacionadas ao receptor P2X7.

Os receptores P2Y2 e P2Y4 são receptores purinérgicos ativados fisiologicamente por UTP, que promovem um aumento do cálcio intracelular através da via de sinalização fosfolipase C β/ inositol trifosfato [131]. Eles são expressos em diversos tipos celulares tais como células epiteliais e leucócitos, além de órgãos como cérebro, coração, rins, fígado, baço e músculos, onde executam importantes funções [145]. A ativação desses receptores também está relacionada ao desenvolvimento de doenças como a fibrose cística, a doença do olho seco, a doença de Alzheimer e o câncer, o que os tornam importantes alvos-terapêuticos [131,252,253]. Entretanto, a escassez de ligantes seletivos ainda compromete a descoberta de novas funções relacionadas a esses receptores assim como a sua aplicação na terapia, o que estimula a busca por novas moléculas que apresentem essa referida atividade [254].

Portanto, na primeira parte do presente trabalho, nós utilizamos uma metodologia de triagem para a identificação de novos antagonistas para os receptores P2Y2 e P2Y4 previamente padronizada pelo nosso grupo de pesquisa, através da qual foi possível realizar o teste de 162 amostras de extratos e substâncias isoladas de produtos naturais. Dessas amostras, um total de 84 foram testadas durante o Doutorado. Os resultados obtidos na triagem encontram-se parcialmente apresentados no Artigo 1 ou no Apêndice 1. Dentre as amostras testadas, observamos que três extratos foram capazes de inibir significativamente e de forma concentração-dependente a mobilização de cálcio intracelular induzida pelo UTP, apresentando IC_{50s} abaixo da concentração de 50 μg/mL. São eles: o extrato de galhos da

espécie botânica *Joannesia princeps* Vell., e os dois extratos (flores e folhas) do gênero botânico *Peixotoa* A. Juss. Entretanto, devido a problemas de escassez de amostra, bem como a dificuldades de caracterização da espécie, nos detivemos em estudar a resposta da espécie *J. princeps* Vell. de forma mais detalhada.

O segundo passo que demos quanto ao estudo da atividade antagonista do extrato de *J. princeps* Vell. foi realizar a sua caracterização farmacológica. Para isso, investigamos qual seria o perfil de resposta desse extrato frente à ativação de outros receptores P2 que estão associados com a sinalização de cálcio, isto é, P2X em geral, além de P2Y1, P2Y6 e P2Y11 [15]. A fim de estimular os receptores P2X de forma mais ampla, utilizamos o agonista fisiológico ATP, contudo não observamos nenhuma resposta inibitória. O ATP é um agonista não-seletivo dos receptores P2X e também é capaz de ativar os receptores P2Y2 e P2Y11. A faixa de EC_{50} para ativação desses receptores varia de 0,1 a 10 μM, com exceção do P2X7, cuja ativação ocorre em concentrações superiores a 100 μM [15]. No entanto, como a linhagem celular utilizada (J774.G8) expressa diversos subtipos de receptores P2 [218,255], a resposta de cálcio observada após o estímulo com o ATP poderia ser uma espécie de somatório da ativação de vários receptores simultaneamente. Assim, mesmo que o extrato fosse capaz de inibir somente o subtipo P2Y2, através da "adição" da resposta de outros receptores, esse efeito poderia tornar-se imperceptível. Na tentativa de selecionar parte dessa resposta somente para os receptores P2X, utilizamos o BzATP, o qual é um análogo do ATP. Apesar do BzATP ser considerado um agonista potente do receptor P2X7, ele também é capaz de ativar o receptor P2X4, além de funcionar como um agonista parcial para os receptores P2X1, P2X2, P2X3 e P2X5 [75]. Apesar disso, o perfil de resposta obtido foi similar ao observado para o ATP. A técnica de permeabilização celular também foi empregada na verificação de uma possível atividade do extrato sobre o P2X7, o qual é amplamente expresso nessa linhagem celular. Entretanto, nenhuma resposta inibitória do extrato sobre o receptor P2X7 foi observada.

Os agonistas fisiológicos dos receptores P2Y1 e P2Y6 (ADP e UDP, respectivamente) também foram utilizados, todavia, o extrato apenas inibiu parcialmente a resposta do UDP. Interessantemente, o UDP é um produto da degradação do UTP a partir da ação de enzimas ectonucleotidases. Por partilharem de estrutura semelhante, é provável que os sítios de ligação de ambos os ligantes (UTP e UDP) aos receptores P2Y possuam algum grau de homologia, o que explicaria facilmente a capacidade de inibição somente para esses subtipos. Contudo, alguns estudos recentes demonstraram que a ligação do UDP ao receptor P2Y6 resulta em modificações conformacionais a nível quaternário da proteína, enquanto que a ativação dos receptores P2Y2 e P2Y4 pelo UTP não resulta em nenhuma modificação estrutural [256,257].

Uma vez que não podemos descartar a possibilidade do extrato funcionar como um modulador alostérico negativo, essas modificações conformacionais poderiam continuar ocorrendo mesmo em um nível menor, mas suficiente para que o receptor P2Y6 ainda permaneça em estado ativo.

Visto que o UTP é capaz de ativar tanto o P2Y2 quanto o P2Y4, foram utilizados agonistas seletivos para cada um desses receptores com o objetivo de tentar identificar se o extrato conseguiria inibir um único subtipo ou mesmo ambos [22]. Contudo, os dados sugerem que o extrato é capaz de inibir a resposta de ambos receptores. Embora eles sejam considerados agonistas seletivos, sabe-se que dependendo da concentração utilizada (faixa de submicromolar), eles podem apresentar uma determinada perda de seletividade [258,259]. Com todos esses desafios, o experimento que deve ser feito na tentativa de concluir essa caracterização farmacológica é a utilização de células transfectadas com um único subtipo de receptor P2Y a fim de se obter uma resposta mais seletiva.

