FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa

TESE DE DOUTORADO

EFEITO INIBITÓRIO DE QUINOLINAS E FISALINA F EM CÉLULAS DE INDIVÍDUOS INFECTADOS PELO HTLV-1

LORENA ANA PINTO

Salvador – Bahia - Brasil
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Tese submetida a coordenação do curso em Pós-graduação em Biotecnologia em Saúde e Medicina Investigativa, Fundação Oswaldo Cruz para a obtenção do Título de doutor.

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"EFEITO INIBITÓRIO DE QUINOLINAS E FISALINA F EM CÉLULAS DE INDIVÍDUOS INFECTADOS PELO HTLV-1."

LORENA ANA PINTO

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Aos meus pais e meus irmãos Anselmo e Érica, minha razão de viver;

A Giulliano, porque seu amor renova meus dias;

A meus amigos-irmãos, companheiros fiéis;

...sem vocês a vida para.

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À Deus, porque a minha fé se renova e me transforma a cada dia, me dando a certeza que o amanhã sempre será melhor que o hoje.

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“Existe um paradoxo no crescimento do conhecimento científico. Enquanto a informação é acumulada em quantidades que nos intimidam, fatos desconexos e mistérios impenetráveis abrem caminho para explicações racionais, a simplicidade aflora do caos.”

Bruce Alberts

PINTO, Lorena Ana. Efeito inibitório de quinolinas e fisalina f em células de indivíduos infectados pelo HTLV-1. 124 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2014.

RESUMO

A proliferação espontânea de linfócitos é um marcador da infecção pelo Vírus Linfotrópico de Célula T Humana do tipo 1 (HTLV-1). Esta é mais elevada em pacientes com paraparesia espástica tropical/mielopatia associada ao HTLV (HAM/TSP) que em indivíduos assintomáticos. Embora o seu papel na patogênese da HAM/TSP ainda seja desconhecido, a identificação de drogas capazes de modular a proliferação espontânea pode ser relevante para o tratamento da HAM/TSP. Neste estudo nós avaliamos os efeitos dos derivados quinolínicos BS373, Q1 e Q2 e da fisalina F em culturas de células mononucleares do sangue periférico (PBMC) de indivíduos infectados pelo HTLV com HAM/TSP. Estes compostos inibiram, *ex vivo*, a proliferação espontânea em culturas de PBMC, conforme avaliado pela incorporação de ^{3}H-timidina. Além disso, a produção espontânea, *ex vivo*, de citocinas inflamatórias foi significativamente inibida pelo composto BS373 (25 μM) e pela fisalina F (10 μM). A expressão da proteína viral Tax foi reduzida cerca de 80% após incubação de PBMC com BS373 (25 uM). BS373 e fisalina F induziram um aumento na porcentagem de células em apoptose, como demonstrado por análise da marcação do PBMC com anexina V por citometria de fluxo. A análise ultraestrutural de células cultivadas na presença destes compostos mostrou vacúolos apresentando membranas de mielina, que se assemelham a compartimentos autofágicos. Em conclusão, as quinolinas e a fisalina F foram capaz de inibir a proliferação espontânea de células de indivíduos infectados pelo HTLV-1. Outros estudos são necessários para compreendermos os mecanismos pelos quais estes compostos agem no PBMC de indivíduos infectados pelo HTLV-1.

Palavras-chave: HTLV-1, proliferação espontânea, inibição, apoptose, quinolina, fisalina F.

PINTO, Lorena Ana. Inhibitory effect of quinolines and physalin f in cells of individuals infected with HTLV-1. 124 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2014.

ABSTRACT

Spontaneous lymphocyte proliferation, a hallmark of Human-T Lymphocyte Virus Type 1 (HTLV-1) infection, is particularly high in HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients compared to asymptomatic carriers. Although its role in the pathogenesis of HAM/TSP is still unknown, the identification of drugs capable of modulating the spontaneous proliferation may be relevant for the treatment of HAM/TSP. Here we evaluated the effects of the quinoline derivative BS373, Q1 and Q2 and physalin F in cultures of peripheral blood mononuclear cells (PBMC) obtained from HTLV-infected subjects with HAM/TSP. Compounds inhibited of spontaneous proliferation in PBMC cultures, as assessed by ^{3}H-thymidine incorporation. Additionally, the spontaneous production of inflammatory cytokines by PBMC was significantly inhibited by BS373 (25 μM) and physalin F (10 μM). The expression of the viral transcription factor Tax was reduced about 80% after incubation of PBMC with BS373 (25 μM). BS373 and physalin F induced an increase in the percentage of apoptotic cells, as shown by flow cytometry analysis of annexin V-stained PBMC. Ultrastructural analysis of cultured cells in the presence of compounds showed vacuoles presented with myelin-like membranes, resembling autophagic vacuole-like compartments. In conclusion, quinolines and physalin F was able to inhibit the spontaneous proliferation of cells from HTLV-1-infected individuals. Further studies are required to understand the mechanisms by which the compounds affect HTLV-1 PBMC.

Keywords: HTLV-1, spontaneous proliferation, inhibition, apoptosis, quinoline, physalin F.

SUMÁRIO

1 INTRODUÇÃO

O Vírus Linfotrópico de Célula T Humana do tipo 1 (HTLV-1) é um retrovírus envelopado incluído na família Retroviridae, descrito originalmente em 1980 após ser isolado de células mononucleares do sangue periférico (PBMC) de um paciente com linfoma cutâneo (POIESZ *et al.*, 1980). O HTLV possui uma estrutura genética que consiste em três genes estruturais clássicos similares à dos demais retrovírus: gag (grupo antígeno específico do core), pol (polimerase) e env (envelope), e apresenta região pX - que codifica proteínas acessórias e regulatórias. As proteínas regulatórias são Tax, Rex e HBZ. Duas sequências terminais longas idênticas (LTR) flanqueiam o genoma viral (SEIKI *et al.*, 1982) (Figura 1a).

Seu genoma possui duas fitas de RNA simples envolvidas por um core e constituído pela glicoproteína gp21, codificada pelo gene env, que também codifica gp46, uma glicoproteína presente na superfície do envelope viral. As proteínas de matriz (p19), do capsídeo (p24) e do nucleocapsídeo (p15) são codificadas pelo gene gag. O gene pol codifica as enzimas transcriptase reversa (p55), endonucleases e integrases (p32) (Figura 1b).

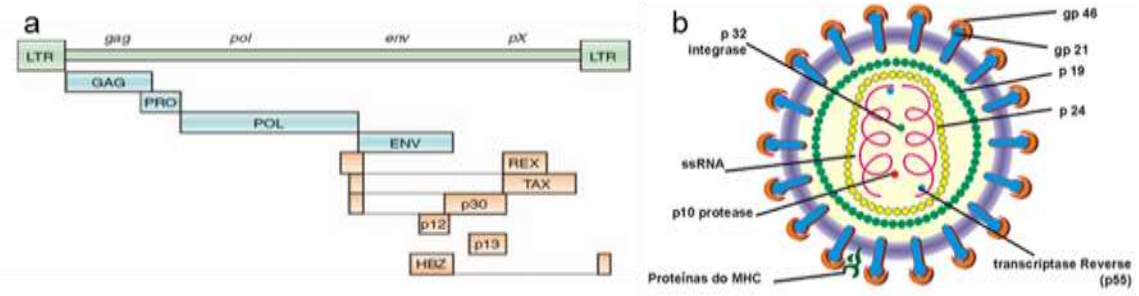

Figura 1: HTLV-1: (a) organização genética, Fonte: BOXUS e WILLEMS, 2009, e (b) estrutura morfológica, Fonte: *www.htlv.com.br*.

Estima-se em 5-10 milhões o número de indivíduos infectados pelo HTLV-1 no mundo. Áreas endêmicas da infecção incluem ilhas do Sudeste do Japão, Caribe, África Ocidental e América Latina, especialmente Brasil que tem o maior número absoluto de infectados (GESSAIN e CASSAR, 2012) (Figura 2).

No Brasil, a infecção foi avaliada em cinco capitais de diferentes regiões, em um estudo multicêntrico com doadores de sangue (GALVÃO-CASTRO *et al.*, 1997, PROEITTI *et al.*, 2005). Além da Bahia, outros estados como o Pará e o Maranhão apresentam elevadas taxas de prevalência (CATALAN-SOARES *et al.*, 2005). Na cidade de Salvador, a prevalência da infecção pelo de HTLV-1 na população geral é de cerca de 2%, aumentando

com a idade e podendo chegar a 10% em mulheres acima de 50 anos (DOURADO *et al.*, 2003). O HTLV-1 foi igualmente relatado em outras localidades da Bahia. Em um estudo com gestantes em Cruz das Almas-Bahia, foi encontrada 0,98% de prevalência (MAGALHÃES *et al.*, 2008). Rego *et al.*, em 2008 sugerem que a infecção pelo HTLV-1 está se espalhando de Salvador para outras regiões do estado da Bahia, com prevalências variando entre 1,23% - 3,85% em cidades da região do Vale do São Francisco.

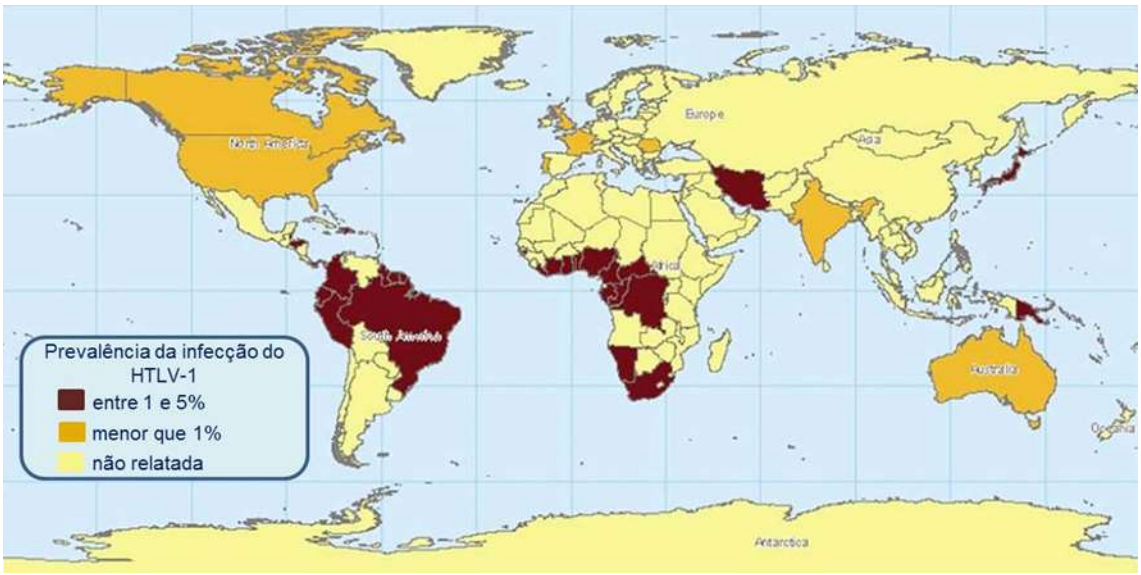

Figura 2: Distribuição geográfica da infecção pelo HTLV-1 no mundo. Fonte: adaptado de PROEITTI *et al.*, 2005.

A transmissão do HTLV pode acontecer por três vias: vertical, da mãe para o filho, principalmente pela amamentação; horizontal, através do contato sexual; e parenteral, ocorrendo através da transfusão de sangue contaminado e seus derivados que contenham células, assim como pelo uso de seringas ou perfuro-cortantes contaminados (MANNS *et al.*, 1999).

1.1 Doenças Associadas

O HTLV é o agente etiológico da paraparesia espástica tropical/mielopatia associada ao HTLV (HAM/TSP), uma mieloneuropatia progressiva caracterizada por lesão inflamatória e desmielinizante dos neurônios motores longos da medula, e da leucemia/linfoma de células T do adulto (ATLL), caracterizada por uma expansão clonal de linfócitos T ativados (OSAME *et al.*, 1986, HINUMA *et al.*, 1981). Além da HAM/TSP e da ATLL, outras doenças foram associadas a este retrovírus como a uveíte associada ao HTLV-1

(MOCHIZUKI *et al.*, 1992) e a dermatite infecciosa associada ao HTLV-1 (DIH) (LaGRENADE *et al.*, 1990).

A HAM/TSP é mais comum em mulheres do que em homens e a chance de desenvolver a doença torna-se maior quando a infecção é adquirida por via sexual (KRAMER *et al.*, 1995). Esta desenvolve-se em aproximadamente 4% das pessoas infectadas com o HTLV-1 (ORLAND *et al.*, 2003). Esta doença ocorre principalmente em adultos e a média da idade de início da doença é de 40-50 anos (MORENO-CARVALHO *et al.*, 1992;). Estudos realizados principalmente no Brasil, em alguns países africanos e na Guiana Francesa mostram que a prevalência da HAM/TSP pode estar subestimada (CASSEB, 2009). Isso se deveria a vários fatores como, a dificuldade de diagnóstico e a possível confusão entre HAM/TSP e neuromielopatia causada por outras etiologias.

O tempo entre a infecção primária, e o início dos sinais da mielopatia, principalmente em adultos, pode variar de anos a décadas. A HAM/TSP também pode desenvolver-se dentro de 3,3 anos, em 50% dos casos associados transfusão sanguínea (OSAME *et al.*, 1986, GOUT *et al*, 1990;. KAPLAN *et al*, 1991).

No início da doença, as principais queixas podem envolver fraqueza dos membros inferiores e dor lombar, alterações sensoriais, bem como distúrbios urinários (noctúria, incontinência urinária) e disfunção sexual, a exemplo de disfunção erétil. Após anos de evolução, as características neurológicas da HAM/TSP incluem espasticidade e/ou hiperreflexia predominantemente em membros inferiores, distúrbios da bexiga, fraqueza muscular das extremidades inferiores em mais de 90% dos casos, e distúrbios sensoriais com dor lombar, em 50% dos casos (NAKAGAWA *et al.*, 1995).

O diagnóstico da HAM/TSP é complexo, pois envolve além dos sinais e sintomas clínicos, exames laboratoriais e de imagem. A Organização Mundial da Saúde (OMS) (World Health Organization – WHO, OSAME, 1990) elaborou uma relação de critérios para o diagnóstico desta doença. No entanto, em muitos países onde a doença é endêmica, os médicos não dispõem de todos os exames complementares necessários. Além disso, muitos pacientes apresentam sinais isolados de mielopatia, sem preencher completamente os critérios para HAM/TSP. Assim, em 2006, um grupo de especialistas neurologistas se reuniu em Belém (Brasil) para propor um novo critério diagnóstico, estabelecendo três níveis de certeza para o diagnóstico de HAM/TSP: provável, possível e definido. Estes níveis de certeza levam em consideração a presença de sinais e sintomas neurológicos, resultados sorológicos e/ou detecção do HTLV-1/DNA e a exclusão de outras causas de mielopatia, que se assemelhem a HAM/TSP. O diagnóstico definido segue os critérios estabelecidos pela OMS, enquanto o

diagnóstico provável é atribuído aos pacientes monossintomáticos (principalmente com bexiga hiperativa, ou sinal de Babisnky) para os quais outras causas de mieolopatia foram excluídas. O diagnóstico de HAM/TSP possível ocorre quando outras patologias que causam mielopatia não foram/ou não puderam ser excluídas. Nestes casos, o paciente pode apresentar a síndrome completa ou ser oligossintomático (De CASTRO E COSTA *et al.*, 2006).

1.2 Alterações imunológicas e imunopatogênese da HAM/TSP

O HTLV-1 infecta linfócitos T CD4+ e T CD8+, natural Killer (NK) e células dendríticas, com tropismo preferencial pelos linfócitos T CD4+CD45RO+CD25+ (NAGAI *et al.*, 2001; PRINCE *et al.*, 1995; MAKINO *et al.*, 1999). *In vivo*, a infecção pelo HTLV-1 requer contato célula-célula, via formação de uma sinapse virológica entre a célula infectada e a célula alvo (IGAKURA *et al.*, 2003). A infecção ainda pode ocorrer quando a partícula viral se liga à superfície da célula, através da interação entre glicoproteínas do envelope viral e o receptor de superfície celular GLUT-1 (MANEL *et al.*, 2003). Com essa interação, o vírus torna-se capaz de penetrar na célula, liberando todo seu conteúdo no citoplasma. A propagação do vírus se dá também por divisão mitótica, com a expansão clonal das células infectadas (WATTEL *et al.*, 1996). Essa expansão clonal resulta na proliferação de linfócitos T CD4+ CD45RO+ e o acúmulo de células T CD8+, infectadas pelo vírus (ASQUITH *et al.*, 2007; ZANE *et al.*, 2009).

O vírus estimula a expansão clonal das células infectadas através dos produtos de alguns genes virais que se encontram na região pX. Os principais produtos relacionados com a proliferação celular são as proteínas Tax e HBZ (MATSUOKA & KUAN-TEH JEANG, 2007; MESNARD *et al.*, 2006).

A proteína viral Tax ativa um grande número de genes virais e do hospedeiro, promovendo desregulação do ciclo celular, e uma significante atividade mitogênica (AZRAN *et al.*, 2004). Dentre as alterações mediadas por Tax refere-se uma instabilidade genética que pode propiciar uma resistência a apoptose e indução autócrina e parácrina da secreção de IL-2 e seu receptor IL-2R, bem como aumento na secreção de citocinas (AZRAN *et al.*, 2004). Tax ao modular a transcrição do genoma viral (LTR) e de diversos genes celulares do HTLV, incluindo as sequências para IL-2, IL-13, IL-15, IL-2R, fator estimulador de colônia de granulócitos e monócitos (GM-CSF), fator de necrose tumoral (TNF-alfa), entre outros, e protoncogenes como c-Fos e c-Jun. A proteína Tax encontra-se diretamente relacionada à

proliferação celular espontânea e é considerada indutora principal da patogênese viral (SODROSKI 1992; SUZUKI *et al.*, 1999; AZRAN *et al.*, 2004; MATSUOKA *et al.*, 2005).

A proteína HBZ também contribui para a transformação e proliferação celular. HBZ suprime a expressão de Tax e tem capacidade de regular a transcrição viral mediada por esse gene (SATOU *et al*., 2006). A proteína HBZ também interfere nas vias de transcrição da célula hospedeira, interagindo na via do NF-KB. Além disso a proteína HBZ também regula fatores de transcrição humanos, entre eles, a família de Jun/Fos (MATSUOKA *et al.,* 2009; BASBOUS *et al.,* 2003). A expressão do RNA mensageiro de HBZ aumenta a expressão de genes incentivando assim a proliferação de células T (MESNARD *et al.,* 2006).