Outro ponto importante que nos questionamos foi se a inibição causada pelo extrato poderia ser decorrente de um efeito *quenching*. Uma vez que algumas substâncias oriundas de plantas são capazes de emitir fluorescência ou apresentar efeito *quenching* (ex: quercetina e vitexina), deve-se analisar os resultados obtidos com cautela a fim de verificar se eles não são frutos de artefatos experimentais [260]. Nesse sentido, foi realizado um teste com a ionomicina, um ionóforo de cálcio que promove uma elevação dos níveis intracelulares desse íon, resultando em um aumento da intensidade de fluorescência proporcional a esse efeito. Com isso, foi observado que a presença do extrato de *J. princeps* Vell. não resultou em nenhuma mudança no perfil de resposta da ionomicina, eliminando assim, a possibilidade de um efeito *quenching*.

Perguntamo-nos ainda se a abertura dos canais de cálcio operados por estoque (SOC) poderia influenciar a resposta do extrato [261]. Essa elevação dos níveis de cálcio mediante a abertura de canais SOC poderia estar dificultando a observação da mobilização de cálcio através da via da fosfolipase C/ IP_3. Portanto, através da remoção do cálcio extracelular da solução tampão, esperávamos continuar observando a resposta inibitória do UTP. Surpreendentemente, a mobilização de cálcio reduziu drasticamente e a resposta do extrato foi similar à resposta basal do UTP. Isso nos levou a questionar dois fatos. O primeiro foi a possibilidade de o extrato estar inibindo a resposta de canais SOC ao invés de inibir os receptores. A segunda questão foi se de fato, o extrato inibisse canais SOC, não observaríamos uma resposta de inibição mesmo que parcial sobre a mobilização de cálcio induzida pelos demais agonistas como ATP, ADP, BzATP e UDP? Outra hipótese é de que a

ausência de cálcio extracelular poderia ter produzido algum tipo de artefato experimental ou algum fenômeno não esclarecido que poderia ter afetado a resposta observada.

Outra preocupação que tivemos foi quanto à aplicabilidade do extrato como possível fitoterápico. A primeira amostra de extrato utilizada no presente trabalho foi extraída a partir de uma mistura dos solventes metanol e diclorometano. Entretanto, de acordo com as regulamentações brasileira (Agência Nacional de Vigilância Sanitária – ANVISA) e americana (*Food and Drug Administration* - FDA), para que um fármaco de origem vegetal seja aprovado como um fitoterápico, ele necessita ter sido extraído em solução aquosa ou etanólica [214,262]. Com isso, foi realizada uma nova extração utilizando o etanol como solvente. Semelhantemente ao que já havia sido observado com o extrato metanólico, esse novo extrato etanólico também foi capaz de inibir a resposta do UTP em células J774.G8 e macrófagos peritoneais, além de não produzir citotoxicidade em até 24h de tratamento.

J. princeps Vell. é uma espécie botânica encontrada na Mata Atlântica e Caatinga no Brasil, bem como em algumas regiões nos continentes Africano e Asiático. Ela pertence à família Euphorbiaceae, cujos indivíduos medem em torno de 20 m de altura. No Brasil, ela é popularmente conhecida como "cotieira" e possui valor econômico no paisagismo e extração de madeira [263,264]. Essa espécie também possui uso medicinal, contudo, a única parte da planta que é utilizada para essa finalidade são as sementes. O óleo presente nas sementes é utilizado como laxante e cicatrizante, enquanto que o extrato dessa parte da planta possui atividades anti-helmínticas [264,265]. Por outo lado, segundo relatos da medicina popular, a *J. princeps* Vell. ainda é utilizada para o tratamento de desordens menstruais e digestivas, febre perniciosa, doenças microbianas, sífilis e inchaço [264]. Apesar disso, há uma escassez de estudos que demonstram o potencial uso medicinal de outras partes dessa planta, como folha, caule, raiz e flor. *J. princeps* Vell. apresenta moléculas pertencentes às classes de sesquiterpenos, bis-sesquiterpenos, diterpenos, triterpenos e esteróides [266]. No entanto, os poucos trabalhos que descrevem a fitoquímica e as propriedades medicinais dessa espécie detêm-se apenas nas sementes e folhas. Nesse sentido, o estudo da composição química e aplicação medicinal de extratos dos galhos ainda são escassos, sendo o nosso grupo pioneiro nesse cenário. Como resultado preliminar acerca da obtenção do perfil químico da *J. princeps* Vell., observamos a presença de pelo menos 45 substâncias no extrato através de um ensaio de cromatografia líquida de alta performance (*high performance liquid chromatography* – HPLC). Entretanto, a caracterização da molécula ativa, responsável pela atividade antagonista, encontra-se entre um dos objetivos do nosso grupo de pesquisa, uma vez que é imprescindível para o desenvolvimento de um novo fármaco, embora não seja necessária para a elaboração de um medicamento fitoterápico [267].

A caracterização da molécula ativa responsável pela inibição do P2Y2 e P2Y4 certamente contribuirá para a farmacologia desses receptores. Apesar do P2Y2 já possuir um antagonista seletivo descrito, este não conseguiu avançar para a aplicação clínica devido a algumas propriedades farmacológicas desfavoráveis, como alta polaridade e baixa disponibilidade pela via oral [49]. O P2Y4, por outro lado, ainda não possui antagonistas seletivos, o que dificulta a caracterização de suas funções fisiológicas. Nesse sentido, o antagonista RB2 geralmente é utilizado na obtenção de alguma referência acerca de um possível efeito inibitório. Todavia, devido à sua capacidade de inibir outros receptores P2, os resultados devem ser avaliados com cautela. Nesse cenário, a descrição de um antagonista que atue sobre ambos receptores, mesmo que sem seletividade, poderá contribuir para o desenvolvimento de diversos estudos *in vitro* e *in vivo*, e ainda pode servir como um modelo estrutural para a síntese e desenvolvimento de novos antagonistas seletivos para esses receptores [49].