A proliferação espontânea, *in vitro*, de células infectadas é uma das alterações imunológicas mais importantes na infecção pelo HTLV (PRINCE *et al*., 1990; ASQUITH *et al.*, 2007). De forma persistente e contínua a proliferação celular poderia aumentar a probabilidade de migração celular e a inflamação, aumentando assim o risco de HAM/TSP e de outras doenças inflamatórias (ASQUITH *et al.*, 2007).

A proliferação espontânea de PBMC é observada em cerca de 70% dos indivíduos infectados pelos HTLV-1 (MASCARENHAS *et al.*, 2006). As subpopulações de linfócitos T CD4+ e T CD8+ de memória (CD45RO) são as principais células envolvidas neste fenômeno. Estas expressam os marcadores de ativação celular CD25 e HLA-DR. Diversos autores relatam uma maior intensidade dos níveis de proliferação espontânea em pacientes com HAM/TSP (ITOYAMA *et al.,* 1988) e intensa ativação do sistema imune (PRINCE *et al.*, 1995). Essa intensa ativação está relacionada à elevada produção de citocinas como IL-2, TNF-α, IFN-γ, IL-6, IL-10, tanto em pacientes com HAM/TSP, quanto em indivíduos assintomáticos (FURUKAWA *et al.,* 2003; SANTOS *et al.,* 2004; MONTANHEIRO *et al.,* 2009; CARVALHO *et al.,* 2001; COUTINHO *et al.,* 2014). Essa resposta imune celular à infecção viral não representa um controle da infecção e não têm um papel claramente estabelecido na patogênese de HAM/TSP.

A carga proviral do HTLV-1 (número de células contendo cópia do provirus) tem sido considerada um possível marcador de progressão da doença. Uma alta carga proviral (acima de 5% de células infectadas) é frequentemente observada nos pacientes com HAM/TSP, comparada aos indivíduos assintomáticos (OLINDO *et al.,* 2005; GRASSI *et al.,* 2011). A carga proviral poderia representar um dado adicional para apoiar o diagnóstico de HAM/TSP e contribuir para o diagnóstico diferencial. A carga proviral parece ser mantida, principalmente, pela proliferação de células T CD4+ contendo provirus (CAVROIS *et al.,* 1996; EIRAKU *et al.,* 1998).

A patogênese da HAM/TSP ainda é pouco compreendida e fatores virais e do hospedeiro, tais como a carga proviral e a resposta imune podem desempenhar um papel importante na evolução da doença (BANGHAM e OSAME, 2005; LEPOUTRE *et al*, 2009). Não está claro se as lesões da mielina observadas na HAM/TSP é fruto de um processo primário ou secundário, se resulta de um efeito causado pela persistência viral ou se ocorre a partir de um processo imune mediado por infiltrações linfocitárias (OZDEN *et al.*, 2001). Para explicar o papel do HTLV-1 no desenvolvimento de HAM/TSP, observa-se o dano neurológico e busca-se entender de que forma o sistema imune está causando ou influenciando este dano. Uma hipótese seria uma consequência direta da resposta mediada por linfócitos T citotóxicos (CTLs). Numa segunda hipótese, observa-se uma resposta autoimune que seria consequência da persistência do vírus e de uma intensa ativação de células T, com infiltração de linfócitos T no sistema nervoso central. Outra possibilidade analisa os danos causados pela intensa ativação de células T CD4+, CD8+ e microglia que liberam citocinas tóxicas à mielina como TNF-α e assim danificam diretamente a medula ou as células da glia (ARAÚJO e SILVA, 2006) (Figura 3).

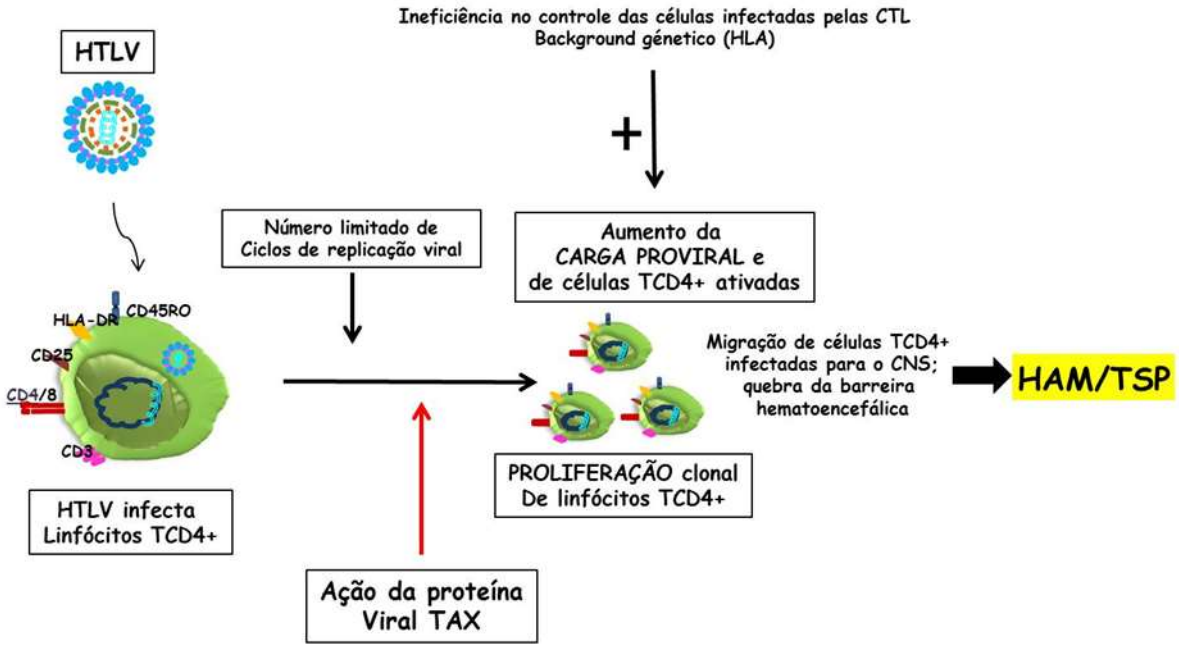

Figura 3: Patogênese de HAM/TSP e papel do sistema imune. Adaptada de GESSAIN e MAHIEUX, 2012.

1.3 Tratamento

Contrariamente à infecção pelo HIV-1, cujos critérios para o tratamento antirretroviral estão bem estabelecidos, não existe um tratamento específico comprovadamente eficaz para a infecção pelo HTLV-1 e poucas drogas são disponíveis. Muitos estudos foram realizados utilizando, entre outras drogas, a terapia a base de corticoteroide, danazol, vitamina C, IFN-α, zidovudina, zidovudina associada à lamivudina com sucesso clínico muito limitado (OSAME *et al.*, 1990; YAMASAKI *et al.*, 1997, OH *et al.*, 2005; TAYLOR *et al.*, 2006; CRODA *et al.*, 2008; TATTERMUSCH *et al.*, 2012; RAFATPANAH *et al.*, 2012). Moens *et al.*, em 2012 relataram que ácido ascórbico em altas doses apresentou um efeito imunomodulador e antiproliferativo superior ao IFN-α, *ex vivo* e *in vitro*. Um estudo recente mostrou que IFN-α combinado a zidovudina suprimiu a expressão de genes do HTLV-1 e controlou o ciclo celular, induzindo apoptose em células infectadas (KINPARA *et al.*, 2013). Estudos realizados com ácido valpróico (VPA) investigaram a modulação da carga proviral e seus efeitos na expressão de Tax e HBZ do HTLV-1 através da exposição das células infectadas ao sistema imune aumentando os níveis de apoptose nessas células (LEZIN *et al*., 2007; BELROSE *et al.*, 2011; OLINDO *et al.*, 2011). Araya *et al.* em 2011 mostram que terapia com fucoidan foi capaz de reduzir a carga proviral em pacientes com HAM/TSP.

Apesar da avaliação de diferentes drogas, a indicação terapêutica ainda é restrita aos grupos sintomáticos. A identificação de novas drogas é de fundamental importância, especialmente nos países em desenvolvimento, onde a infecção é endêmica.

A busca de novas alternativas terapêuticas a partir de compostos naturais ou derivados destes é um tema atual. Os produtos naturais interagem com uma grande variedade de moléculas com fins específicos e ainda possuem uma grande diversidade química (KOEHN e CARTER, 2005). Demonstrando interesse pelos produtos naturais, Fournet *et al*. em 1989 passaram a estudar a composição químico-biológica da *Galipea longiflora*, uma rutácea com propriedades medicinais encontrada na Amazônia boliviana. Purificações bioguiadas resultaram no isolamento de uma nova família de alcaloides com substituição no carbono da posição 2, as quinolinas (FOURNET *et al.*, 1989). Após a extração e identificação, as quinolinas foram produzidas sinteticamente (FAKHFAKH *et al.*, 2001). Desde então diversos estudos vêm sendo realizados, entre eles um demonstra que compostos quinolínicos são capazes de diminuir a proliferação espontânea em células de linhagem transformadas pelo HTLV-I (FOURNET *et al.*, 2003). Os compostos quinolínicos foram inicialmente estudados

para tratamento da leishmaniose (FOURNET *et al.*, 1996; NAKAYAMA *et al.*, 2005; DESRIVOT *et al.*, 2007; VIEIRA *et al.*, 2008). Estes compostos apresentam ainda atividade contra o *Plasmodium vinckei petteri* (GANTIER *et al.*, 1996) e contra o *Trypanosoma cruzi* em camundongos (NAKAYAMA *et al.*, 2001). Quinolinas apresentam também atividade antiviral, impedindo a replicação do HIV-1 em células CEM4xf (FAKHFAKH *et al.*, 2003). Além disso, estes compostos agem inibindo a atividade da enzima integrase do HIV-1 (MEKOUAR *et al.*, 1998; ZOUHIRI *et al.*, 2000). Nosso grupo identificou uma série de novos compostos quinolínicos capazes de inibir mais de 70% a proliferação espontânea de PBMC de indivíduos infectados pelo HTLV-1 (GRASSI *et al.*, 2008).

Physalis angulata L. (Solanaceae), uma erva encontrada em regiões tropicais e subtropicais do mundo, é amplamente utilizada na medicina popular como analgésico, anti-inflamatório e antirreumático (SOARES *et al.*, 2003; NAGAFUJI *et al.*, 2004). As moléculas isoladas *de Physalis spp*., conhecidas como fisalinas, são derivados esteroidais de estruturas complexas, caracterizadas como seco-derivados resultantes da clivagem oxidativa efetuada por um agente oxidante, apresentam atividades anti-inflamatórias e são imunomoduladoras potentes (TOMASSINI *et al.*, 2000; SOARES *et al.*, 2006; ANKRAH *et al.*, 2003; JACOBO-HERRERA *et al.*, 2006; PINTO *et al.*, 2010; JI *et al.*, 2012). Fisalina F previne a mortalidade e inibe a rejeição de transplantes alogênicos em ratos (SOARES *et al.*, 2006; JI *et al.*, 2012). A atividade anti-inflamatória da fisalina F também foi demonstrada em modelos de isquemia intestinal e artrite (VIEIRA *et al.*, 2005; BRUSTOLIM *et al.*, 2010). Além disso, foi demonstrado que vários derivados da fisalina têm efeito leishmanicida (GUIMARÃES *et al.*, 2009), atividade antimalárica (SÁ *et al.*, 2011) e tripanocida (MEIRA *et al.*, 2013). Fisalina F inibi a ativação do NF-kB, e a produção de mediadores pró-inflamatórios, tais como TNF, IL-6 e IL-12 (JACOBO-HERRERA *et al.*, 2006; VIEIRA *et al.*, 2005).

Com base na atividade anti-proliferativa e imunomoduladora destes compostos, a hipótese do presente trabalho é que a fisalina F e os derivados de quinolinas terão efeito imunomodulador em células de pacientes infectados pelo HTLV-1 com diagnóstico de HAM/TSP. Acreditamos poder contribuir com novas estratégias terapêuticas para as patologias associadas ao HTLV, particularmente ligadas a proliferação celular exacerbada.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar, *ex vivo*, o efeito dos compostos quinolínicos Q1, Q2 e BS373 e da Fisalina F sobre a ativação de PBMC de indivíduos infectados pelo HTLV-1.

2.2 Objetivos Específicos

- Quantificar a inibição da proliferação celular;
- Determinar o efeito destes compostos sobre a apoptose e a produção de citocinas Th1 e Th2;
- Quantificar a expressão da proteína viral Tax;
- Avaliar o efeito dos compostos na organização ultraestrutural nas células.

3 RESULTADOS

Os resultados desta tese encontram-se apresentados em três capítulos representando os manuscritos produzidos no período do curso de pós-graduação, tendo como títulos: **(1)** Inhibitory effect of a quinoline derivative in cells from HTLV-1-infected individuals; **(2)** Physalin F, a seco-steroid from Physalis angulata L., has immunosuppressive activity in peripheral blood mononuclear cells from patients with HTLV1-associated myelopathy; **(3)** Effect of two quinoline derivate on spontaneous proliferation and on ultrastructural organization of cells from HTLV-1-infected individuals.

CAPÍTULO 01

Manuscrito 1: Inhibitory effect of a quinoline derivative in cells from HTLV-1-infected

Atualmente não existe nenhuma terapia eficaz para infecção pelo HTLV-1. Diferentes drogas, com distintas formas de administração já foram testadas e a maior parte delas é utilizada no tratamento das patologias associadas como HAM/TSP e ATLL, com pouco benefício. O estabelecimento de novas estratégias terapêuticas para a infecção pelo HTLV-1 torna-se necessária. O objetivo deste estudo foi avaliar a atividade do composto derivado de quinolina, BS373, sobre a proliferação de células mononucleares de sangue periférico (PBMC), a modulação sobre as citocinas, a expressão da proteína viral Tax, a morte celular por apoptose e as alterações ultraestruturais.

Principais resultados encontrados:

1- BS373 inibe a proliferação espontânea das células de pacientes infectados pelo HTLV-1;

2- BS373 reduz a produção de citocinas: IL-2, IL-10, TNF e IFN-γ;

3- BS373 induz, em uma concentração dose dependente, a apoptose celular em células de pacientes infectados;

4- Pode-se observar núcleos em picnose, presença de compartimentos que se assemelham a vacúolos autofágicos e figuras de mielina em células de pacientes infectados tratadas com BS373.

Artigo a ser submetido para publicação.

Inhibitory effect of a quinoline derivative in cells from HTLV-1-infected individuals

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Abstract

Spontaneous lymphocyte proliferation, a hallmark of Human-T Lymphocyte Virus Type 1 (HTLV-1) infection, is particularly high in HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients compared to asymptomatic carriers. Although its role in the pathogenesis of HAM/TSP is still unknown, the identification of drugs capable of modulating the spontaneous proliferation may be relevant for the treatment of HAM/TSP. Here we evaluated the effects of the quinoline derivative BS373 in cultures of peripheral blood mononuclear cells (PBMC) obtained from HTLV-infected subjects with HAM/TSP. BS373 caused a concentration-dependent inhibition of spontaneous proliferation in PBMC cultures, as assessed by ^{3}H-thymidine incorporation. MTT metabolization indicated that BS373 had no toxic effect on PBMC at the concentrations evaluated. The IC_{50} for BS373 was 12.1±8.93 μM. Additionally, the spontaneous production of IL-2, IL-10, TNF-α and IFN-γ by PBMC was significantly inhibited by BS373 (25 μM). The expression of the viral transcription factor Tax was reduced about 80% after incubation of PBMC with BS373 (25 μM). BS373 induced, in a concentration-dependent fashion, the increase in the percentage of apoptotic cells, as shown by flow cytometry analysis of annexin V-stained PBMC. Ultrastructural analysis of cultured cells in the presence of BS373 showed vacuoles presented with myelin-like membranes, resembling autophagic vacuole-like compartments. In conclusion, the quinoline BS373 showed no toxicity and was able to inhibit the spontaneous proliferation and cytokine production of T cells from HTLV-1-infected individuals. Further studies are required to understand the mechanisms by which BS373 affects HTLV-1 PBMC.

Keywords: HTLV-1, spontaneous proliferation, inhibition, apoptosis, quinoline.

1. Introduction

Human T cell lymphotropic type 1 (HTLV-1) is a retrovirus belonging to *Retroviridae* family. The virus causes a number of diseases, including HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1], leukemia/lymphoma adult T-cell (ATLL) [2, 3], uveitis associated with HTLV-1 [4] and infective dermatitis in children [5]. It is estimated that about 10 million individuals are infected by the HTLV-1 worldwide, mainly in Southeastern islands of Japan, the Caribbean, West Africa, and Latin America [6]. Although less than 5% of infected individuals will develop one of these HTLV-associated diseases, an increasing number of rheumatologic, urinary, clinical and neurological manifestations isolated or not directly related to the virus are being reported [7].

HTLV-1 induces a spontaneous proliferation *in vitro* and *in vivo* of $CD4^+$ and $CD8^+$ T-lymphocytes and NK cells [8-11]. Furthermore, it is associated with a strong immune system activation causing an elevated production of inflammatory cytokines such as IL-2, TNF-α, IFN-γ and IL-6 [12] and increased expression of CD25, HLA-DR on both $CD4^+$ and $CD8^+$ T lymphocytes [13]. These findings are described as more intense in patients with HAM/TSP, although they can occur in asymptomatic subjects [14, 15]. HAM/TSP is a myelopathy characterized by a chronic inflammatory disorder resulting in a long-term progressive degeneration. The inflammatory process leads to demyelination of the spinal cord, especially in the lower thoracic area [16]. The spontaneous proliferation of cells and activation of immune system may account for the recruitment of inflammatory cells into the spinal cord, which will cause cell injury and demyelination.

To this moment, there are no available drugs against HTLV-1, being thus a neglected disease. Treatment of HTLV-1 is directed to the related associated diseases, such as HAM/TSP and

ATLL, with little benefit. Therefore, there is an urgent need for developing new treatments able to reduce or prevent the manifestations caused by the virus.

Quinolines are either alkaloids isolated from *Galipea longiflora*, a Bolivian plant with medicinal properties or synthetically produced compounds [17, 18]. Many of its derivatives have potent activities, such as quinine, which possesses antimalarial activity, and 4-hydroxy-2-alkylquinolines, showing antibiotic properties [19-21]. Quinoline is also known as useful scaffold in the discovery of antitumor agents [22]. In the present study, we investigated the effects of a 2-substituted quinoline derivative in cultures of human peripheral blood mononuclear cells obtained from HTLV-1-infected subjects.

2. Materials and Methods

2.1 Subjects of study

Seventeen HTLV-1-infected subjects with HAM/TSP diagnosis defined according to the World Health Organization criteria [23] followed at Bahiana School of Medicine and Public Health reference center for HTLV in Salvador, Brazil, were included in the study. Serum samples were screened for HTLV-1/2 antibodies by an enzyme-linked immunosorbent assay (Ab-Capture ELISA test system; Ortho-Clinical Diagnostics Inc., Raritan, NJ) and confirmed by western blot assay (HTLV Blot 2.4; Genelabs Technologies, Singapore). The group consisted of 10 women (59%) and had a mean age of 58 years. Blood samples from four healthy volunteers from the Gonçalo Moniz Research Center were used as controls. Informed consent was obtained from all enrolled subjects, and the Institutional research boarding of the Oswaldo Cruz Foundation (FIOCRUZ) approved this study.

2.2 PBMC isolation and culture

Peripheral blood mononuclear cells (PBMC) from individuals were obtained from heparinized venous blood samples by density gradient centrifugation SepCell (LGC Biotechnology; São Paulo, Brazil). PBMC were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM L-glutamine (Sigma), 1% nonessential amino acids (Gibco Laboratories, Gaithersburg, MD), 1 mM sodium pyruvate (Sigma), 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), 100 µg/mL HEPES (Invitrogen; Eugene, CA) and 10% fetal calf serum (FCS, Hyclone; Logan, UT).

2.3 Quinoline derivative

The compound used in this study, 2-(2-hydroxyprop-2-enyl)quinoline (BS373), was synthesized according to a methodology described previously [18]. The compound was dissolved in dimethyl sulfoxide (DMSO, Sigma) at a concentration of 100 mM, aliquoted and stored at -20° C. Upon usage, the compound was diluted in medium supplemented culture and adjusted for concentration. The chemical structure of BS373 evaluated in this study is shown in figure 1.

2.4 In vitro cell toxicity assay

PBMC (10^5 cells/well) from HTLV-infected patients and uninfected controls were cultured in 96-well plates in presence of serial dilutions of quinoline BS373 (ranging from 1.56 to 50 µM) at 37°C in a 5% CO^2 humidified atmosphere. After 24 hours of culture, cells were pulsed for three hours with 20 µl of 5 mg/mL MTT (3-[4,5-dimethylthiazol 2yl]-2,5 diphenyltetrazolium bromide; thiazolyl blue; Sigma). The optical density (OD) was determined using Versamax photometer (Molecular Devices, Inc., Menlo Park, CA) at a test

wavelength of 570 nm. The toxicity was evaluated by the ratio of OD of wells containing BS373 with the OD of control wells containing only culture medium.

2.5 HTLV-1-infected PBMC proliferation assay

PBMC (10^5 cells/well) from three patients with HAM/TSP diagnosis were cultured in RPMI 1640 culture medium with 10% FCS. Cells were cultured in 96-well U-bottom culture plates (Costar; Cambridge, MA), in triplicate, at 37°C in a 5% CO^2 humidified atmosphere. To evaluate the cell proliferation, PBMC were cultured for three days and pulsed overnight with 1 μCi ^{3}H-thymidine (ICN; Costa Mesa, CA). After this period, the content of the plate was harvested to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Chameleon, Hydex; Turku, Finland). Results were expressed as mean counts per minute.

2.6 Detection of phosphatidylserine translocation by annexin V staining

PBMC from six HTLV-1-infected and four uninfected subjects were plated in 96-well plates at $2x10^5$ cells/well in the absence or in the presence of 6.25, 12.5 and 25 μM of quinoline BS373 at 37 °C for 18 hours. Cells were then stained with Alexa® Fluor 488 annexin V and PI, using the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen; Eugene, CA) according to the manufacturer's instructions. The data were acquired by flow cytometry (FACSAria II, Becton Dickinson; Mountain View, CA) and the analysis was performed using the FlowJo software (FlowJo; Ashland, OR).

2.7 Detection of intracellular Tax

PBMC from four HTLV-1-infected subjects were plated in 96-well plates at $2x10^5$ cells/well in the presence of 25 μM BS373, at 37 °C, during 18 hours. PBMC were incubated for 15 min at room temperature with monoclonal antibodies anti-$CD3^{PE}$ and anti-$CD4^{PE\text{-}Cy7}$ (BD

Biosciences, San Jose, CA). Cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde during 15 min, and then washed twice with PBS-BSA-saponin 0.2% followed by incubation with 2 mL of PBS-BSA-saponin 0.2% for 15 min at room temperature. Next, cells were incubated with AB serum 1% for 15 min for blocking non-specific binding, and then stained with anti-Tax Lt-4^{FITC} monoclonal antibodies or isotype controls $IgG3^{FITC}$ for 30 min at room temperature. Lastly, the cells were washed and fixed in PBS-paraformaldehyde. Analyses were performed using FACSaria II and the FlowJo software. At least 10^5 events were analyzed per sample.

2.8 Assessment of cytokine production by CBA assay

PBMC from 10 HTLV-1-infected subjects were plated in 96-well plates at $2x10^5$ cells/well in the presence of 25 μM of BS373, at 37 °C, for 24 hours. Supernatants were then harvested and cytokines were measured using BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences; San Jose, CA), according to the manufacturer's instructions. The concentrations of samples were calculated by extrapolating the mean fluorescence intensity (MFI) on the respective standard curves. The data was acquired by flow cytometry and the analysis was performed using FlowJo and GraphPad Prism 5.02 (GraphPad; San Diego, CA) softwares.

2.9 Transmission electron microscopy analysis

For transmission electron microscopy (TEM) analysis, PBMC ($2x10^6$) were cultured in presence or absence of compounds for 18 hours. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and then treated with 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM calcium chloride, for 60 min in the dark. Cells were dehydrated using graded acetone and infiltrated in polybed epoxy resin (Polysciences; Warrington, PA),

which was polymerized at 60 °C for 48 h. Ultrathin sections obtained by ultramicrotomy, using diamond knives (Diatome) were collected on 300 mesh grids and stained with aqueous 5 % uranyl acetate for 40 min. and 3 % lead citrate for 5 min. The ultrastructure analysis was performed in a transmission electron microscope (Jeol JEM 1230 or Zeiss EM109).

Photooxidation analysis was performed in PBCM samples cultured in the presence of compounds for 18 hours. PBMC fixed in 4% paraformaldehyde were pre-loaded for 15 min with 10 mg/mL of diaminobenzidine (DAB) in Tris-HCl, pH 7.2 and incubated for 30 min under ultraviolet light. Then, additional 10 mg/mL of DAB was added, and cultures kept under ultraviolet light for further 30 min. The cells were then washed twice in Tris-HCl, pH 7.2, and once in 0.1M sodium cacodylate at pH 7.4. Then, cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 60 minutes at room temperature and processed for TEM, as described above.

2.10 Statistical analyses

Data are expressed as percentages, means and standard errors. We compared the groups using Mann-Whitney, Wilcoxon signed-rank tests. *P* values below 0.05 were considered statistically significant. GraphPad Prism software was used for all statistical analysis.

3 Results

3.1 BS373 inhibits the spontaneous proliferation of PBMC from HTLV-1-infected subjects

We first tested the effects of quinoline BS373 in cultures of PBMC obtained from HTLV-1 infected subjects with diagnostics of HAM/TSP. Addition of BS373 caused a concentration-dependent reduction of the spontaneous lymphocyte proliferation characteristic of HTLV-1-infected cells (Figure 2). The estimated IC_{50} was 12.1±8.9 µM. BS373 did not cause cell toxicity in cultures of PBMC when tested in concentrations lower than 50 µM (data not shown).[24]

3.2 Reduction of cytokine production by BS373 in PBMC cultures from HTLV-1-infected subjects

Cultures of PBMC obtained from HTLV-1-infected subjects spontaneously produced several cytokines, including IL-2, IL-6, IL-10, IL-17A, TNF and IFN-γ (Figure 3). Addition of 25 µM BS373 caused a significant reduction of IL-2 (68%), IL-10 (97%), TNF-α (90%) and IFN-γ (96%) (Figure 3).

3.3 Decreased expression of viral protein Tax

Tax is a HTLV nuclear protein produced in infected cells, which regulates a number of cellular processes, including cell proliferation [25]. The expression of Tax in PBMC cultures of HTLV-1^+ subjects was reduced about 80% when cultured in the presence of 25 µM BS373 (Table 1).

3.4 Induction of apoptosis by BS373 in cultures of PBMC

Next, we investigated whether BS373 induces apoptosis in PBMC cultures. Addition of BS373 enhanced apoptosis in a concentration-dependent way, in PBMC from HTLV-1-infected and uninfected subjects. The number of apoptotic cells was 16 and 14% higher in cells from infected subjects in the presence of 6.25 and 12.50 μM, respectively (Figure 4).

3.5 Ultrastructural analysis of BS373-treated PBMC

Transmission electron microscopy of PBMC from HTLV-1 infected subjects showed pyknotic nuclei and structural changes in the mitochondria (Figure 5a), as well as increased number of autophagic vacuoles (Figure 5b) after culture in the presence of the compound BS373. Photooxidation analysis by the reaction product detection indicate that BS373 was localized within cell vesicles displaying myelin-like figures, presumably autophagic vacuoles involved in membrane turnover (Figures 5c and d).

4 Discussion

The lack of effective antiviral drugs against HTLV-1 reinforces the need for developing alternative treatments in order to prevent or retard the progression of HTLV-1-associated diseases. In this context, a number of studies have searched for natural and synthetic antiproliferative molecules to be used in the modulation of lymphocyte proliferation induced by HTLV-1, by using both HTLV-infected cell lines or PBMC cultures [17, 24, 26-29].

Here we investigated the effects of a quinoline derivative (BS373) on cultures of PBMC isolated from HTLV-1 infected subjects with diagnostics of HAM/TSP. This class of compounds shows an antiproliferative potential, including for HTLV-1-induced spontaneous proliferation [26]. We found that BS373 presents potent antiproliferative activity, inhibiting in micromolar range the spontaneous proliferation of PBMC from HTLV-1-infected subjects, in non-toxic concentrations. This inhibition correlated with a marked decrease in the production of IL-2, a cytokine that acts as a T-cell growth factor, after 24 hours of incubation with BS373. Thus, the reduced proliferation caused by this quinoline derivative may be in part due to the suppression of IL-2 production observed.

Tax is a viral protein, which plays key roles in the pathogenicity of HTLV-1, activating the transcription of both viral and cellular genes [30]. Tax interacts with transcription factors and promotes cell cycle progression by interacting with cell cycle regulator proteins of cyclin-dependent kinase family [31]. Moreover, Tax induces IL-2 and IL-2Rα expression by activation of the NF-κB/Rel family of transcription factors [32, 33]. Thus, the reduction of Tax expression observed after treatment with BS373 may participate in the inhibition of cell proliferation observed in HTLV-1-infected PBMC cultures.

In addition to promoting cell proliferation, a number of studies have shown that persistent expression of Tax causes apoptosis of HTLV-1-infected cells [34-37]. In our study, we found that PBMC from HTLV-1-infected donors spontaneously presented higher apoptosis rate than those of healthy donors. Using flow cytometry analysis, we showed that BS373 significantly increased the percentage of apoptotic cells in PBMC cultures of both HTLV-infected and uninfected donors. Induction of apoptosis in cancer cells has been described after treatment with other quinoline derivatives, by mechanisms dependent on caspase activation [38, 39]. Thus, it is possible that the quinoline derivative tested in our study acts by caspase activation to promote cell apoptosis.

The mitochondrial damage may be associated to different cell death mechanisms as permeability transition may participate not only in apoptosis but also in necrosis and autophagy [40]. Interestingly, natural product-induced ROS production triggers antineoplastic activity involving apoptosis, necroptosis and autophagy [41]. It is noteworthy that autophagy comprises a scape mechanism and it is required for activated T cell ATP production and proliferation [42]. In addition reduced autophagy down regulates pancreatic cancer cell growth [43]. It is well-known that chloroquine can increase autophagic vacuole/lysosomal pH diminishing metabolic function and proliferative capacity in different cell types. Choloquine-mediated lysosomal alkalinization increases the mRNA expression of the lysosomal/autophagy transcription factor TcFEB [44].The biogenesis of large autophagic vacuoles observed in BS373-treated cells may be due to a positive feed-back mechanism where lysosomal alkalinization leads to accumulation of oxidized lipids which in turn may further elevate lysosomal pH and so on [44]. The quinolinic antimalarial agents chloroquine [45], hydroxychloroquine [46] and amodiaquineincrease [47] down modulate metabolic function and diminish cell proliferation. Autophagy can inhibit apoptosis in carcinoma cells [48]. On the other hand, autophagy blockade may promote apoptosis [49] Therefore,

quinoline compound-mediated inhibited autophagy may diminish PBMC proliferation triggering apoptosis. In accordance with the flow cytometry analysis for annexin V, by performing ultrastructural analysis we observed alterations indicative of apoptotic cells, such as pyknotic nuclei and structural changes in the mitochondria and autophagic vacuoles. Autophagic vacuole formation may lead to metabolic deficit as the accumulation within endocytic.

In summary, we described herein a potent antiproliferative activity of BS373, a -quinoline derivative previously shown to possess activity against *Trichomonas vaginalis* and *Caenorhabditis elegans* [50], in HTLV-1-infected cells. Our results reinforce the potential of quinoline compounds as pharmacological agents, in particular for the development of new treatments for HTLV-1-induced diseases.

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6 Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1: Chemical structure of quinoline compound BS373.

Figure 2: Concentration-dependent inhibition of spontaneous proliferation of PBMC by BS373. PBMC from three HTLV-1^{+} subjects were cultured in the absence or in the presence of different concentrations of BS373. Spontaneous proliferation was assessed by ^{3}H-thymidine incorporation after 3 days of culture. Results are expressed as mean±SEM (n=3) of the percentage of inhibition in relation to untreated controls.

Figure 3: Cytokine production by HTLV-1+ cells treated with BS373. PBMC obtained from 10 HTLV-1+ subjects were cultured in the absence or presence of quinoline BS373 (25 μM). Cell-free supernatants were collected 24 hours later and analyzed by flow cytometry for quantification of IL-2, IL-6, IL-10, IL-17α, TNF-α and IFN-γ using a CBA kit. *P=0.02; **P=0.006; ***P<0.0001. (Wilcoxon signed-rank test)

Figure 4: Apoptosis induced by BS373 in HTLV-1-infected cells. PBMC obtained from HTLV-1-infected subjects were cultured in the absence or presence of quinoline compound BS373 (6.25, 12.50 and 25 μM). Cells were stained with annexin V and propidium iodide and analyzed by flow cytometry 18 hours later. Results are expressed as mean±SEM (n=6). *P=0.02; P=0.01 (Mann-Whitney test).

Figure 5: Transmission electron microscopy of PBMC from HTLV-1-infected subjects after 18 hours of treatment with BS373. Cell presenting pycnotic nucleus (*) and disrupted mitochondria (a- white arrow heads). Cell with normal nucleus (N) intact electrondense

mitochondria and increased of autophagic vacuole formation (b- black arrow heads). Photooxidation detection of BS373 reaction product located within the numerous cytoplasmic autophagic compartments (c, d) presenting membrane profiles (c, arrow). (a, b, c and d - 1 µM).

Table 1: Expression of HTLV-1 Tax protein in PBMC from HTLV-1-infected individuals in the absence or presence of BS373.

Subject #	$CD3^+CD4^+Tax^+$ T lymphocytes (%)[a]		% inhibition
	-BS373	+BS373*	
1389	1.55	0.19	88.0%
3355	1.70	0.12	93.0%
6134	0.49	0.14	71.7%
6023	0.50	0.15	70.6%

[a]PBMC were cultured during 18 hours in the absence or presence of 25 µM BS373. Anti-Tax (Lt4 monoclonal antibody).