Enquanto isso, a segunda parte desse trabalho se deteve na pesquisa por novos candidatos a antagonistas para o receptor P2X7, cuja ativação além de promover um fluxo de cátions através da membrana, está relacionada com a formação de um poro membranar e a indução de uma série de respostas com perfil pró-inflamatório [69,102,167,169–172]. Além disso, diversos estudos vêm demonstrando a participação desse receptor no desenvolvimento de doenças inflamatórias, neurodegenerativas e infecciosas, o que o torna um importante alvo terapêutico [172,268]. Embora o uso de antagonistas em modelos *in vivo* de inflamação e dor tenham demonstrado efeitos anti-inflamatórios e antinociceptivos significativos, as moléculas que avançaram para a etapa de ensaios clínicos foram reprovados nessa fase [172]. Assim, essa lacuna na terapia associada ao P2X7 estimula a pesquisa por novas moléculas com atuação sobre este receptor e que possam ser usadas na clínica para o tratamento dessas doenças.

Nesse contexto, executamos uma mini-campanha de triagem com o objetivo de identificar novos antagonistas para o receptor P2X7 a partir de produtos naturais. Ao longo dessa campanha, identificamos que a molécula CS-15 apresentou-se como um *hit* promissor devido à sua habilidade de inibir as funções do P2X7 *in vitro* e *in vivo*.

Entretanto, para realizarmos essa triagem utilizamos uma metodologia de permeabilização celular previamente padronizada pelo nosso grupo de pesquisa para ser aplicada à descoberta de novos antagonistas para o receptor P2X7 [247]. O corante utilizado na padronização desse protocolo foi o iodeto de propídeo. Já no presente trabalho, nós reproduzimos o mesmo protocolo, com exceção do corante que foi substituído pelo YO-PRO-

1, cujo uso vem se popularizando em ensaios de permeabilização celular. Essa modificação no protocolo nos permitiu verificar a reprodutibilidade da metodologia previamente padronizada.

Uma vez que realizamos uma pequena modificação no protocolo, algumas observações precisaram ser feitas antes da sua implementação no processo de triagem. A primeira foi em relação à escolha da concentração do corante que seria utilizada. Em um teste realizado preliminarmente foram avaliadas diferentes concentrações do corante (YO-PRO-1) na faixa de 62,5 nM a 8 μM (dados não mostrados). Contudo, observamos que o equipamento utilizado era capaz somente de distinguir diferenças entre a fluorescência basal e a relacionada à captação do corante via ativação do P2X7 a partir da concentração de 1 μM. Assim, adotamos essa concentração como ponto de partida para estudo da relação sinal-ruído. Com isso, observamos que a relação sinal-ruído para a concentração de 1 μM de corante era de 1,42, enquanto que com o dobro dessa concentração (2 μM), a relação aumentava para 1,65. Apesar de haver pouca diferença entre a relação sinal-ruído das duas concentrações estudadas, a concentração de 2 μM foi adotada para uso por possuir respaldo da literatura, uma vez que é utilizada em diversos trabalhos científicos [248,269–271]. Além disso, o valor obtido para a relação sinal-ruído a 2 μM foi similar ao detectado por Rat et al. (2017) para o YO-PRO-1 (2 μM) em linhagem celular de retina humana (ARPE-19) e queratinócitos (HaCat) (1,7 e 1,45, respectivamente) na presença de 1 e 10 mM de ATP [248]. Outra vantagem de ter escolhido essa concentração foi a relação custo-benefício, ou seja, optar por uma concentração maior do que essa (2 μM), certamente iria gerar um aumento do consumo e, consequentemente, maior gasto com reagentes.

Com a concentração do YO-PRO-1 definida, os experimentos em espectrofotômetro prosseguiram com a avaliação do perfil de captação do corante na presença do ATP. O EC_{50} detectado para o ATP foi de 2,18 mM, o qual se encontra dentro da faixa do EC_{50} descrito para o receptor P2X7 (2 a 4 mM) [75]. Contudo, em nosso trabalho prévio em que foi utilizado o iodeto de propídeo, o EC_{50} encontrado ficou entre 0,7114 e 0,7663 mM [247]. Essa variação pode ser decorrente da diferença de sensibilidade entre os equipamentos, os corante ou mesmo os subclones de células J774.G8 utilizados. Um subclone distinto de uma mesma linhagem celular pode por alguma razão, expressar o receptor P2X7 em menor densidade ou ainda apresentar um variante de *splice* diferente, que pode possuir uma determinada resistência em formar o poro membranar [272]. Apesar disso, o fator z', uma medida de robustez do ensaio, foi calculado e o valor encontrado foi de 0,59, indicando que o protocolo estava adequado para uso. Esse valor foi semelhante ao encontrado em nosso protocolo com o iodeto de propídeo (0,635), porém menor do que o identificado para o corante amarelo de

lúcifer (0,876), sugerindo que possam existir variações quanto à sensibilidade dos corantes [247].

O perfil de inibição foi avaliado utilizando-se dois antagonistas do P2X7: o BBG e o A-740003. O IC_{50} detectado para o antagonista BBG foi de 106 nM e encontra-se dentro da faixa de IC_{50} descrita na literatura, a qual varia entre 15 a 250 nM [75]. Por outro lado, esse valor foi menor do que o identificado em nosso protocolo com o iodeto de propídeo, que variou entre 1,3 a 2,6 μM [247]. Enquanto isso, o antagonista seletivo do receptor P2X7, o A-740003, apresentou um IC_{50} de 511 nM, o qual também encontra-se dentro da faixa de IC_{50} descrita na literatura (entre 20 a 700 nM) [75].