Figure 1

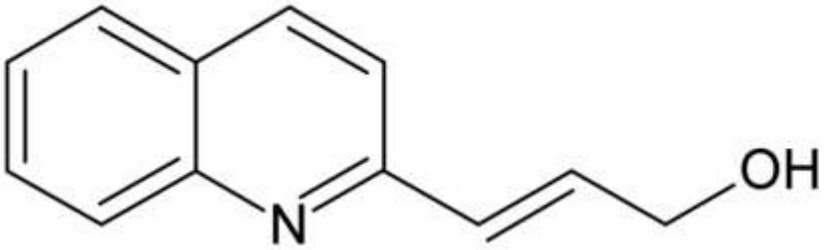


Figure 2

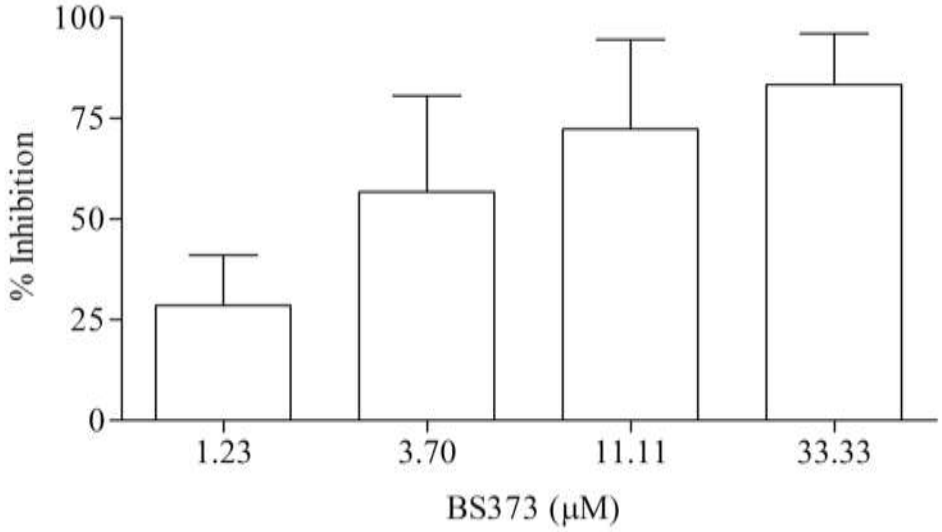


Figure 3

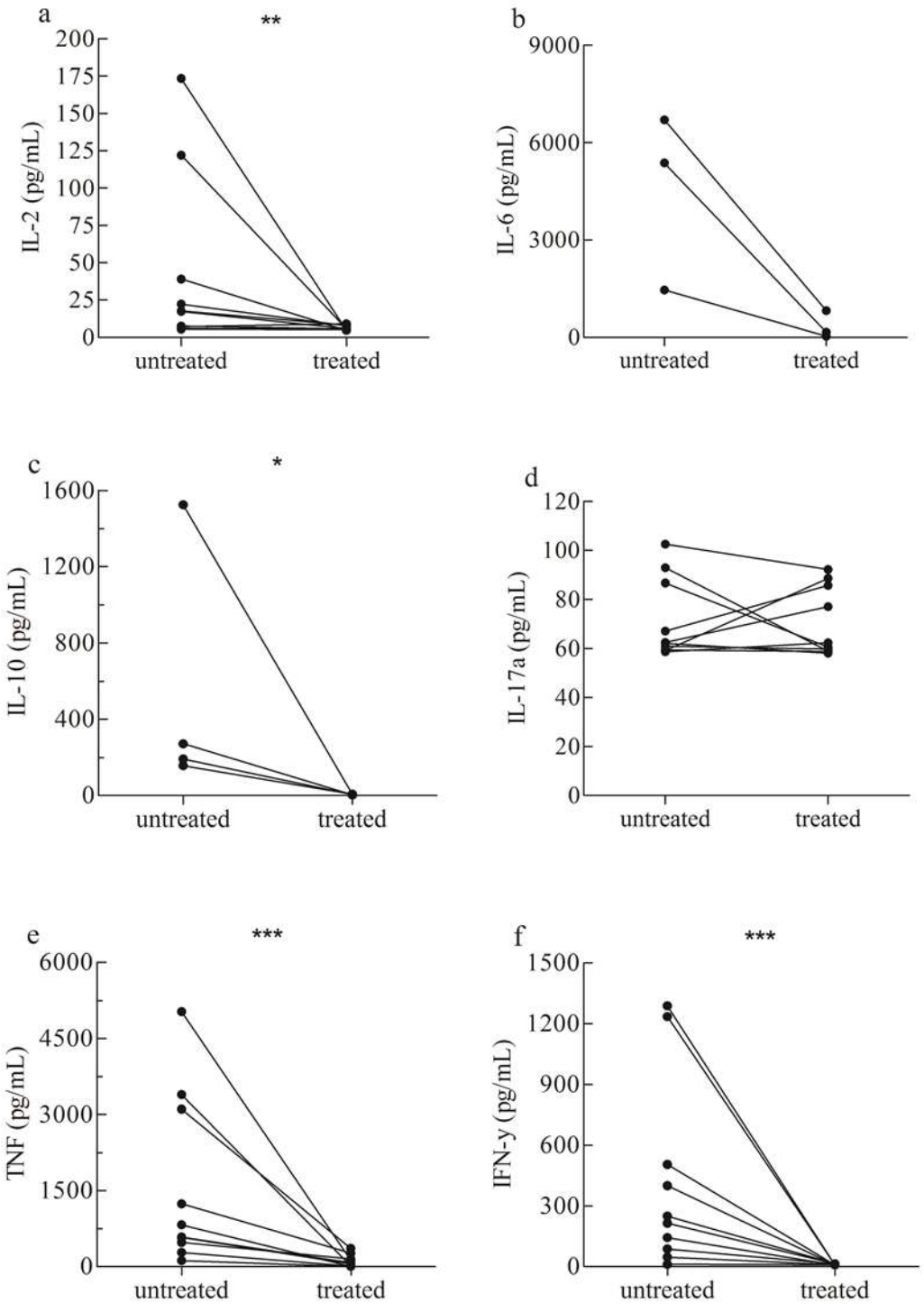

a
**
IL-2 (pg/mL)
200
175
150
125
100
75
50
25
0
untreated
treated
b
IL-6 (pg/mL)
9000
6000
3000
0
untreated
treated
c
*
IL-10 (pg/mL)
1600
1200
800
400
0
untreated
treated
d
IL-17a (pg/mL)
120
100
80
60
40
20
0
untreated
treated
e
***
TNF (pg/mL)
6000
4500
3000
1500
0
untreated
treated
f
***
IFN-y (pg/mL)
1500
1200
900
600
300
0
untreated
treated

Figure 4

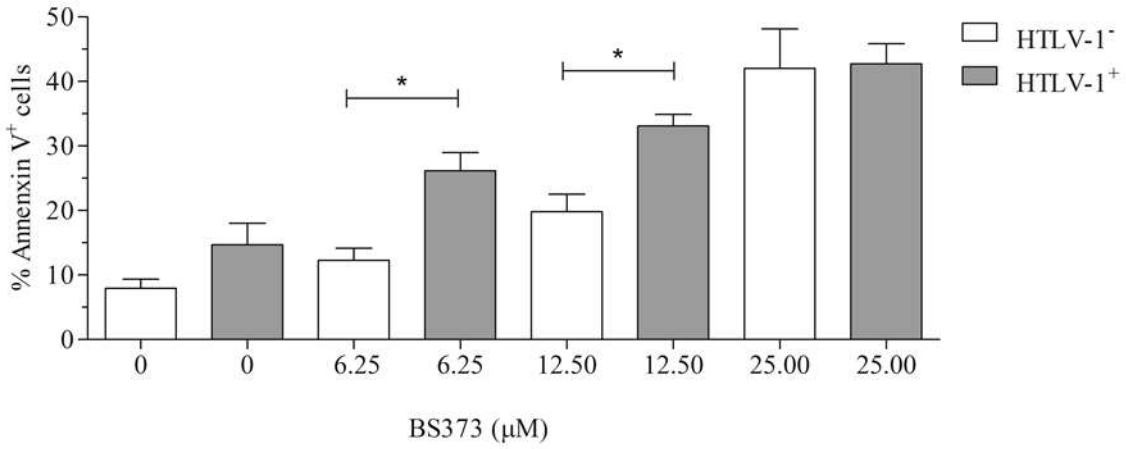
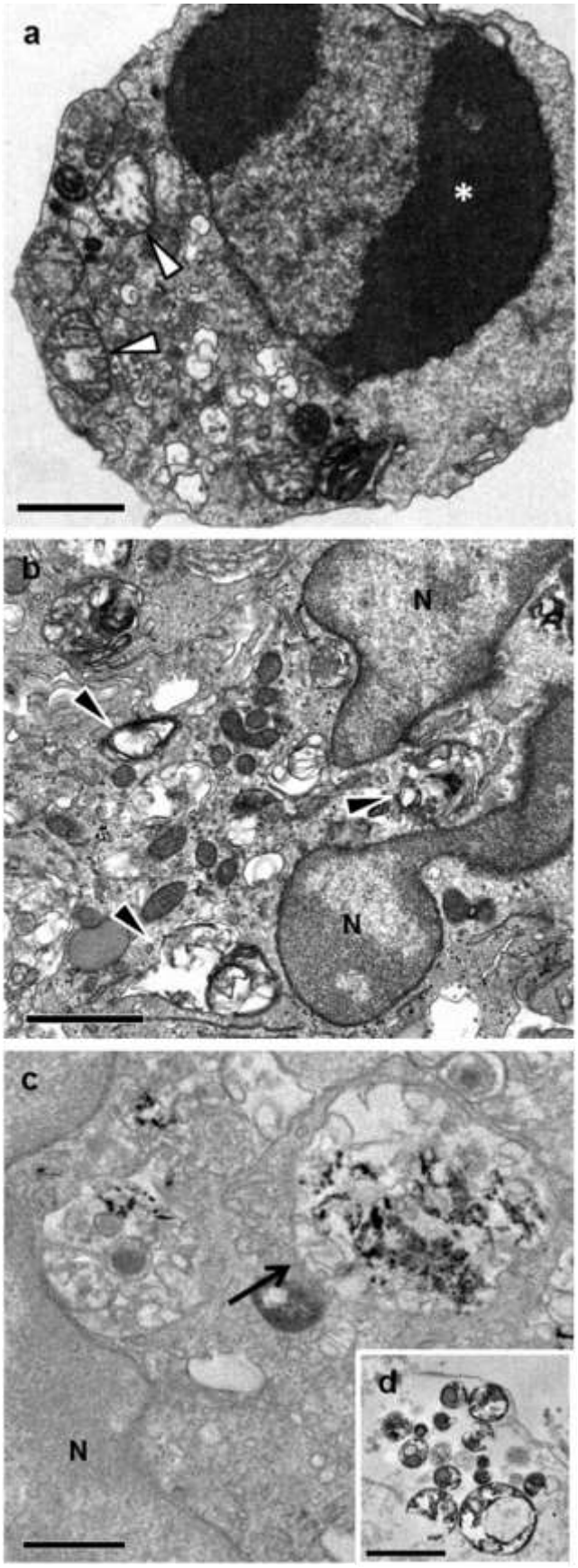

50
40
30
20
10
0
% Annexin V+ cells
*
*
0
0
6.25
6.25
12.50
12.50
25.00
25.00
BS373 (μM)
HTLV-1-
HTLV-1+

Figure 5



CAPÍTULO 02

Manuscrito 2: Physalin F, a seco-steroid from Physalis angulata L., has immunosuppressive activity in peripheral blood mononuclear cells from patients with HTLV1-associated myelopathy

As fisalinas são derivados esteroidais de estruturas complexas com atividades anti-inflamatórias e imunomoduladoras potentes. Resultados mostram que a fisalina F tem atividade anti-inflamatória, inibitória em tripanossomatídeos, promovendo apoptose e autofagia. Com base na atividade anti-proliferativa e imunomoduladora deste composto, o objetivo deste estudo foi avaliar o efeito da fisalina F sobre a proliferação de células mononucleares de sangue periférico (PBMC) e a modulação sobre as citocinas inflamatórias em pacientes com HAM/TSP.

Principais resultados encontrados:

1- Fisalina F inibiu a proliferação espontânea das células de pacientes com HAM/TSP infectados pelo HTLV-1;

2- Fisalina F reduziu a produção de citocinas como IL-2, IL-6, IL-10, TNF e IFN-γ;

3- Fisalina F induziu a apoptose celular em células de pacientes infectados;

4- Foram observados núcleos em picnose, presença de compartimentos semelhantes a vacúolos autofágicos, vacúolos com figuras de mielina, alterações na mitocôndria e no retículo células de pacientes tratadas com a fisalina F.

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Physalin F, a seco-steroid from *Physalis angulata* L., has immunosuppressive activity in peripheral blood mononuclear cells from patients with HTLV1-associated myelopathy

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ABSTRACT

Human T cell lymphotropic virus 1 (HTLV-1) induces a strong activation of the immune system in infected individuals with HTLV1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). A role for increased immunological response in the progression from asymptomatic to HAM/TSP has been proposed. Physalin F is a secosteroid isolated from *Physalis* spp. with potent anti-inflammatory and immunomodulatory activities. The present study investigated the effects of physalin F on cytokine profile, spontaneous proliferation, apoptosis and ultrastructure of peripheral blood mononuclear cells (PBMC) of HAM/TSP subjects. Physalin F (0.37-10 μM) exhibited a concentration-dependent inhibition of spontaneous proliferation of PBMC from HAM/TSP subjects, as evaluated by ^{3}H-thymidine uptake. The IC50 was 0.97±0.11 μM. Flow cytometry analysis using CBA showed that physalin F (10 μM) reduced significantly the levels of IL-2, IL-6, IL-10, TNF-α and IFN-γ, but not IL-17A, on supernatant of PBMC cultures. Next, apoptosis induction was addressed by flow cytometry after annexin V staining. Treatment with physalin F (10 μM) increased the apoptotic population in PBMC of HAM/TSP subjects. Ultrastructural analysis showed that physalin F induced ultrastructural changes in cells, such as pyknotic nuclei, abnormal mitochondria, autophagic vacuoles and the presence of myelin figures and endoplasmic reticulum dilation. In conclusion, physalin F induces apoptosis of PBMC, decreasing the spontaneous proliferation and cytokine production caused by HTLV-1 infection.

Keywords: HTLV1, physalin F, immunomodulation, cytokines, spontaneous proliferation, apoptosis.

Introduction

Human T cell lymphotropic virus type 1 (HTLV-1) infects 5-10 million people, mainly in Latin America, Caribbean Sea, south and center of Africa, and Japan (1). The virus is the etiological agent of two major diseases, adult T-cell leukemia and lymphoma (ATL) and a progressive neurologic disease, known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (2, 3), which occur in less than 5% of infected individuals. The virus also causes in a lower frequency uveitis and infective dermatitis (4, 5). In addition, HTLV-1-infected individuals are more prone to have other infectious diseases such as strongyloidiasis (6, 7), scabies (8) and tuberculosis (9, 10), which suggests an impairment the immune response.

HTLV-1 preferentially infects memory $CD45RO^+$ $CD4^+$ and $CD8^+$ T-lymphocytes, monocytes and dendritic cells (11-13) leading to persistent infection and strong immune activation. Spontaneous proliferation of $CD4^+$ and $CD8^+$ T-cell subsets, as well as NK cells are found both *in vitro* (14-16) and *in vivo* in HTLV-1-infected individuals (17). T-lymphocyte activation, reduced lymphoproliferative response *in vitro* to recall antigens (14) and high levels of proinflammatory cytokines such as IFNγ, TNFα, IL2, IL6 and IL-10 are mainly reported in patients with HAM/TSP (18, 19). A role for immune activation and increased levels of cytokines in both pathology and progression to HAM/TSP disease has been proposed (18, 20).

Since HAM/TSP was first described, little progress has been made in the development of treatment options. Steroids are still widely used for treatment since it has anti-inflammatory properties, yet little benefit is observed (21).

Physalis angulata (Solanaceae) is a widespread indigenous herb found in areas of Africa, Asia, and Americas, widely used in popular medicine as analgesic, anti-inflammatory

and antirheumatic. Physalins are steroid derivatives isolated from *Physalis spp*. with potent anti-inflammatory and immunomodulatory activities (22-26). Physalin F prevents mortality induced by a lethal injection of lipopolysaccharide - LPS, and inhibits rejection of allogeneic transplants in mice (22, 23). The anti-inflammatory activity of physalin F was also demonstrated in models of intestinal ischemia and reperfusion injury and arthritis (27, 28). In addition, physalin F inhibit NF-kB activation, a key inflammatory transcription factor, and the production of proinflammatory mediators, such as TNFα, IL-6 and IL-12 (26, 27). The purpose of this study was to investigate the immunomodulatory effects of physalin F on PBMC of HAM/TSP patients. Given the immunopathological mechanisms of HTLV1 infection, the effects of physalin F were evaluated on spontaneous cell proliferation, cytokine profile, apoptosis and ultrastructural changes of PBMC.

Results

Physalin F inhibits the spontaneous proliferation of PBMC from HTLV-1$^+$ subjects

We first tested the effects of physalin F (Figure 1) in cultures of PBMC obtained from HTLV-1$^+$ subjects with diagnostics of HAM/TSP. Addition of physalin F caused a concentration-dependent reduction of spontaneous proliferation characteristic of HTLV-1-infected cells (Figure 2). The estimated IC_{50} for physalin F was 0.97±0.11 μM, and this compound did not cause cell toxicity in cultures of PBMC when tested in concentrations lower than 20 μM (data not shown).

Reduction of cytokine production by physalin F in PBMC cultures from HTLV-1$^+$ subjects

Cultures of PBMC obtained from HTLV-1$^+$ subjects spontaneously produced several cytokines, including IL-2, IL-6, IL-10, IL-17a, TNF-α and IFN-γ (Figure 3). Addition of 10 μM physalin F caused a significant reduction of IL-2, IL-6, IL-10, TNF-α and IFN-γ. No differences in the levels of IL-17A were observed in cultures treated or not with physalin F (Figure 3). IL-4 levels were below the detection limit of the assay (data not shown).

Induction of apoptosis by physalin F in cultures of PBMC from HTLV-1$^+$ subjects

Next we investigated the effects of physalin F on apoptosis in PBMC cultures. Addition of physalin F enhanced the percentage of apoptotic cells in a concentration-dependent way, in PBMC cultures from HTLV-1-infected subjects, as shown by annexin V staining (Figure 4). Transmission electron microscopy analysis revealed that, in the absence of physalin F, PBMC from HTLV-1-infected subjects had morphological alterations, including ultrastructural

changes in the mitochondria and presence of pyknotic nuclei (Figure 5 a-c). When incubated with 10 µM of physalin F, and a higher frequency of the changes already described, a remarkable swelling of the endoplasmic reticulum cisternae was observed, culminating in long ridges of cytoplasm, and autophagic vacuole-like compartments (Figure 5 d-g). Physalin-treated cell nuclei also presented myelin-like membranes and intranuclear membranes, presumably nucleoplasmic reticulum.

Discussion

HTLV-1 induces cell activation and intense proliferation of both subpopulations of $CD4^+$ and $CD8^+$ T lymphocytes (14, 16). It has been reported that cells from subjects with HAM/TSP present proliferation and production of proinflammatory cytokines higher than those of asymptomatic carriers (18, 19). Thus, the development of drugs of immunomodulatory strategies to reduce the immunological alterations promoted by the virus may be beneficial for HTLV-1-infected individuals.

The present study shows that physalin F, a secosteroid isolated from plants from *Physalis* genus, has a potent inhibitory activity on the alterations induced by HTLV-1 in PBMC cultures obtained from HAM/TSP individuals. This activity was evidenced by inhibition of spontaneous proliferation, reduction of cytokine production and induction of apoptosis after incubation with atoxic concentrations of physalin F.

Physalin F has been extensively investigated regarding its immunomodulatory potential. This compound has a potent inhibitory activity on mouse lymphocytes, causing the reduction of proliferation and cytokine production in cultures stimulated with mitogen or mixed lymphocyte reaction (23). Additionally, its effects were investigated *in vivo* in mouse models of collagen-induced arthritis, allergic airway inflammation and allogeneic transplant (23, 28). Physalin F also showed inhibitory effects on the proliferation of cancer cells (29). Thus, our study in human cells infected with HTLV-1 reinforces the antiproliferative potential of physalin F.

The proviral load of HTLV-1 (amount of provirus incorporated to the genome) is maintained largely by mitotic division of infected cells, as well as by the activation and secretion of T cell growth factors cytokines such as IL-2 and IL-15 (30). Here we found that

IL-2 is strongly inhibited, which suggests that the reduced proliferation may be due in part by lack of IL-2 production.

We also observed an increase in the percentage of apoptotic cells after treatment with physalin F, which may contribute to the reduction of proliferation in PBMC cultures. This was observed by flow cytometry (increased annexin V staining), as well as ultrastructural alterations indicative of apoptosis and autophagy (thickening of the mitochondrial membrane, presence of pyknotic nuclei and myelin figures). A previous report has shown that physalin F triggers apoptosis of cancer cells by activating caspase 3 and c-myc pathways (31), as well as inhibition of NF-kB activation and accumulation of reactive oxygen species (29). Further studies are required to demonstrate the mechanisms by which physalin F induces apoptosis of HTLV-1-infected cells. The demonstration that HTLV-1-infected cells have increased resistance to apoptosis (32) suggests that apoptosis-inducing drugs, such as observed herein with physalin F, is a relevant therapeutic approach.

In conclusion, we have found a potent immunosupressive effect of the natural compound physalin F on HTLV-1-infected cells, which reinforces this class of compounds as antiproliferative agents. Due to the lack of therapeutic approaches for HTLV-1-infected patients, our results also support the search for complementary therapies, in addition to the development of antiretroviral drugs.

Experimental Section

Subjects. Twenty one HTLV-1-infected patients with HAM/TSP diagnosis defined according to World Health Organization (33) criteria followed at Bahiana School of Medicine and Public Health reference center for HTLV in Salvador, Northeast Brazil were included in the study. Samples were screened for HTLV-1/2 antibodies by an enzyme-linked immunosorbent assay (Ab-Capture ELISA test system; Ortho-Clinical Diagnostics, Inc., Raritan, NJ) and confirmed by using a Western blot assay (HTLV Blot 2.4; Genelabs Technologies, Singapore). The group consisted of 14 women (63%) and had a mean age of 58 years. Informed consent was obtained from all enrolled subjects, and the Institutional research boarding of the Oswaldo Cruz Foundation (FIOCRUZ) approved this study.

Culture conditions and PBMC isolation. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood samples by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech; Uppsala, Sweden). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM L-glutamine (Sigma), 1% nonessential amino acids (Gibco Laboratories, Gaithersburg, MD), 1 mM sodium pyruvate (Sigma), 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), 100 µg/mL HEPES (Invitrogen; Eugene, OR) , and 10 % fetal bovine serum (FBS; Gibco).

Drug. Physalin F was isolated from *Physalis angulata* L. collected in Belém do Pará, Brazil, as described previously (22). Preparation of physalin F (97.8 % purity by HPLC) was dissolved in DMSO (Sigma) and then diluted in cell culture medium. The final concentration of DMSO was less than 1 % in all experiments.

In vitro cellular toxicity assay. PBMC (10^5 cells/well) from HTLV-infected patients and uninfected controls were cultured in 96-well plates in the absence or presence of serial dilutions of physalin F (ranging from 0.62 to 20 µM) at 37°C in a 5%CO_2 humidified atmosphere. After 24 hours of culture, cells were pulsed for three hours with 20 µl of 5 mg/mL MTT (3-[4,5-Dimethylthiazol 2yl]-2,5 diphenyltetrazolium bromide; Thiazolyl blue; Sigma). The optical density (OD) was determined by Versamax photometer (Molecular Devices Inc.; Menlo Park, CA) at a test wavelength of 570 nm. The toxicity was evaluated by the ratio of OD of a well in the presence of physalin F with the OD of control wells in the presence of medium. The cellular viability of at least 80% was considered to indicate a non-toxic concentration.

Lymphoproliferation assay. PBMC (10^5 cells/well) from three subjects with HAM/TSP diagnosis were cultured in the absence or presence of different concentrations of physalin F on RPMI 1640 medium supplemented with 10% FBS. Cells were seeded in triplicate on 96-well plates and cultured at 37°C in a 5% CO_2 humidified atmosphere for 72 hours. A 1µCi/well amount of [methyl-3H]thymidine (Perkin Elmer, Waltham, MA) was added to the cultures, which were then incubated for 18 hours at 37 °C and 5 % CO_2. After this period, the content of the plate was harvested to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Chameleon, Hydex; Turku, Finland). Results of cell proliferation were expressed as mean counts per minute.

Detection of phosphatidylserine translocation by annexin V. PBMC from six HTLV-1-infected subjects were seeded on 96-well plates at a cell number of $2x10^5$ and cultured in the absence or presence of 10 µM of physalin F for 18 hours at 37 °C and 5 % CO_2. Cells were then stained with Alexa Fluor® 488 annexin V and propidium iodide (PI), using the Alexa

Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer's instructions. The cells were acquired by flow cytometry (FACSAria, Becton Dickinson, Mountain View, CA) and the analysis was performed using the FlowJo software (Ashland, OR). A total of 50,000 events were acquired in the region previously established as that corresponding to PBMC.

CBA assay. The PBMC from six HTLV-1-infected patients were plated in 96-well plates at $2x10^5$ cells/well in the presence of 10 µM of physalin F at 37 °C for 24 hours. Supernatants were then harvested and cytokines were measured using BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit, according to the manufacturer's instructions (BD Biosciences, San Jose, CA). The concentrations of cytokines in the samples were calculated by extrapolating the mean fluorescence intensity (MFI) on the respective standard curves. The cells were acquired by flow cytometry (FACSAria) and analysis was performed FlowJo and Graphpad Prism 5.02 softwares (Graph Pad Software, San Diego, CA).

Transmission electron microscopy analysis. For transmission electron microscopy (TEM) analysis, PBMC ($2x10^6$) were treated with physalin F (10 µM) and incubated for 18 hours at 37 °C. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and then treated with 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM calcium chloride, for 60 min in the dark. Cells were dehydrated in acetone series and infiltrated in polybed epoxy resin (Polysciences, Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate. The ultrastructure analysis was performed in a transmission electron microscope (Jeol JEM 1230 or Zeiss EM109).

Statistical analyses. Data are expressed as percentages, means and standard errors. Groups were compared using the Mann-Whitney test and Wilcoxon signed-rank test. A P value of less than 0.05 denoted a statistically significant difference. Graphpad Prism 5.02 software was used for all statistical analyses.

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

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Legend of figures

Figure 1. Chemical structure of physalin F.

Figure 2. *In vitro* inhibition of spontaneous proliferation of PBMC from subjects with HAM/TSP by physalin F. PBMC isolated from three patients with HAM/TSP diagnosis were cultured in the presence or absence of different concentrations of physalin F for three days. Cell proliferation was then measured by ^{3}H-thymidine incorporation using a β-radiation counter. Values represent the means ± SEM of three patients.

Figure 3. Assessment of cytokines in culture supernatants of HTLV-1-infected cells. PBMC cultures were treated or not with physalin F (10 μM) for 18 hours. Cell-free supernatants were analyzed by flow cytometry using CBA method. (a) IL-2; (b) IL-6; (c) IL-10; (d), IL-17α, (e), TNF, (f), IFN-γ.

Figure 4. Analysis of phosphatidylserine surface exposure in cells from HTLV-infected subjects. PBMC cultures were treated or not with physalin F (10 μM) for 18 hours. Cells were incubated with propidium iodide and annexin V and analysed by flow cytometry. Values represent the mean ± SEM of six determinations. *P=0.01 (Wilcoxon and Mann Whitney tests).

Figure 5. Transmission electron microscopy of PBMC obtained from HTLV-infected subjects before (a-c) and after (d-j) physalin F treatment. The majority of the cells (a) displayed intact mitochondria (M) and normal nuclei (N), whereas some cells showed disrupted mitochondria (b, arrows) and pyknotic nuclei (c, *). Physalin F triggered remarkable swelling of the endoplasmic reticulum cisternae (d, e arrows), culminating in long ridges of cytoplasm (f arrowheads, g). The nuclear envelope formed thin (h arrow head) or broad (i, arrows) reticulum cisternae, presumably sectioned transversally and tangentially, respectively

as well as autophagic vacuole-like compartments (i, arrowhead). Physalin F-treated cell nuclei also presented myelin-like membranes (j arrowhead) and intranuclear membranes, presumably nucleoplasmic reticulum (j arrow). Scale bars: a, c, d and e = 2 µM; b, f, h, i and k = 1 µM; g and j = 0.5 µM.

Figure 1

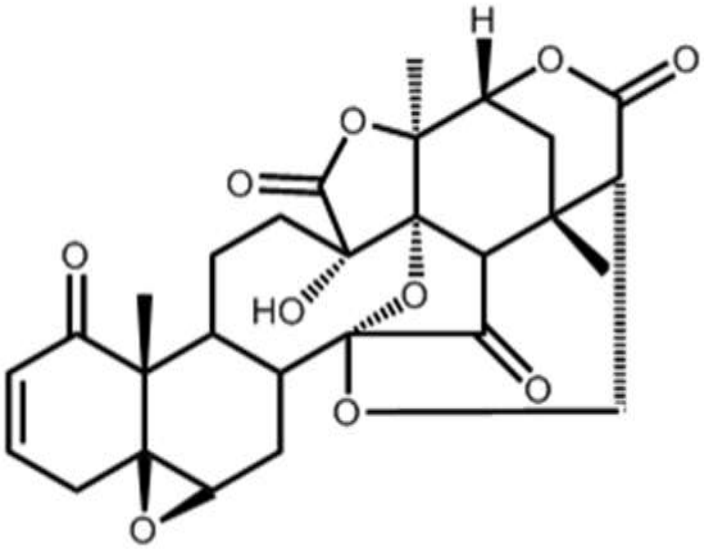


Figure 2

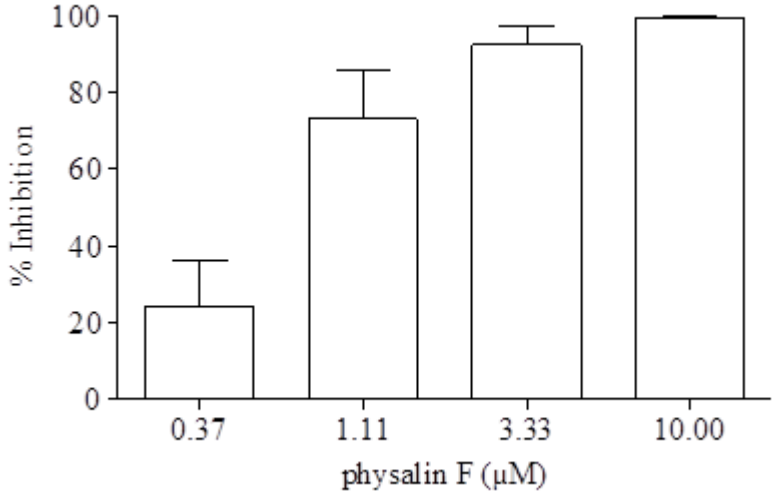
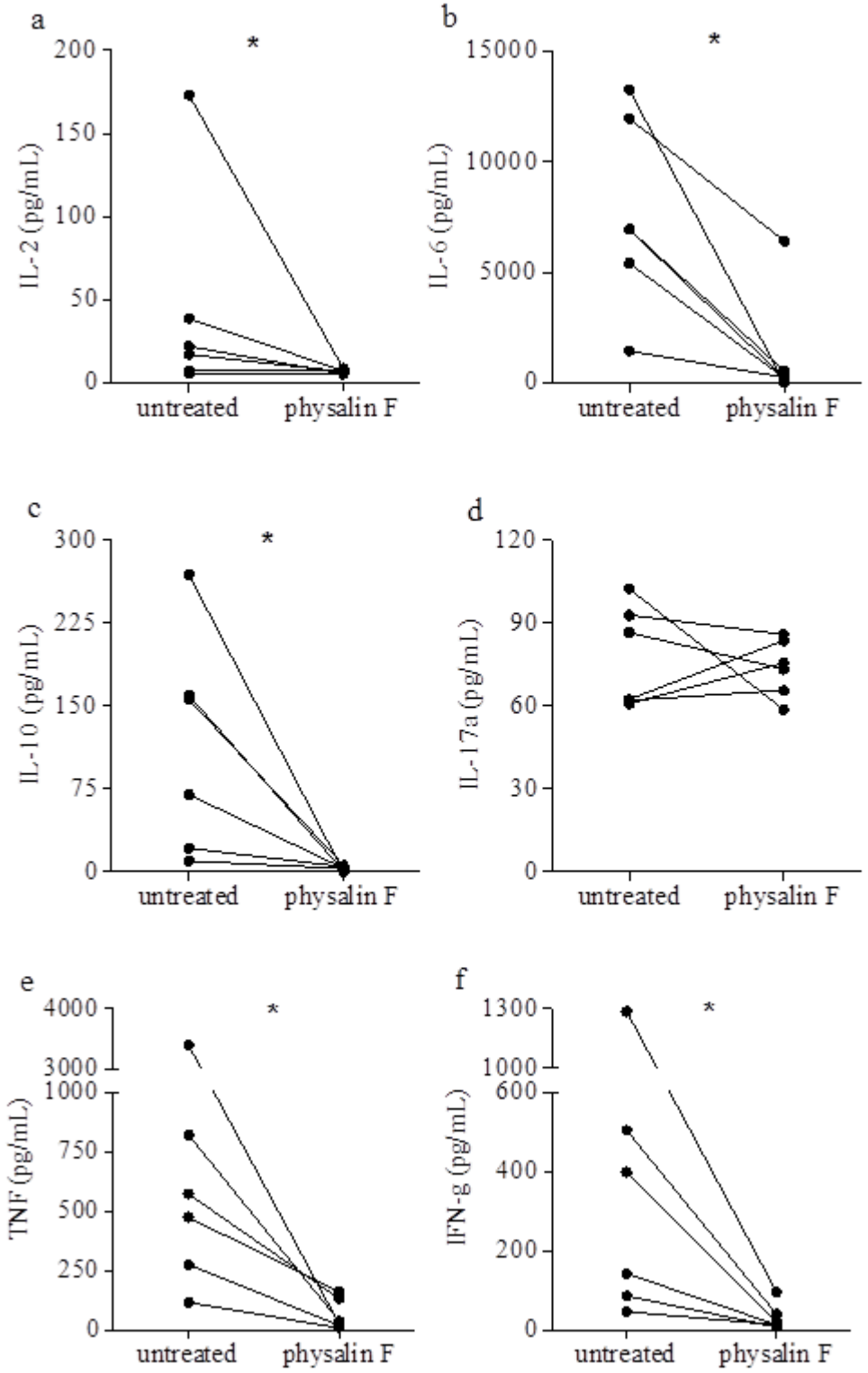


Figure 3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physalin F
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150
75
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IL-10 (pg/mL)
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IL-17a (pg/mL)
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TNF (pg/mL)
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IFN-g (pg/mL)
untreated
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Figure 4

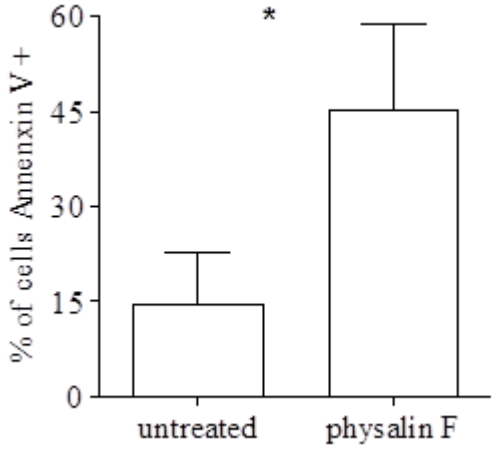
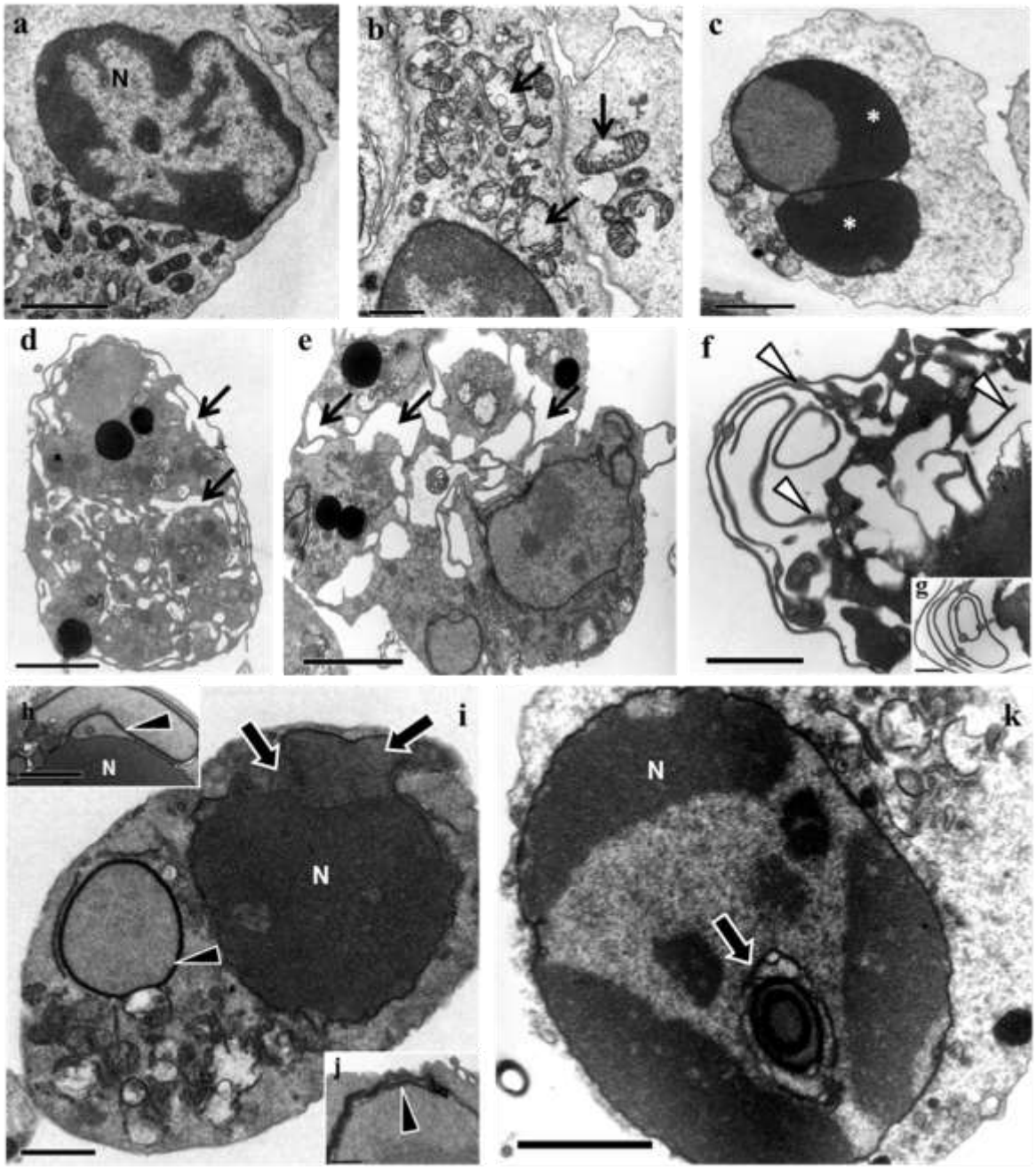


Figure 5



CAPÍTULO 03

Manuscrito 3: Effect of two quinoline derivate on spontaneous proliferation and on ultrastructural organization of cells from HTLV-1-infected individuals.