Com a caracterização do perfil do YO-PRO-1 aplicado a ensaios de permeabilização celular, foi realizada uma mini-campanha de triagem, na qual 134 amostras foram testadas, cujos resultados estão apresentados no Apêndice 2. Durante essa campanha, a molécula CS-15, um triterpeno isolado da planta *Clusia studartiana* C. M. Vieira & Gomes da Silva, foi identificada como principal *hit*, uma vez que ela foi capaz de inibir a captação do YO-PRO-1 de forma concentração-dependente. Além disso, ela exibiu um IC_{50} na faixa de nanomolar, enquanto que a maioria dos *hits* apresenta a atividade almejada entre 1 a 10 μM [273]. O referida molécula também não afetou a viabilidade celular mesmo após 24h de tratamento.

Nós ainda tentamos verificar se a molécula seria capaz de inibir a mobilização de cálcio induzida por ATP. Entretanto, com a finalidade de ativar o receptor P2X7, foi necessário utilizar uma alta concentração de ATP (100 μM), a qual é capaz de ativar outros receptores P2. Uma vez que as células J774.G8 expressam diversos subtipos de receptores P2, incluindo P2X e P2Y [218,255], não fomos capazes de observar a inibição esperada utilizando esse modelo. Uma alternativa para alcançar uma maior taxa de sucesso nesse experimento seria o uso de células transfectadas com o P2X7 ou o uso do BzATP, um agonista mais potente, porém não seletivo para esse receptor [75]. Por fim, também avaliamos outro nucleotídeo, o UTP, a fim de observar se essa inibição se estenderia para receptores P2Y, porém a molécula não foi capaz de bloquear a sua resposta de cálcio. Ainda assim, novos ensaios com agonistas seletivos de outros receptores P2 e com células transfectadas com o receptor P2X7 humano (hP2X7) devem ser feitos para confirmar se de fato a molécula possui seletividade para o esse receptor. Além disso, experimentos utilizando a técnica de *patch-clamp* na modalidade *whole cell*, que atualmente é considerada o padrão-ouro na investigação de canais iônicos, podem contribuir significantemente para caracterizar a atividade da molécula CS-15 sobre o P2X7.

Finalmente, o efeito da molécula foi testado em modelo experimental de dor produzido pela injeção intraplantar de formalina. Esse modelo experimental foi desenvolvido

na década de 1970 e ainda hoje é útil para a triagem de novas moléculas com atividade analgésica e anti-inflamatória [274,275]. Através da aplicação desse modelo, é possível avaliar o comportamento nociceptivo dos animais em duas fases distintas. A primeira fase inicia-se logo após a injeção da formalina e dura cerca de 5 minutos, sendo caracterizada pela ativação química dos nociceptores. Já a segunda fase inicia-se entre 15 a 30 minutos após a injeção da formalina e é caracterizada pelo envolvimento do processo inflamatório no desenvolvimento da dor [275]. Utilizando esse modelo, alguns grupos de pesquisa observaram o efeito analgésico e anti-inflamatório de algumas plantas medicinais, inclusive com ação sobre o P2X7 [222,274]. Apesar disso, em nossos ensaios, a molécula CS-15 reduziu significativamente os sintomas da dor nociceptiva e inflamatória. Ainda não sabemos se esse efeito é dependente ou independente da ativação do P2X7. Para isso, novos experimentos nos quais a análise de citocinas como a IL-1β e outros medidadores inflamatórios relacionados à ativação do P2X7 necessitam ser investigados *in vivo*. Em todo caso, resultados similares a esses já foram observados em experimentos com outros antagonistas do P2X7, o que demonstra o potencial farmacológico dessa molécula [119,222].

C. studartiana C.M. Vieira & Gomes da Silva é uma espécie botânica pertencente à família Clusiaceae. Ela é uma árvore de hábito terrestre, que ocasionalmente pode apresentar forma hemiepífita. Essa espécie é endêmica da Mata Atlântica brasileira na região Sudeste do país [276]. Embora essa espécie tenha sido descrita no início dos anos 1990, até o momento não se tem nenhuma informação na literatura acerca de suas propriedades medicinais, o que torna o nosso trabalho pioneiro nesse sentido. Apesar disso, a família Clusiaceae possui alguns indivíduos com conhecidas propriedades medicinais. O látex presente nos frutos de *Clusia* sp. são popularmente utilizados no tratamento de doenças reumáticas e candidíase oral infantil, enquanto que as sementes de *C. grandiflora* apresentam moléculas com atividade antimicrobiana [277–280]. Em todo o caso, o nosso estudo demonstrou pela primeira vez o efeito analgésico de uma molécula isolada da planta *C. studartiana*, possivelmente causado pela inibição do receptor P2X7. Com isso, acreditamos que esses achados contribuirão para o escasso campo da terapia purinérgica no futuro assim para o conhecimento de propriedades medicinais oriundas de produtos naturais.

V. CONCLUSÃO

Os resultados apontaram que o extrato de galhos da espécie botânica *Joannesia princeps* Vell. foi capaz de inibir a mobilização de cálcio intracelular induzida por UTP de forma concentração-dependente, sendo que esse efeito não foi provocado por citotoxicidade ou fenômeno *quenching*. Em virtude de o extrato ter sido capaz de inibir parcialmente a mobilização de cálcio induzida por UDP, os resultados sugerem uma possível seletividade quanto à sua ação antagonista sobre os receptores P2Y ativados por nucleotídeos derivados da uridina.

Enquanto isso, os resultados obtidos com a pesquisa de novos antagonistas para o receptor P2X7 apontaram para a descoberta de um novo candidato a antagonista para esse receptor. A molécula CS-15, um triterpeno isolado da planta *Clusia studartiana* C. M. Vieira & Gomes da Silva, foi capaz de inibir a captação do YO-PRO-1, função relacionada com a ativação do P2X7 de forma concentração-dependente, apresentando atividade na faixa de nanomolar. Além disso, essa molécula não demonstrou toxicidade *in vitro* e foi capaz de aliviar os sintomas de nocicepção em modelo experimental animal.