Na infecção pelo HTLV-1 a indicação de terapêutica é restrita aos grupos sintomáticos. Compostos quinolínicos são capazes de diminuir a proliferação espontânea em células de linhagem transformadas pelo HTLV-I. Em 2008, nosso grupo identificou novos compostos quinolínicos capazes de inibir a proliferação espontânea em PBMC de indivíduos infectados pelo HTLV-1. Neste estudo, nos propomos a identificar os mecanismos de ação de dois diferentes compostos quinolínicos em PBMC de células de pacientes infectados pelo HTLV-1. Acreditamos poder contribuir com novas estratégias terapêuticas para as patologias associadas ao HTLV, particularmente ligadas a proliferação celular exacerbada.

Principais resultados encontrados:

1- Q1 e Q2 inibiram a proliferação espontânea das células de pacientes com HAM/TSP infectados pelo HTLV-1;

3- Pode-se observar presença de compartimentos semelhantes a vacúolos autofágicos e com figuras de mielina em células de pacientes tratados com Q1 e Q2.

A ser submetido para publicação na Biomedicine & Pharmacotherapy.

SHORT COMMUNICATION

Effect of two quinoline derivate on spontaneous proliferation and on ultrastructural organization of cells from HTLV-1-infected individuals.

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ABSTRACT

Spontaneous proliferation, a hallmark of Human-T Lymphocyte Virus Type 1 (HTLV-1) infection, is particularly higher in HAM/TSP patients compared to asymptomatic carriers. The identification of drugs that modulate the spontaneous proliferation may be useful for the treatment of HAM/TSP disease. This study evaluated the effect of two different quinoline compounds (Q1 and Q2) on the modulation of spontaneous proliferation of cells from HTLV-1 patients with HAM/TSP. The cells were cultivated in the presence of serial concentrations of quinolone compounds. Cell proliferation was assessed by ^{3}[H]thymidine incorporation. Cell viability was measured by optical density in the presence of MTT. The ultrastructure analysis was done using a transmission electron microscope. Quinoline compounds were not toxic at the concentrations evaluated. The IC50 was 38 µM for compound Q1 and 82 µM for Q2. Both compounds inhibited spontaneous proliferation. In addition, compounds induced vacuoles with myelin-like membranes, probably autophagic vacuole-like compartments, observed by electronic microscopy. In conclusion, the quinoline compounds were able to inhibit the spontaneous proliferation of T cells from HTLV-1-infected individuals. Further studies should be conduct to understand how these compounds act on cells by decreasing cell proliferation.

Keywords: HTLV-1, HAM/TSP, spontaneous proliferation, inhibition, quinolones.

1. Introduction

Spontaneous proliferation of peripheral blood mononuclear cells (PBMCs) is an immunological hallmark of Human-T Lymphocyte Virus Type 1 (HTLV-1) infection. Cells from HTLV-1-infected individuals proliferate *in vitro* in absence of exogenous addition of antigens [1]. *In vivo*, the proliferation rate of memory $CD45RO^+$ $CD4^+$ and $CD8^+$ T-lymphocytes can reach an extra 10^{12} T-lymphocytes produced in one year in HTLV-1-infected individuals, compared with an uninfected control [2]. In adult T cell Leukemia/lymphoma (ATLL), one of the HTLV-1-associated diseases, cell proliferation results from the expansion of a malignant monoclonal clone [3]. In contrast, in asymptomatic carriers and in patients with myelopathy associated with HTLV-1/tropical spastic paraparesis (HAM/TSP) a polyclonal expansion of both $CD4^+$ and $CD8^+$-T lymphocytes and NK cells is observed [4, 5]. It is assumed that the magnitude of the spontaneous proliferation is greater in HAM/TSP patients, indicating a possible pathogenic role of this phenomenon. However, spontaneous proliferation of PBMCs is observed in almost 70% of HTLV-1-infected asymptomatic individuals [6].

In contrast to the infection with human immunodeficiency virus (HIV), another retrovirus, there are no treatment specific to HTLV-1 infection. Treatment is directed to the associated ATLL or HAM/TSP diseases with limited efficacy. Quinoline compounds are alkaloids isolated from *Galipea longiflora*, a Bolivian plant with medicinal properties [7]. Synthetically produced quinolones have demonstrated an inhibitory effect on proliferation of HTLV-1 transformed cell lines [8], and on PBMCs from HTLV-1-infected individuals with low toxicity [9]. This study evaluated the inhibitory effect of two quinolines on spontaneous proliferation of cells from HTLV-1-infected individuals with HAM/TSP and described the main ultrastructural changes induced by these compounds in PBMCs.

2. Materials and methods

Peripheral blood mononuclear cells (PBMCs) were obtained from HTLV-1-infected patients (ELISA and Western blot) with HAM/TSP diagnosis [10] followed reference center for HTLV at Bahiana School of Medicine and Public Health in Salvador, Northeast Brazil. Informed consent was obtained from all subjects, and the Institutional research board of the Oswaldo Cruz Foundation (FIOCRUZ) approved this study. Evaluated compound were 2-(2-methoxyethenyl) quinoline derivate (Q1) and 2-(2-furylethenyl) quinoline derivate (Q2) (Figure 1). To assess cellular toxicity, 10^5 cells/well were cultured in presence of serial dilutions of Q1 and Q2 (1.56 to 100 μM) for 24 hours. Then, cells were pulsed for three hours with 20 μl of MTT (3-[4,5-Dimethylthiazol 2yl]-2,5 diphenyltetrazolium bromide; Thiazolyl blue, Sigma Chemical Co., St. Louis, MO). Low-toxicity of a compound was indicated by cellular viability over 80%. In order to evaluate PBMC proliferation, cells were cultured for three days and pulsed overnight with 1 μCi ^{3}H-thymidine (ICN; Costa Mesa, CA). Results were expressed as mean counts per minute (cpm). For transmission electron microscopy (TEM) analysis, PBMC ($2x10^6$) were cultured in presence or absence of compounds for 18 hours. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and then treated with 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM calcium chloride, for 60 min in the dark. Cells were dehydrated in acetone series and infiltrated in polybed epoxy resin (Polysciences; Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate. The ultrastructure analysis was performed in a transmission electron microscope (Jeol JEM 1230 or Zeiss EM109). Photooxidation analysis was performed in PBCM samples cultured in the presence of compounds for 18 hours. PBMC fixed in 4% paraformaldehyde were pre-loaded for 15 min with 10 mg/mL of diaminobenzidine (DAB) in Tris-HCl, pH 7.2 and incubated for 30 min under ultraviolet light. Then, additional 10 mg/mL of DAB was added, and cultures kept under ultraviolet light

for further 30 min. The cells were then washed twice in Tris-HCl, pH 7.2, and once in 0.1M sodium cacodylate at pH 7.4. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 60 minutes at room temperature and processed for TEM.

Data were expressed as percentages, means and standard deviations. Compared groups using the Friedman test. A P value of less than 0.05 denoted a statistically significant difference. Graphpad Prism 5.02 (GraphPad; San Diego, CA) software was used for statistical analyses.

3. Results

Addition of both Q1 and Q2 caused a concentration-dependent reduction of spontaneous proliferation of PBMCs from HTLV-1-infected individuals (Figure 2). The inhibitory effect persisted for 168 hours (data not shown). The estimated IC50 was 53±38 µM (95% IC, R2=0.99) and 68±44 µM (95%IC, R^2=0.99) for Q1 and Q2, respectively. Q1 and Q2 did not cause cell toxicity in cultures of PBMC when tested in concentrations lower than 100 µM (data not shown).

Compounds Q1 and Q2 were detected in cytoplasmic membrane-bounded compartments, eventually displaying myelin-like figures, presumably comprising autophagic vacuoles (Figure 3). Enlarged de Golgi apparatus cisternae were observed Q2-treated cells (Figure 3d).

4. Discussion

Taken together these findings demonstrated that both Q1 and Q2 compounds strongly inhibit spontaneous proliferation of cells from HTLV-1-infected patients with HAM/TSP. Cells treated with both compounds presented vesicles containing the drug and membranes arranged in myelin-like figures, suggestive of autophagic vacuoles. It is possible that cells are under oxidative stress.

Spontaneous proliferation is one of the most remarkable immunological changes in individuals infected with HTLV-1, and might play a role in the pathogenesis of the disease, since higher intensity of proliferation are described in patients with HAM/TSP [11], yet asymptomatic carriers also may have an elevated intensity of cell proliferation [1, 6]. The subpopulations of memory ($CD45RO^+$) $CD4^+$ and $CD8^+$ T-lymphocytes are the main subsets involved in this phenomenon [4]. In the past decade, HTLV-1 proviral load has been suggested as a biomarker for the developing HTLV-1 associated diseases, especially HAM/TSP [12, 13]. A cutoff of 5% infected cells was found as the best value to differentiate HAM/TSP from asymptomatic carriers, with high specificity [14]. High HTLV-1 proviral load has also been found in HTLV-1-infected individuals with other clinical manifestations such as infective dermatitis [15] and keratoconjuntivitis sicca [16]. HTLV-1 proviral load is mainly maintained by mitotic division of infected $CD4^+$ T cells [17], although virological synapses can also disseminated the virus. Therefore, decreasing spontaneous proliferation might constitute a strategy to contribute to the control of proviral load. However, in the present study the effect of these compounds on the HTLV-1 proviral load could not be assessed.

Yet, it is possible to suggest that Q1 and Q2 by inducing changes in the machinery of cells produce a cytostatic effect, reducing then the spontaneous proliferation. Further studies should be conducted to better understand the mechanisms of action of these quinoline compounds.

5. Acknowledgments

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6. Conflict of Interest

The authors declare no conflict of interest.

7. References

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Figure legends

Figure 1: Chemical structure of quinoline compound Q1 (a) and Q2 (b).

Figure 2: Concentration-dependent inhibition of spontaneous proliferation of PBMC by Q1 and Q2. PBMC from three HTLV-1-infected subjects were cultured in the absence or in the presence of different concentrations of Q1 and Q2. Spontaneous proliferation was assessed by ^{3}H-thymidine incorporation after 3 days of culture. Results are expressed as mean±SEM (n=3) of the percentage of inhibition in relation to untreated controls. (a) Q1; (b) Q2.

Figure 3: Transmission electron microscopy of PBMC from HTLV-1-infected subjects after 18 hours of treatment with Q1(a-b) and Q2(c-f). Cell presenting normal metabolism observed by mitochondria and endoplasmic reticulum (white arrowhead - c). Photooxidation of fluorescent compounds in the presence of diaminobenzidine with ultraviolet revealed the presence of these compartments of endocytic vesicles (b, d, e) and autophagic (a, f), indicated by degradation material and membranes, including myelin-like figures (f). Golgi apparatus having expansion in the presence of quinolone Q2 (black arrowhead - d). Compounds were found within cells in autophagic vesicles (black arrow). Scale bars: a, b and c – 2 μm; d, e – 1 μm; f – 0,2 μm

Figure 1

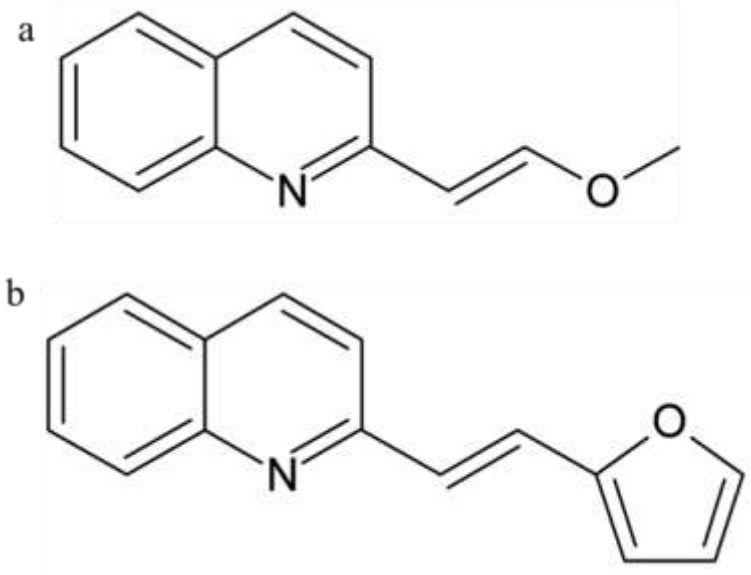
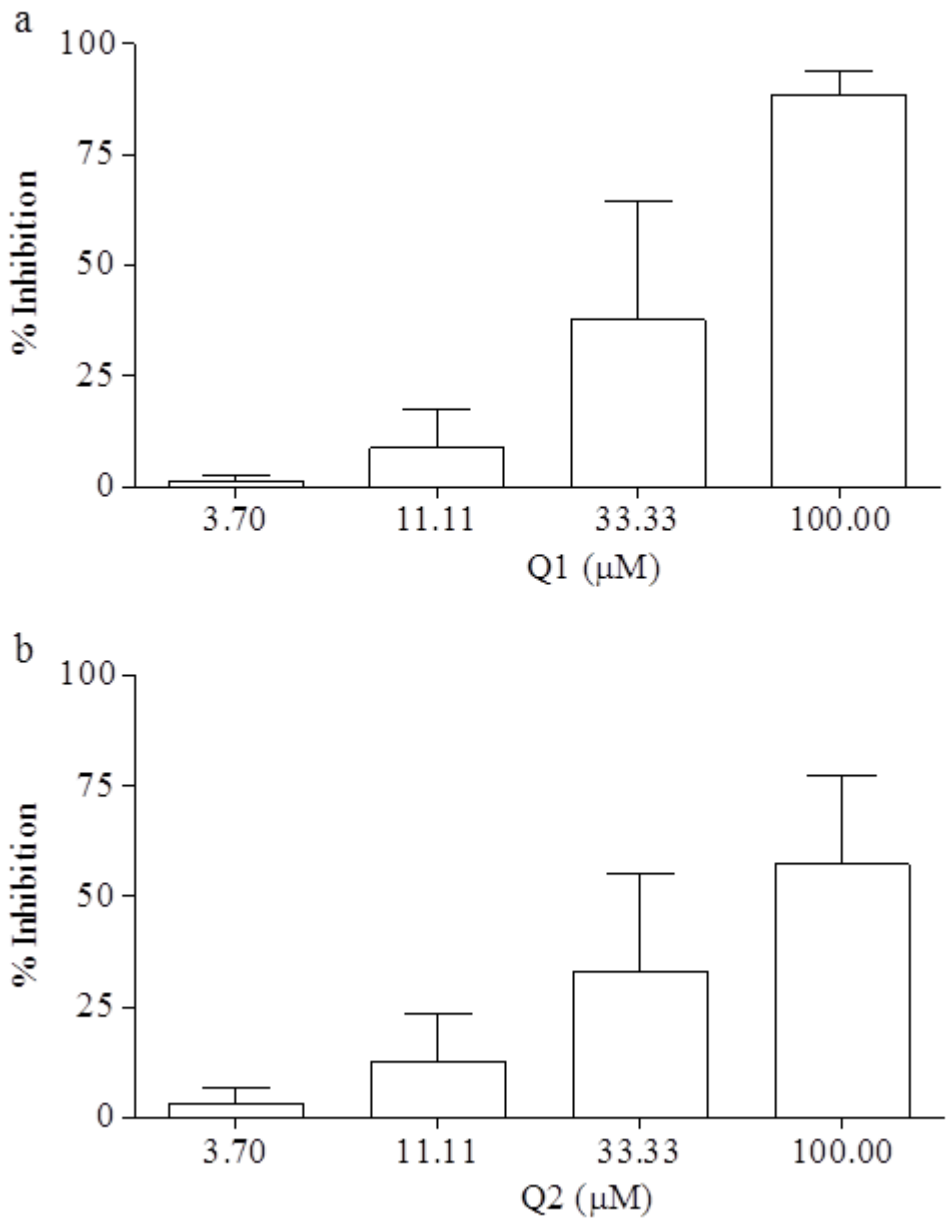