VI. REFERÊNCIAS

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Tabela: Efeito dos extratos sobre a mobilização de cálcio induzida pelo UTP

Nº	Rack	Código do extrato	Média com UTP	Controle (UTP)	Efeito	Significância	Nº de replicatas
01	FarMang	PN-1	100,2 ± 21,58	104,6 ± 17,34	↓ 4%	ns	9
02	FarMang	PN-2	98,39 ± 27,38	104,6 ± 17,34	↓ 5%	ns	9
03	FarMang	PN-3	103,2 ± 23,89	104,6 ± 17,34	↓ 1%	ns	9
04	FarMang	PN-4	97,50 ± 19,93	104,6 ± 17,34	↓ 7%	ns	9
05	FarMang	PN-5	112,8 ± 38,70	104,6 ± 17,34	↑ 8%	ns	9
06	FarMang	PN-6	104,1 ± 22,67	104,6 ± 17,34	↓ 0,5%	ns	9
07	FarMang	PN-7	104,7 ± 21,72	104,6 ± 17,34	↑ 0,1%	ns	9
08	FarMang	PN-8	105,9 ± 25,01	104,6 ± 17,34	↑ 1%	ns	9
09	FarMang	PN-9	100,3 ± 24,62	104,6 ± 17,34	↓ 4%	ns	9
10	FarMang	PN-10	112,6 ± 40,28	104,6 ± 17,34	↑ 8%	ns	9
11	FarMang	CS-3	110,3 ± 28,12	104,6 ± 17,34	↑ 5%	ns	9
12	FarMang	CS-4	116,3 ± 26,74	104,6 ± 17,34	↑ 11%	ns	9
13	FarMang	CS-5	110,3 ± 26,46	104,6 ± 17,34	↑ 5%	ns	9
14	FarMang	CS-15	118,7 ± 27,21	104,6 ± 17,34	↑ 13%	ns	9
15	FarMang	AG	124,9 ± 27,32	104,6 ± 17,34	↑ 19%	ns	9
16	FarMang	GM	129,1 ± 27,32	104,6 ± 17,34	↑ 23%	ns	9
17	FarMang	OXO	125,2 ± 21,75	104,6 ± 17,34	↑ 20%	ns	9
18	FarMang	LNFLSE	89,83 ± 46,46	93,10 ± 24,49	↓ 4%	ns	9
19	FarMang	LNFLSEH	91,58 ± 35,95	93,10 ± 24,49	↓ 2%	ns	9
20	FarMang	LNFLSED	79,30 ± 33,02	93,10 ± 24,49	↓ 15%	ns	9
21	FarMang	LNFLSEA	93,01 ± 48,61	93,10 ± 24,49	↓ 0,1%	ns	9
22	FarMang	LNFLSEAc	87,70 ± 49,53	93,10 ± 24,49	↓ 6%	ns	9
23	FarMang	LNFLSEB	30,61 ± 34,62	93,10 ± 24,49	↓ 67%	**	9
24	FarMang	LNGSC	54,97 ± 33,96	93,10 ± 24,49	↓ 41%	ns	9
25	FarMang	LNGSE	21,35 ± 11,26	93,10 ± 24,49	↓ 77%	***	9
26	FarMang	LNGSH	82,50 ± 24,16	93,10 ± 24,49	↓ 11%	ns	9
27	FarMang	LNGED	36,79 ± 22,26	93,10 ± 24,49	↓ 60%	**	9
28	FarMang	LNGSEA	28,62 ± 30,99	93,10 ± 24,49	↓ 69%	***	9
29	FarMang	LNGSEAc	90,47 ± 49,06	93,10 ± 24,49	↓ 3%	ns	9
30	FarMang	LNGSEB	85,75 ± 49,34	93,10 ± 24,49	↓ 8%	ns	9
31	FarMang	MGEF	85,04 ± 27,07	86,52 ± 17,86	↓ 2%	ns	9
32	FarMang	MGEFD	77,69 ± 33,60	86,52 ± 17,86	↓ 10%	ns	9
33	FarMang	MGEFB	82,84 ± 26,99	86,52 ± 17,86	↓ 4%	ns	9
34	FarMang	MGEFAc	93,12 ± 22,34	86,52 ± 17,86	↑ 8%	ns	9
35	FarMang	MGEFH	70,12 ± 27,69	86,52 ± 17,86	↓ 19%	ns	9
36	FarMang	SBFE3	48,94 ± 20,92	75,68 ± 23,51	↓ 35%	ns	12
37	FarMang	SBFE3H	89,72 ± 26,08	75,68 ± 23,51	↑ 19%	ns	12
38	FarMang	SBFE3D	89,38 ± 25,13	75,68 ± 23,51	↑ 18%	ns	12
39	FarMang	SBFE3Bu	40,76 ± 13,72	75,68 ± 23,51	↓ 46%	*	12
40	FarMang	SBFE3Aq	19,70 ± 14,00	75,68 ± 23,51	↓ 74%	***	12
41	FarMang	STFLSE70	15,51 ± 13,04	76,12 ± 26,93	↓ 80%	***	12
42	FarMang	STFLSE70D	51,46 ± 30,60	76,12 ± 26,93	↓ 32%	ns	12
43	FarMang	STFLSE70A	7,136 ± 7,445	76,12 ± 26,93	↓ 91%	***	12
44	FarMang	STFLSE70PPT	57,70 ± 18,01	76,12 ± 26,93	↓ 24%	ns	11
45	FarMang	TCIE	73,34 ± 23,40	76,12 ± 26,93	↓ 4%	ns	11
46	FarMang	TCIEH	85,87 ± 31,57	76,12 ± 26,93	↑ 13%	ns	12
47	FarMang	TCIED	71,32 ± 20,46	76,12 ± 26,93	↓ 6%	ns	12
48	FarMang	TCIEBu	46,10 ± 22,73	76,12 ± 26,93	↓ 39%	ns	12
49	FarMang	TCIEAc	57,28 ± 25,94	76,12 ± 26,93	↓ 25%	ns	12