Figure 2


a
100
75
50
25
0
% Inhibition
3.70
11.11
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100.00
Q1 (µM)
b
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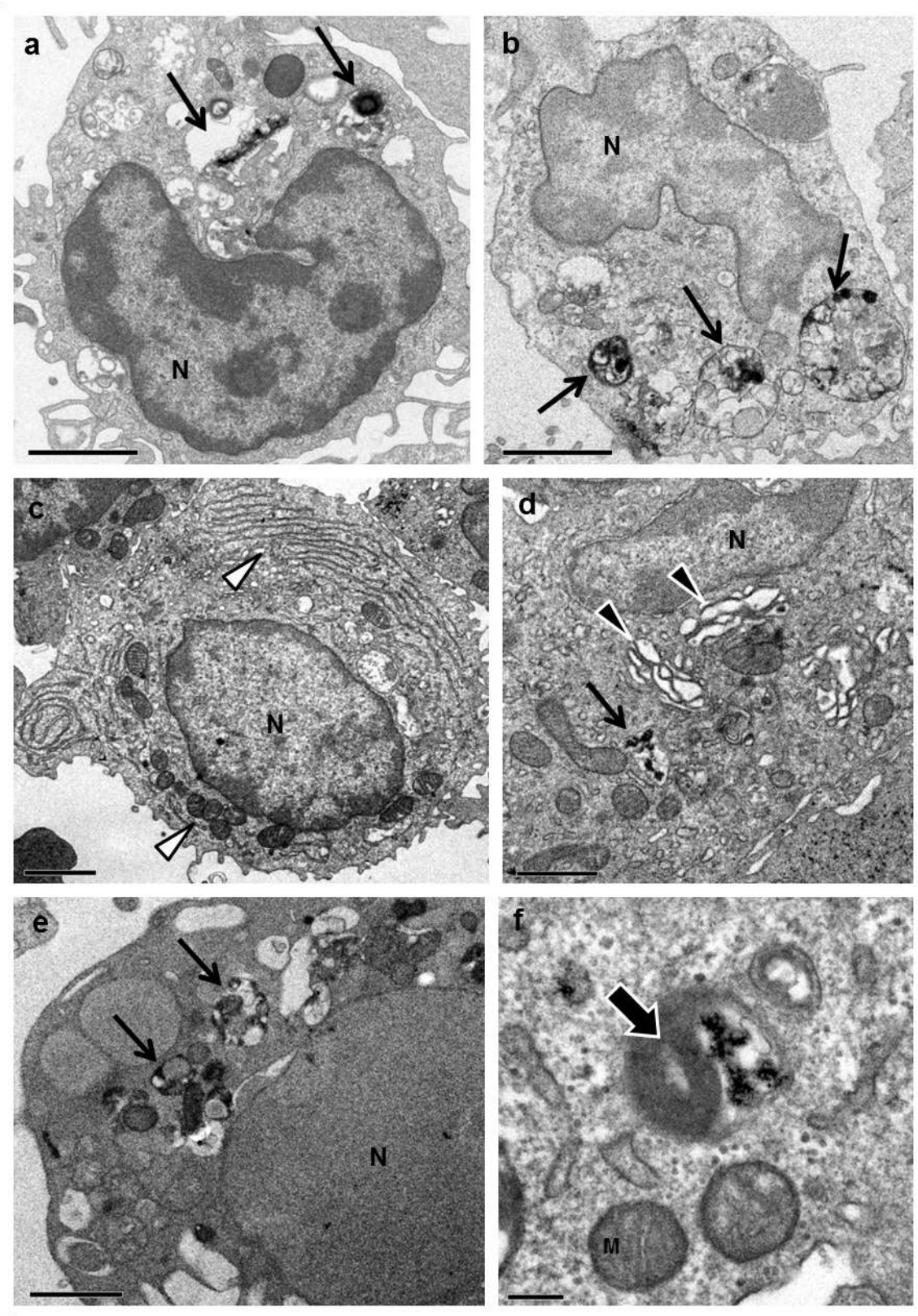
25
0
% Inhibition
3.70
11.11
33.33
100.00
Q2 (µM)

Figure 3



4 DISCUSSÃO

Os resultados apresentados neste trabalho mostram que as quatro moléculas avaliadas (três compostos derivados de quinolinas e a fisalina F) foram capazes de reduzir a proliferação espontânea dos PBMCs de pacientes com HAM/TSP e promoveram alterações relevantes na ultraestrutura das células infectadas tratadas. O derivado de quinolina, 2-(2-hydroxyprop-2-enyl) quinoline (BS373), e a fisalina F induziram aumento da morte celular por apoptose e reduziram a produção de citocinas Th1 e Th2, além disso a quinolina BS373 inibiu a expressão da proteína viral Tax. A autofluorescência apresentada pelos compostos quinolínicos 2-(2-methoxyethenyl)quinoline (BS318) e 2-(2-furylethenyl)quinoline (BS260) representou um fator limitante no avanço dos parâmetros avaliados para essas moléculas. Ao sumarizar estes resultados consideramos os quatro compostos testados como ativos e alvos de investigação para melhor entendimento das vias celulares envolvidas em sua ação.

O HTLV-1 induz uma ativação celular *in vitro* e *in vivo* de ambas as subpopulações de linfócitos T CD4+ e T CD8+ (PRINCE, *et al*., 1995; MASCARENHAS, *et al*., 2006, ASQUITH, *et al*., 2007). Tem sido descrito que em indivíduos com HAM/SP a intensidade de proliferação e a produção de citocinas proinflamatórias são maiores que a de portadores assintomáticos (NISHIURA, *et al*., 1996; BRITO-MELO, *et al*., 2004, ASQUIT *et al.*,. 2007). A inibição da proliferação espontânea nas células infectadas reflete um controle inicial do desequilíbrio imune ao qual estar submetido os indivíduos HTLV-1+. Além de inibir a proliferação, dois dos compostos avaliados, BS373 e fisalina F, reduziram a produção de citocinas inflamatórias, e este controle pode direcionar a um retardo na propagação viral no indivíduo infectado.

Elevada carga proviral é observada em indivíduos infectados pelo HTLV-1 com HAM/TSP, especialmente naqueles com valores superiores a 5% de células infectadas (GRASSI, *et al*., 2011). A ativação celular e proliferação espontânea estão igualmente presentes em portadores assintomáticos com carga proviral menor que 1% de células infectadas pelo vírus (COUTINHO *et al.*, 2014). O controle da proliferação é uma estratégia que interfere na manutenção da carga proviral já que esta é mantida em grande parte pela divisão mitótica das células infectadas, assim como pela própria ativação e secreção de citocinas como IL-2 (BALLARD, *et al*., 1988).

Já foi demonstrado que quinolinas apresentam efeito antiproliferativo em células de linhagem transformadas pelo HTLV e células de pacientes infectados, e inibem a replicação do HIV em células de linhagem (FOURNET *et al.*, 2003, FAKHFAKH *et al.*, 2003; GRASSI,

et al., 2008), o que fortalece os achados para os compostos quinolínicos avaliados neste estudo. Dentre eles, o BS373, demonstrou atividade antiviral, impedindo a replicação do HIV-1 em células de linhagem CEM4fx com IC_{50} de 3,6 μM (FAKHFAKH *et al.*, 2003), porém 10 μM de BS373 não apresentou atividade inibitória em células de linhagem transformadas pelo HTLV (FOURNET *et al.*, 2003). Em relação às células dos pacientes com HAM/TSP, baixas concentrações desse composto foram suficientes para inibir a proliferação. Esta diferença na concentração necessária para inibir linhagens e células de pacientes, poderia ser justificada por características inerentes a esses dois tipos celulares, já que a proliferação de células de linhagem representa a expansão de um único clone celular, enquanto a proliferação de PBMC de indivíduos infectados é policlonal (PRINCE *et al.*, 1991; MASCARENHAS *et al.*, 2006). Além da avaliação em modelos de infecção viral, originalmente as quinolinas foram avaliadas e demonstram importante efeito leishmanicida (FOURNET *et al.*, 1993, NAKAYAMA *et al*., 2005, DESRIVOT *et al*., 2007, VIEIRA *et al*. 2008).

Fisalinas são potentes inibidores da ativação de macrófagos, que bloqueiam a produção de citocinas pró-inflamatórias (SOARES *et al.*, 2003). A fisalina F apresentou ainda efeito imunossupressor em modelos de artrite e mostrou ser uma indutora de apoptose em células de câncer (BRUSTOLIM *et al*., 2010; WU *et al*., 2012). Nesse estudo, tanto a fisalina F como a quinolina BS373 reduziram a produção de citocinas. Observando os achados de ativação celular dos pacientes com HAM/TSP, que apresentam uma maior produção de citocinas inflamatórias IL-6, INF-γ e TNF-α, e que esses altos níveis de TNF-α podem estar relacionadas ao dano medular, e consequentemente a ativação do sistema imune, podemos contribuir para reduzir a ativação celular causada pelo vírus nas células (SANTOS *et al*., 2004, MUNIZ *et al*., 2006, ARAÚJO e SILVA, 2006). O tratamento de células HTLV-1 infectadas com altas doses de ácido ascórbico também foi capaz de reduzir a proliferação e produção de INF-γ e TNF-α de PBMC HTLV-1 infectadas (MOENS *et al.*, 2012). A redução dos níveis de IL-2 e IL-10 observados neste estudo poderia refletir uma desativação do sistema imune, e a diminuição nos níveis de IL-2 poderia estar diretamente relacionados à redução da proliferação, já que a proliferação é dependente IL-2 e da maior expressão de seu receptor (AZRAN *et al.*, 2004).

No nosso estudo foi possível também avaliar o efeito de BS373 na expressão da proteína viral Tax. Esta proteína está relacionada com a proliferação espontânea e ativação celular (AZRAN *et al.*, 2004), e a redução observada na produção da Tax ratificam os achados de inibição da proliferação e redução da produção de citocinas. WANG *et al*. em

2002 demonstraram que a fluoroquinolina K-37 foi capaz de inibir a replicação do HTLV-1 em células de linhagem MT-2 e MT-4, em PBMC de indivíduos com HAM/TSP, ao inibir a expressão do gene Tax. Resultados semelhantes foram obtidos com doses elevadas ácido ascórbico, que demonstraram ser superior ao IFN-α em inibir a proliferação celular, reduzindo a ativação e induzindo morte celular (MOENS, *et al.* 2012).

Ao observar a inibição da proliferação e o efeito imunomodulador da quinolina BS373 entendemos que estas alterações podem ser uma consequência da morte celular por apoptose. De fato, as alterações ultraestruturais evidenciam núcleos com cromatina condensada, indicativos de apoptose e presença de figuras de mielina, podem sugerir além da apoptose, um processo de autofagia. Essas alterações ultraestruturais estavam presentes igualmente nas células tratadas com a fisalina F. As quinolinas testadas encontravam-se no citoplasma da célula dentro de vesículas, no interior das quais se observava além da droga fotooxidada, um excesso de membranas, indicando um processo de digestão celular. Estes dados são sugestivos de uma autofagia, que poderia ser desencadeada por um estresse oxidativo.

A fisalina F, além de induzir a formação de figuras de mielina, promoveu a dilatação do retículo endoplasmático, que pode indicar também um estresse oxidativo. De fato, já foi demostrado que a fisalina F induziu apoptose de células tumorais através da via mitocondrial mediada por ROS- e suprimiu a ativação do NF-kB (WU *et al.*, 2012). As fisalinas B e F são descritas como potentes inibidores da proliferação celular e como capazes de aumentar a autofagia celular (MEIRA *et al.*, 2013; HE *et al*. 2013a,b). No entanto, no presente trabalho, uma limitação foi a impossibilidade de avaliar qual a via de indução da apoptose e as propriedades antioxidantes das moléculas.

Uma possível explicação para as redução da proliferação celular observada pode ser uma modulação dos níveis de glutationa intracelular ou de seu receptor. Os níveis celulares da glutationa são importantes para a manutenção da proliferação celular e a glutationa tem uma importante atividade antioxidante (MESSINA e LAWRENCE, 1989; HAMILOS *et al*, 1989;. SUTHANTHIRAN *et al*, 1990; KUBBIES *et al*. , 1991;. MARTIN *et al*, 2000). Na infecção pelo HTLV-1, a proteína viral Tax está envolvida na diminuição dos níveis de glutationa intracelular (LOS *et al*., 1998). A modulação dos níveis de glutationa poderia interferir na intensidade da proliferação das células de induzida pelo HTLV-1 (NOVAES *et al.*, 2003). Estudos futuros devem ser feitos para avaliar se a quinolina BS373, ao reduzir em 80% a expressão da proteína Tax, poderia modular os níveis de glutationa celular e dessa forma, reduzir a proliferação espontânea das células.

Células tratadas com BS373 e fisalina F apresentam alterações na mitocôndria. O dano mitocondrial poderia estar associado a diferentes mecanismos de morte celular, não só apoptose, mas também necrose e autofagia (LEMASTERS *et al.*, 1998). Curiosamente a produção natural de espécies reativas de oxigênio (ROS) induzidas por produtos antineoplásicos desencadeiam apoptose e autofagia (MÖHLER *et al.*, 2014). A autofagia compreende um mecanismo de escape necessário para a produção de ATP e proliferação de células T. Além disso, a redução da autofagia regula o crescimento de células de câncer de pâncreas (YANG *et al.*, 2014).

É conhecido que a cloroquina pode aumentar o pH do vacúolo autofágico/lisossomal, diminuindo a função metabólica e capacidade proliferativa em diferentes tipos de células (RAMSER *et al.*, 2009; QIAO *et al*., 2013; GUHA *et al.*, 2014; CHEN *et al.*, 2014).A biogênese de grandes vacúolos autofágicos observado nas células tratadas com as quinolinas pode ser devido a um mecanismo de feed-back positivo, no qual a alcalinização lisossomal levaria ao acúmulo de lipídeos que por sua vez, poderia elevar ainda mais o pH lisossomal. A autofagia pode inibir a apoptose em células de carcinoma (ZHOU *et al.*, 2014). Por outro lado, o bloqueio da autofagia pode promover a apoptose (WANG *et al.*, 2013). Por conseguinte, autofagia inibida mediada pelo composto quinolínico poderia diminuir a proliferação de PBMC desencadeando a apoptose. No entanto, não foi possível confirmar em nosso estudo se o processo de morte celular era devido a autofagia.

Utilizando citometria de fluxo, mostrou-se que BS373 aumentou significativamente a porcentagem de células em apoptose em cultura de PBMC de doadores infectados com HTLV-1 e de doadores não infectados. A indução de apoptose em células de câncer foi descrita após o tratamento com outros derivados de quinolina, por mecanismos dependentes da ativação da caspase (DING and NGUYEN, 2013; KIM, *et al.*, 2005). É possível também que o derivado de quinolina testado no nosso estudo atue através da ativação de caspases para promover a apoptose celular. Estudos futuros devem ser conduzidos para confirmar esta hipótese.

Os estudos atuais buscam compreender a patogênese de HAM/TSP e a ação do HTLV-1 nas células dos indivíduos infectados, esses resultados indicam as alterações imunológicas importantes a serem moduladas, e nesse contexto pesquisas com compostos sintéticos e naturais têm sido conduzidas (WANG, *et al*., 2002; FOURNET, *et al.*, 2003; GRASSI *et al*., 2008; LUNA, *et al*., 2011; OLINDO *et al*., 2011; ARAYA *et al*., 2011; GUIMARÃES-CÔRREA, *et al*., 2011; RAFATPANAH, *et al*., 2012; MOENS, *et al*., 2012; NAKAMURA, *et al*., 2013). Ainda assim, é necessário maior avanço na busca por

tratamentos que evitem ou atrasem a progressão das doenças associadas à infecção pelo HTLV. Estudos futuros precisam ser conduzidos para melhor esclarecer a forma de ação dos compostos avaliados nesse estudo.

5 CONCLUSÃO

Os três compostos quinolínicos avaliados e a fisalina F foram capazes de inibir a proliferação espontânea das células de pacientes infectados pelo HTLV-1. Além de inibir a proliferação, as células tratadas com os quatro compostos apresentaram alterações ultraestruturais, como acúmulo de membranas em vesículas, sugestivo de autofagia. A quinolina BS373 e a fisalina F induziram apoptose celular nas células infectadas pelo HTLV-1 e reduziram a produção de citocinas inflamatórias. O composto quinolínico BS373 foi ainda capaz de reduzir em 80% a produção da proteína viral Tax.

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ANEXO A

Comprovante de submissão do artigo para a revista Journal of Naturals Products

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RE: Manuscript Submission Successfully Submitted
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ANEXO B

Article: C7a, a Biphosphinic Cyclopalladated Compound, Efficiently Controls the Development of a Patient-Derived Xenograft Model of Adult T Cell Leukemia/Lymphoma

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Article

C7a, a Biphosphinic Cyclopalladated Compound, Efficiently Controls the Development of a Patient-Derived Xenograft Model of Adult T Cell Leukemia/Lymphoma

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Abstract: Adult T-cell leukemia/lymphoma (ATLL) is a highly aggressive disease that occurs in individuals infected with the human T lymphotropic virus type 1 (HTLV-1). Patients with aggressive ATLL have a poor prognosis because the leukemic cells are resistant to conventional chemotherapy. We have investigated the therapeutic efficacy of a biphosphinic cyclopalladated complex {Pd_2 [$S_{(-)}C^2$, N-dmpa]$_2$ (μ-dppe)Cl_2}, termed C7a, in a patient-derived xenograft model of ATLL, and investigated the mechanism of C7a action in HTLV-1-positive and negative transformed T cell lines *in vitro*. *In vivo* survival studies in immunocompromised mice inoculated with human RV-ATL cells and intraperitoneally treated with C7a led to significantly increased survival of the treated mice. We investigated the mechanism of C7a activity *in vitro* and found that it induced mitochondrial release of cytochrome c, caspase activation, nuclear condensation and DNA degradation. These results suggest that C7a triggers apoptotic cell death in both HTLV-1 infected and uninfected human transformed T-cell lines. Significantly, C7a was not cytotoxic to peripheral blood mononuclear cells (PBMC) from healthy donors and HTLV-1-infected individuals. C7a inhibited more than 60% of the *ex vivo* spontaneous proliferation of PBMC from HTLV-1-infected individuals. These results support a potential therapeutic role for C7a in both ATLL and HTLV-1-negative T-cell lymphomas.

Keywords: cyclopalladated compound; HTLV-1; ATLL; chemotherapy; xenograft model; apoptosis

1. Introduction

About 44,000 new cases of leukemia are expected in the United States in 2011. Nearly half of these will be acute leukemias, including adult T-cell leukemia/lymphoma (ATLL). ATLL is a highly aggressive disease characterized by the rapid and uncontrolled clonal proliferation of mature transformed $CD25^+CD4^+$ T-cells. Infection with human T lymphotropic virus type 1 (HTLV-1) is the major risk factor for the development of ATLL. Currently, approximately 15 million people in the world are infected with HTLV-1. Conventional chemotherapy or a combination of zidovudine (AZT) and interferon-alpha are the currently favored treatments for patients with ATLL, but are effective in only 50% of cases [1]. Additionally, the mean survival of patients in the acute phase of ATLL is approximately six months even with aggressive chemotherapy. Although targeting HTLV-1-infected cells for apoptosis is an attractive approach to treating ATLL, HTLV-1 infected cells are generally resistant to apoptosis induced by available drugs. Thus, it is important to develop new approaches to treat HTLV-1-infected patients diagnosed with ATLL and to specifically induce cell death in HTLV-1-infected T-cells *in vivo*.