50	FarMang	JPF	50,66 ± 21,73	76,12 ± 26,93	↓ 33%	ns	12
51	FarMang	JPFH	83,05 ± 45,18	76,12 ± 26,93	↑ 9%	ns	10
52	FarMang	JPFD	49,04 ± 25,47	76,12 ± 26,93	↓ 36%	ns	12
53	FarMang	JPFAc	63,42 ± 23,75	76,12 ± 26,93	↓ 17%	ns	12
54	FarMang	JPFB	51,20 ± 21,40	76,12 ± 26,93	↓ 33%	ns	12
55	FarMang	JPFAq	60,39 ± 19,86	76,12 ± 26,93	↓ 21%	ns	12
56	FarMang	JPG MeOH	49,91 ± 18,79	76,12 ± 26,93	↓ 34%	ns	12
57	FarMang	JPG	37,19 ± 13,15	76,12 ± 26,93	↓ 51%	*	12
58	FarMang	JPGH	86,70 ± 49,15	76,12 ± 26,93	↑ 14%	ns	12
59	FarMang	JPGD	45,39 ± 30,86	76,12 ± 26,93	↓ 40%	ns	12
60	FarMang	JPGAc	67,14 ± 39,25	76,12 ± 26,93	↓ 12%	ns	12
61	FarMang	JPGB	66,50 ± 18,84	76,12 ± 26,93	↓ 13%	ns	12
62	FarMang	JPGAq	75,58 ± 42,88	76,12 ± 26,93	↓ 1%	ns	12

Tabela: Efeito dos extratos sobre a permeabilização celular induzida pelo ATP

Nº	Rack	Código do extrato	Média com ATP	Controle (ATP)	Efeito	Significância	Nº de replicatas
01	FarMang	PN-1	17,59 ± 4,46	28,92 ± 6,12	↓ 39%	**	9
02	FarMang	PN-2	18,12 ± 4,46	28,92 ± 6,12	↓ 37%	*	9
03	FarMang	PN-3	20,42 ± 5,47	28,92 ± 6,12	↓ 29%	ns	9
04	FarMang	PN-4	17,90 ± 6,34	28,92 ± 6,12	↓ 38%	**	9
05	FarMang	PN-5	22,44 ± 8,74	28,92 ± 6,12	↓ 22%	ns	9
06	FarMang	PN-6	28,59 ± 12,14	28,92 ± 6,12	↓ 1%	ns	9
07	FarMang	PN-7	28,52 ± 8,82	28,92 ± 6,12	↓ 1%	ns	9
08	FarMang	PN-8	27,49 ± 10,19	28,92 ± 6,12	↓ 4%	ns	9
09	FarMang	PN-9	29,24 ± 6,92	28,92 ± 6,12	↑ 1%	ns	9
10	FarMang	PN-10	32,46 ± 14,85	28,92 ± 6,12	↑ 12%	ns	9
11	FarMang	CS-3	22,75 ± 7,80	39,12 ± 11,66	↓ 41%	***	9
12	FarMang	CS-4	17, 05 ± 3,67	39,12 ± 11,66	↓ 56%	***	9
13	FarMang	CS-5	21,56 ± 7, 06	39,12 ± 11,66	↓ 44%	***	9
14	FarMang	CS-15	18, 46 ± 6,45	39,12 ± 11,66	↓ 52%	***	9
15	FarMang	AG	17,98 ± 4,95	39,12 ± 11,66	↓ 54%	***	9
16	FarMang	GM	15,76 ± 3,60	39,12 ± 11,66	↓ 59%	***	9
17	FarMang	OXO	30,86 ± 11, 80	39,12 ± 11,66	↓ 21%	ns	9
18	J774 04	JA7	21,70 ± 6,94	39,12 ± 11,66	↓ 44%	*	9
19	J774 04	JA8	25,73 ± 16,15	39,12 ± 11,66	↓ 34%	ns	9
20	J774 04	JA9	29,58 ± 8,85	39,12 ± 11,66	↓ 24%	ns	9
21	J774 04	JA10	25,99 ± 8,86	39,12 ± 11,66	↓ 33%	ns	9
22	J774 04	JA11	28,83 ± 7,99	39,12 ± 11,66	↓ 26%	ns	9
23	J774 04	JB7	22,82 ± 8,83	39,12 ± 11,66	↓ 41%	*	9
24	J774 04	JB8	24,44 ± 12,69	39,12 ± 11,66	↓ 37%	ns	9
25	J774 04	JB9	27,12 ± 11,67	39,12 ± 11,66	↓ 30%	ns	9
26	J774 04	JC7	23,52 ± 7,88	39,12 ± 11,66	↓ 39%	*	9
27	113 J774	RA2	16,05 ± 4,29	42,70 ± 12,82	↓ 62%	***	9
28	113 J774	RA4	18,39 ± 2,13	42,70 ± 12,82	↓ 56%	***	9
29	113 J774	RA5	48,24 ± 13,07	42,70 ± 12,82	↑ 12%	ns	9
30	113 J774	RA6	38,60 ± 25,86	42,70 ± 12,82	↓ 9%	ns	9
31	113 J774	RA7	35,41 ± 13,55	42,70 ± 12,82	↓ 17%	ns	9
32	113 J774	RA8	33,87 ± 20,05	42,70 ± 12,82	↓ 20%	ns	9
33	113 J774	RA9	17,13 ± 4,69	42,70 ± 12,82	↓ 59%	***	9
34	113 J774	RA10	17,98 ± 1,54	42,70 ± 12,82	↓ 57%	***	9
35	113 J774	RA11	29,62 ± 10,62	42,70 ± 12,82	↓ 30%	*	9
36	113 J774	RB2	25,51 ± 16,14	40,55 ± 9,68	↓ 37%	ns	9
37	113 J774	RB4	28,43 ± 11,32	40,55 ± 9,68	↓ 29%	ns	9
38	113 J774	RB5	22,27 ± 5,29	40,55 ± 9,68	↓ 45%	*	9
39	113 J774	RB6	22,59 ± 4,46	40,55 ± 9,68	↓ 44%	*	9
40	113 J774	RB7	20,49 ± 1,21	40,55 ± 9,68	↓ 49%	*	9
41	113 J774	RB8	22,41 ± 5,38	40,55 ± 9,68	↓ 44%	*	9
42	113 J774	RB9	56,92 ± 28,86	40,55 ± 9,68	↑ 40%	ns	9
43	113 J774	RB10	47,03 ± 25,33	40,55 ± 9,68	↑ 15%	ns	9
44	113 J774	RB11	41,46 ± 18,63	40,55 ± 9,68	↑ 2%	ns	9
45	113 J774	RC2	32,73 ± 15,13	41,93 ± 10,77	↓ 21%	ns	9
46	113 J774	RC4	40,54 ± 12,42	41,93 ± 10,77	↓ 3%	ns	9
47	113 J774	RC5	36,16 ± 12,72	41,93 ± 10,77	↓ 13%	ns	9
48	113 J774	RC6	38,89 ± 13,24	41,93 ± 10,77	↓ 7%	ns	9
49	113 J774	RC7	29,18 ± 5,06	41,93 ± 10,77	↓ 30%	ns	9

50	113 J774	RC8	27,45 ± 6,35	41,93 ± 10,77	↓ 34%	ns	9
51	Rack 80	MA2	18,43 ± 4,90	22,99 ± 7,156	↓ 19%	ns	9
52	Rack 80	MA3	17,62 ± 2,846	22,99 ± 7,156	↓ 23%	ns	9
53	Rack 80	MA4	16,07 ± 2,169	22,99 ± 7,156	↓ 30%	*	9
54	Rack 80	MA5	17,83 ± 4,841	22,99 ± 7,156	↓ 22%	ns	9
55	Rack 80	MA6	13,51 ± 3,288	22,99 ± 7,156	↓ 41%	***	9
56	Rack 80	MA7	24,94 ± 20,19	22,99 ± 7,156	↑ 8%	ns	9
57	Rack 80	MA8	25,05 ± 13,95	22,99 ± 7,156	↑ 8%	ns	9
58	Rack 80	MA9	20,71 ± 9,429	22,99 ± 7,156	↓ 9%	ns	9
59	Rack 80	MA10	17,68 ± 6,088	22,99 ± 7,156	↓ 23%	ns	9
60	Rack 80	MA11	22,09 ± 8,255	22,99 ± 7,156	↓ 3%	ns	9
61	Rack 80	MB2	19,23 ± 6,731	22,99 ± 7,156	↓ 16%	ns	9
62	Rack 80	MB3	24,74 ± 11,77	22,99 ± 7,156	↑ 7%	ns	9
63	Rack 80	MB4	14,70 ± 3,54	20,51 ± 3,98	↓ 28%	ns	9
64	Rack 80	MB5	14,62 ± 3,043	20,51 ± 3,98	↓ 28%	ns	9
65	Rack 80	MB7	12,88 ± 3,717	20,51 ± 3,98	↓ 37%	**	9
66	Rack 80	MB8	13,56 ± 2,36	20,51 ± 3,98	↓ 33%	**	9
67	Rack 80	MB9	13,56 ± 1,89	20,51 ± 3,98	↓ 33%	**	9
68	Rack 80	MB10	16,50 ± 6,03	20,51 ± 3,98	↓ 19%	ns	9
69	Rack 80	MB11	18,04 ± 3,39	20,51 ± 3,98	↓ 12%	ns	9
70	Rack 80	MC2	20,94 ± 10,04	20,51 ± 3,98	↑ 2%	ns	9
71	Rack 80	MC3	18,07 ± 4,32	20,51 ± 3,98	↓ 11%	ns	9
72	Rack 80	MC4	22,32 ± 4,63	20,51 ± 3,98	↑ 8%	ns	9
73	Rack 80	MC5	18,81 ± 5,22	20,51 ± 3,98	↓ 8%	ns	9
74	Rack 80	MC6	17,10 ± 4,55	27,77 ± 4,719	↓ 38%	***	9
75	Rack 80	MC7	15,70 ± 3,16	27,77 ± 4,719	↓ 43%	***	9
76	Rack 80	MC8	15,39 ± 8,412	27,77 ± 4,719	↓ 44%	*	9
77	Rack 80	MC9	14,90 ± 1,503	27,77 ± 4,719	↓ 46%	***	9
78	Rack 80	MC10	14,98 ± 2,347	27,77 ± 4,719	↓ 46%	***	9
79	Rack 80	MC11	19,45 ± 15,81	27,77 ± 4,719	↓ 29%	ns	9
80	Rack 80	MD2	23,12 ± 6,538	27,77 ± 4,719	↓ 16%	ns	9
81	Rack 80	MD3	24,59 ± 6,166	27,77 ± 4,719	↓ 11%	ns	9
82	Rack 80	MD4	26,06 ± 6,722	27,77 ± 4,719	↓ 6%	ns	9
83	Rack 80	MD5	23,30 ± 9,857	27,77 ± 4,719	↓ 16%	ns	9
84	Rack 80	MD6	17,95 ± 6,88	27,77 ± 4,719	↓ 35%	ns	9
85	Rack 80	MD8	13,44 ± 3,62	27,77 ± 4,719	↓ 34%	**	9
86	Rack 80	MD9	19,71 ± 8,245	27,77 ± 4,719	↓ 29%	ns	9
87	FarMang	LNFLSE	13,21 ± 4,266	18,83 ± 6,40	↓ 29%	ns	9
88	FarMang	LNFLSEH	13,72 ± 2,147	18,83 ± 6,40	↓ 27%	*	9
89	FarMang	LNFLSED	13,22 ± 5,56	18,83 ± 6,40	↓ 29%	ns	9
90	FarMang	LNFLSEA	18,11 ± 9,449	18,83 ± 6,40	↓ 3%	ns	9
91	FarMang	LNFLSEAc	16,78 ± 8,77	18,83 ± 6,40	↓ 10%	ns	9
92	FarMang	LNFLSEB	27,73 ± 11,81	18,83 ± 6,40	↑ 47%	ns	9
93	FarMang	LNGSC	23,30 ± 8,39	18,83 ± 6,40	↑ 3%	ns	9
94	FarMang	LNGSE	18,80 ± 9,321	18,83 ± 6,40	↓ 0,1%	ns	9
95	FarMang	LNGSH	22,11 ± 11,94	18,83 ± 6,40	↑ 17%	ns	9
96	FarMang	LNGED	23,41 ± 13,88	18,83 ± 6,40	↑ 24%	ns	9
97	FarMang	LNGSEA	17,86 ± 9,319	18,83 ± 6,40	↓ 5%	ns	9
98	FarMang	LNGSEAc	20,19 ± 11,38	18,83 ± 6,40	↑ 7%	ns	9
99	FarMang	LNGSEB	13,26 ± 3,053	18,83 ± 6,40	↓ 29%	*	9
100	FarMang	MGEF	16,03 ± 3,477	25,07 ± 5,744	↓ 36%	***	9

101	FarMang	MGEFD	16,08 ± 3,538	25,07 ± 5,744	↓ 35%	***	9
102	FarMang	MGEFB	16,18 ± 4,572	25,07 ± 5,744	↓ 35%	***	9
103	FarMang	MGEFAc	17,13 ± 4,324	25,07 ± 5,744	↓ 31%	ns	9
104	FarMang	MGEFH	36,66 ± 7,604	25,07 ± 5,744	↑ 46%	ns	9
105	FarMang	SBFE3	21,10 ± 4,328	25,07 ± 5,744	↓ 15%	ns	9
106	FarMang	SBFE3H	22,89 ± 5,154	25,07 ± 5,744	↓ 8%	ns	9
107	FarMang	SBFE3D	24,90 ± 6,008	25,07 ± 5,744	↓ 0,6%	ns	9
108	FarMang	SBFE3Bu	19,76 ± 5,466	25,07 ± 5,744	↓ 21%	ns	9
109	FarMang	SBFE3Aq	18,98 ± 5,565	25,07 ± 5,744	↓ 24%	ns	9
110	FarMang	STFLSE70	8,188 ± 0,9077	19,09 ± 3,465	↓ 57%	***	9
111	FarMang	STFLSE70D	11,39 ± 1,609	19,09 ± 3,465	↓ 40%	***	9
112	FarMang	STFLSE70A	9,266 ± 2,419	19,09 ± 3,465	↓ 51%	***	9
113	FarMang	STFLSE70PPT	8,593 ± 2,395	19,09 ± 3,465	↓ 54%	***	9
114	FarMang	TCIE	14,63 ± 5,973	19,09 ± 3,465	↓ 23%	ns	9
115	FarMang	TCIEH	18,86 ± 5,047	19,09 ± 3,465	↓ 1%	ns	9
116	FarMang	TCIED	16,69 ± 3,24	19,09 ± 3,465	↓ 12%	ns	9
117	FarMang	TCIEBu	14,70 ± 2,255	19,09 ± 3,465	↓ 22%	ns	9
118	FarMang	TCIEAc	16,06 ± 6,475	19,09 ± 3,465	↓ 15%	ns	9
119	Rack 80	MD10	34,81 ± 6,02	25,07 ± 5,744	↑ 38%	ns	9
120	Rack 80	MD11	20,20 ± 8,266	19,09 ± 3,465	↑ 5%	ns	9
121	FarMang	JPF	9,450 ± 1,771	16,70 ± 3,080	↓ 43%	***	9
122	FarMang	JPFH	11,77 ± 3,882	16,70 ± 3,080	↓ 29%	ns	9
123	FarMang	JPFD	12,41 ± 2,982	16,70 ± 3,080	↓ 25%	ns	9
124	FarMang	JPFAc	8,77 ± 2,082	16,70 ± 3,080	↓ 47%	***	9
125	FarMang	JPFB	12 ± 4,821	16,70 ± 3,080	↓ 28%	ns	9
126	FarMang	JPFAq	11,10 ± 6,332	16,70 ± 3,080	↓ 33%	*	9
127	FarMang	JPG MeOH	15,61 ± 3,504	17,23 ± 2,615	↓ 9%	ns	9
128	FarMang	JPG	15,20 ± 5,389	17,23 ± 2,615	↓ 11%	ns	9
129	FarMang	JPGH	17,22 ± 5,124	17,23 ± 2,615	↓ 0%	ns	9
130	FarMang	JPGD	18,63 ± 8,756	17,23 ± 2,615	↑ 8%	ns	9
131	FarMang	JPGAc	18,90 ± 7,147	17,23 ± 2,615	↑ 9%	ns	9
132	FarMang	JPGB	11,89 ± 5,587	17,23 ± 2,615	↓ 30%	**	9
133	FarMang	JPGAq	19,06 ± 7,757	17,23 ± 2,615	↑ 10%	ns	9
134	Rack 80	ME10	17,84 ± 6,135	19,09 ± 3,465	↓ 6%	ns	9

Instituto Oswaldo Cruz
Comissão de Ética no Uso de Animais - CEUA/ IOC

LICENÇA L-037/2017

Certificamos que o protocolo (CEUA/IOC-034/2017), intitulado "Investigação de novos compostos com ação antagonista sobre receptores P2 em modelo murino", sob a responsabilidade de **LUIZ ANASTÁCIO ALVES** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais**,** inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.
Esta licença tem validade até 31/01/2021 e inclui o uso total de:

Animal	espécie ou linhagem	quant (total)	♂	idade	peso	origem (*)
Camundongo	Swiss	627	X	6 a 8 semanas	25g	ICTB

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 24 de novembro de 2017.

Flávio Alves Lara
Coordenador da CEUA/Instituto Oswaldo Cruz
Fundação Oswaldo Cruz

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