Novel antitumor compounds that are cytotoxic to tumor cells *in vitro* must be tested in preclinical animal models to determine their availability and effectiveness *in vivo*. In the absence of a syngeneic murine model for ATLL, xenogeneic experimental models have been developed, using immunocompromised mice. Mice homozygous for the SCID mutation lack functional T and B lymphocytes, fail to generate humoral and cell-mediated immunity and have been widely used as hosts for both normal and

malignant human cells. Engraftment of SCID mice with patient-derived tumor cells provides an *in vivo* model in which to investigate the tumorigenic potential of HTLV-1-infected human lymphocytes and cell lines. Feuer and colleagues initially reported the development of lymphoma in C.B.-17 scid/scid mice inoculated with peripheral blood mononuclear cells (PBMC) from an ATLL patient [2,3]. This patient-derived ATLL line, termed RV-ATL, can be propagated and expanded in SCID mice and is a useful platform to test the efficacy of novel therapeutic treatments on ATLL. Further refinement of the SCID mouse model by removing the interleukin (IL)-2 receptor generated NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice. NSG mice lack functional NK cells and are deficient in cytokine signaling, which supports better engraftment of human hematopoietic stem cells and PBMCs [4,5]. These mice also support the propagation and expansion of RV-ATL-cells.

Recently, several palladium (Pd) complexes have been evaluated as antitumor agents [6,7], but only a few of them have been tested in preclinical animal models. Cyclization of Pd complexes by cyclometallation reactions not only increased the stability but also produced less toxic complexes, making them promising antitumor compounds [8]. A group of biphosphinic cyclopalladated compounds, obtained from the cyclometallation agents, *N*,*N*-dimethyl-1-phenethyl-amine (dmpa), phenyl-2-pyridinyl-acetylene or 1-phenyl-3-*N*,*N*-dimethylamine-propyne and containing the biphosphinic ligand 1, ethanebis(diphenyl-phosphine) (dppe), were synthesized and tested *in vitro* and *in vivo* in a syngeneic murine melanoma B16F10-Nex2 model. One complex, [Pd(C2,N-(S(-) dmpa)(dppe)].Cl, named C7a, was cytotoxic to murine B16F10 melanoma cells *in vitro* at concentrations lower than 1.25 μM, and was the most active *in vivo*, delaying subcutaneous tumor growth and increasing animal survival [9]. More recently, we demonstrated that C7a reduced the number of pulmonary nodules in the murine metastatic melanoma model, and interacted with mitochondrial membrane thiol-groups to induce the intrinsic apoptotic death pathway in murine and cisplatin-resistant human tumor cells [10]. Hebeler-Barbosa *et al.* demonstrated the additive anti-melanoma protective effect of C7a in a gene therapy protocol with plasmids encoding IL-12 and an Fc-chimera of the soluble α-chain of IL-13 receptor. The combined therapy significantly reduced the subcutaneous tumor evolution with 30% tumor-free mice [11].

The present study investigates the effect of C7a in an ATLL mouse model and the mechanism of cell death induced by C7a in HTLV-1-infected and uninfected T cell leukemia lines. We show that treatment with C7a significantly increased the survival of RV-ATL engrafted mice and that C7a induced caspase-mediated apoptosis of human transformed T-cell lines and HTLV-1 infected T cells. These results support a potential therapeutic role for C7a in both ATLL and HTLV-1-negative T-cell lymphomas.

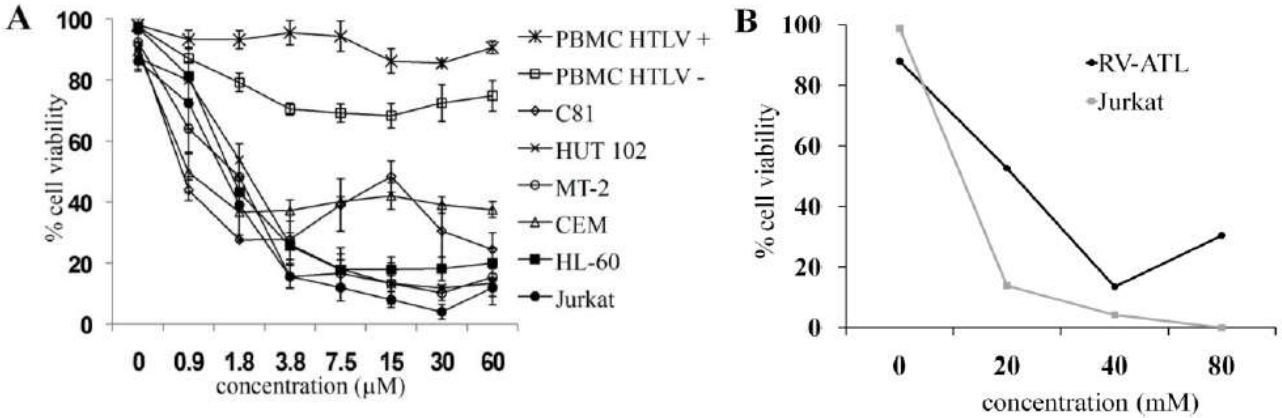
2. Results

2.1. C7a Is Cytotoxic to Human Leukemia T Cell Lines

Because HTLV-1 infected transformed T cells are difficult to control with available chemotherapeutic strategies, we evaluated the cytotoxic effect of C7a on HTLV infected cell lines *in vitro*. C7a was cytotoxic to all cell lines in a dose-dependent manner (Figure 1A) suggesting that established human T cell lines are susceptible to C7a whether or not they are infected with HTLV-1.

Although C7a did not kill 100% of any of the cell lines analyzed, even at the highest concentration tested, 7.5 μM C7a reduced the viability of most cell lines by 90% after 48 h. Uninfected CEM T cells were most resistant to C7a cytotoxicity, with 40% of the cells remaining viable at 60 μM C7a. Importantly, C7a had minimal cytotoxic effect on the viability of HTLV-1 infected or uninfected primary human PBMC, with >80% of the cells remaining viable even at 60 μM C7a (Figure 1A). In a similar dose-dependent cytotoxicity study to examine the cytotoxicity of C7a, 40 μM C7a reduced the viability of RV-ATL cells, a patient-derived HTLV-1-infected ATLL cell line, by 90% after 48 h (Figure 1B).

Figure 1. Cyclopalladated C7a is cytotoxic to human leukemia cell lines, but has little effect on human T lymphotropic virus type 1 (HTLV-1)-infected or uninfected peripheral blood mononuclear cells (PBMC). (**A**) 5×10^5 PBMC (previously stimulated with PHA and IL-2) from three donors (HTLV-1 uninfected), 10^5 cells from human T cell leukemia lines, and 10^5 PBMC from four different HTLV-1-infected individuals were seeded in 96-well plates in triplicate. Cells were incubated with concentrations of C7a as indicated for 48 h, and cytotoxicity was determined by Trypan blue exclusion in an automated cell counter. The bars represent the means and SD of three different experiments; (**B**) RV-ATL cells were expanded in a SCID mouse for three weeks. Freshly harvested cells were seeded at 10^5 cells per well in a 24-well plate. The cells were incubated with concentrations of C7a as indicated for 48 h. Uninfected human Jurkat T cells were used as a control and incubated in the same conditions. Cytotoxicity was determined by Trypan blue exclusion and manual counting. A representative experiment is shown. The experiment was repeated twice, using two independent frozen tissue culture stocks of RV-ATL cells and without refreshing the media or drug, with comparable results. In (A) and (B), the viability of treated cells was expressed as percent of control untreated cells.



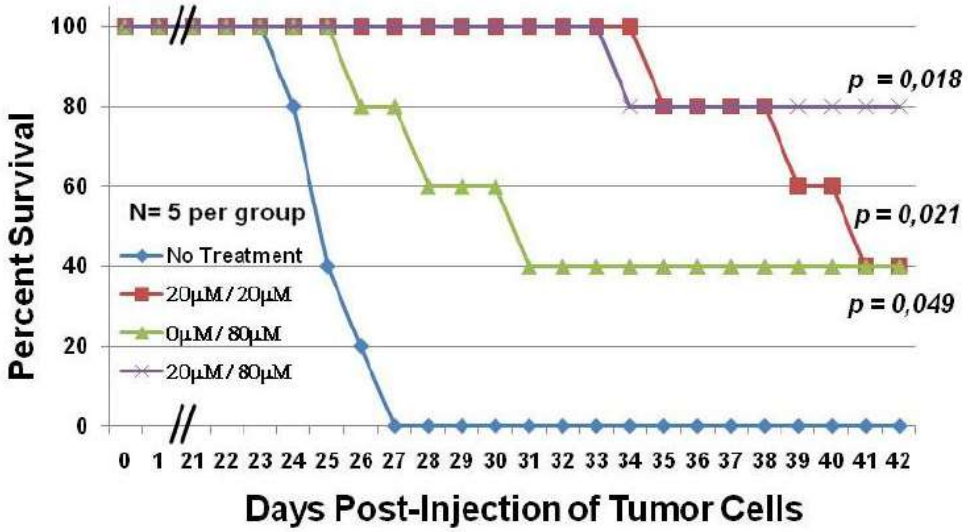
2.2. C7a Is Effective in a Preclinical Model of ATLL

Since C7a could kill RV-ATL cells *in vitro*, we next evaluated the effect of C7a on the development of leukemia *in vivo* using a xenogeneic murine model [2,3]. RV-ATL cells (10^7), previously expanded *in vivo* in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice, were intraperitoneally inoculated on day 0 in 23-week old NSG mice. The engraftment of mice with RV-ATL cells was monitored by peritoneal lavage at days 14, 28 and 42. Collected cells were analyzed by flow cytometry for previously

determined phenotypic markers and were huCD45^{+}, huCD4^{+}, huCD8^{-} and huCD25^{low} (data not shown). All mice were successfully engrafted.

NSG mice engrafted with RV-ATL cells were divided into four groups (five mice per group). Each group of mice was inoculated with the same dose of C7a diluted in 100 μL of PBS, regardless of body weight, and drug concentration per gram of mouse body weight was not statistically different based on weekly weight measurements (data not shown). All mice were monitored daily for clinical assessment and survival. Animals in group 1, which did not receive any C7a, had an average survival of 24 days post-inoculation (Figure 2), and none of the mice survived beyond day 27 (blue line). Animals in group 2, which received 20 μM C7a every other day beginning on day 4 through day 40, survived significantly longer than animals in group 1 (p value = 0.049), with all animals surviving to day 34 and two animals still alive at termination of the study (red line). Animals in group 3 did not receive C7a until day 22, which allowed tumor expansion prior to treatment. These animals then received 80 μM C7a every other day from day 22 through day 40 (green line). Animals in group 3 survived longer than animals in group 1 that did not receive any C7a. Although three animals did not survive past day 31, the remaining two animals in group 3 were still alive at termination of the study suggesting that a higher dose of C7a late in tumor development can protect a significant fraction of animals.

Figure 2. C7a significantly increases the survival of RV-ATL tumor bearing mice. Adult female NSG mice were intraperitoneally (IP) injected with 10^7 fresh, *in vivo* expanded RV-ATL cells on Day 0. Mice were treated with either C7a (20 μM diluted in PBS) (red line and purple line) or PBS alone (blue line and green line) by IP injection every other day starting on Day 4. Starting on day 22, half the mice from each group were treated with 80 μM C7a (green line and purple line). N = 5 per group for four treatment groups. All mice were treated by IP injections through day 40, and survival was monitored daily. p values (Kaplan Meier test) are shown for each group, compared to no treatment group.



Animals in group 4 received 20 μM C7a every other day from day 4 to day 20, and 80 μM C7a every other day from day 22 to day 40 (purple line). These animals showed the best survival, with four of the five animals in this group still alive at termination of the study. Pairwise comparison of the

treatment groups showed that the survival of animals in group 4 was significantly better than those in group 1 (p value = 0.018). In all groups, animal deaths were attributed to tumor burden. Clinical signs prior to death included distended abdomen due to tumor growth, weight loss, dragging of hind limbs (necropsy showed tumor pressing on thigh muscles and/or spinal cord). There was no evidence of clinical signs that were not tumor related.

2.3. C7a Induces the Intrinsic Apoptotic Pathway in Human T Cell Leukemia Lines

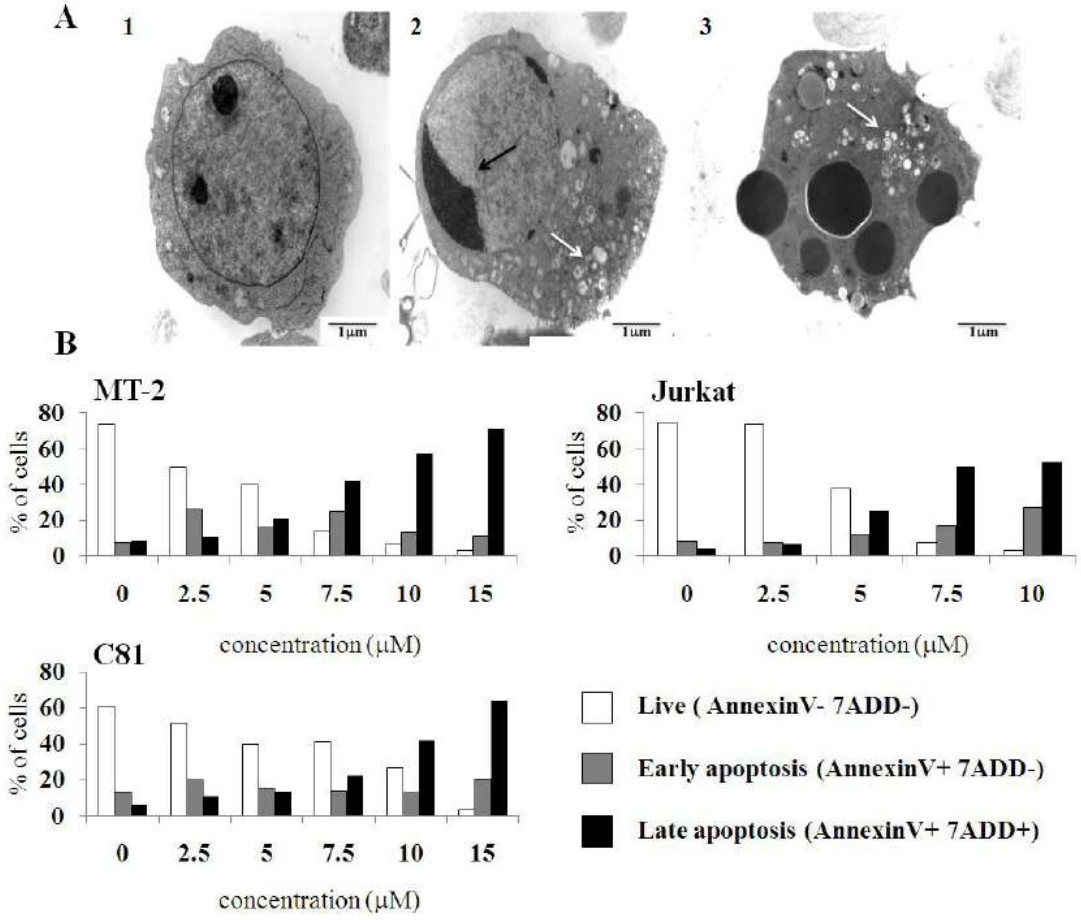
Treatment of HL-60 cells, which were effectively killed by 7.5 μM C7a (Figure 1), with 2 μM C7a for 7 h resulted in profound morphologic alterations observed by transmission electron microscopy. Treated cells showed chromatin margination, and an increase in enlarged vacuoles and electron-dense material in the cytoplasm (Figure 3A). Externalization of phosphatidylserine (PS), which is normally restricted to the inner leaflet of the plasma membrane, is a hallmark of mammalian apoptosis. Cell lines were treated with C7a for 3 h, and PS exposure was analyzed by double staining the cells with 7-amino-actinomycin (7-ADD), a vital dye, and PE-annexin V, a Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS (Figure 3B). A dose-dependent decrease in the number of live cells (PE-annexin negative and 7-ADD negative) and an increase in the number of cells in late apoptosis (PE-annexin positive and 7-ADD positive) were observed in MT-2, C81, and Jurkat cell lines treated with C7a for 3 h (Figure 3B). The morphological changes, and the PS exposure observed in C7a-treated T cell leukemia lines suggested that this cyclopalladated complex can induce apoptosis in these cells, as observed previously for murine melanoma cells.

To determine the effect of C7a on mitochondria of intact HTLV-1-infected T cell leukemia lines, MT-2 cells were incubated *in vitro* with 5 μM C7a for 3 h. In untreated cells, cytochrome c colocalized with the mitochondrial marker (top row, see white arrow) (Figure 4A). In contrast, in cells treated with C7a, cytochrome c was predominately localized in the cytoplasm and did not colocalize with the mitochondrial marker (bottom rows). These results suggest that C7a can induce mitochondrial permeabilization and release of mitochondrial contents.

C7a-induced apoptosis was associated with caspase activation in MT-2 cells, as the caspase-3 precursor was cleaved into the 17 and 19 kDa death-associated fragments (Figure 4B). C7a similarly induced activation of caspase 3 in HL-60 cells as well as activated caspases -8 and -9 (data not shown). Although DTT showed a partial toxicity to MT-2 cells, C7a -mediated cytotoxicity was abolished by DTT treatment in both cell lines (Figure 4C), suggesting that this cyclopalladated complex can induce thiol-cross-linking of mitochondrial membrane proteins resulting in mitochondrial permeabilization in leukemia cells, as previously described in murine melanoma cells treated with the *R*(+) enantiomer of C7a [12].

To investigate whether the lysosomal pathway plays a role in apoptotic cell death induced in leukemia cells by C7a, the cytotoxic effect of C7a was evaluated in MT-2 and HL-60 cells after preincubating with a cathepsin B inhibitor (CA-074). The cytotoxic effect of C7a was not influenced by the inhibitor, indicating that the lysosomal pathway is not involved in C7a-mediated apoptosis of malignant T cells (data not shown).

Figure 3. C7a induces morphological and phenotypic alterations compatible with apoptosis in human leukemia cell lines. (**A**) Transmission electron microscopy. (1) HL-60 cells cultivated in complete RPMI medium for 6 h; (2 and 3) HL-60 cells cultivated in presence of 2 μM C7a for 7 h. Black arrows: nuclear condensation; White arrows: vacuoles. This assay was repeated twice, and representative images are shown; (**B**) Membrane phosphatidylserine expression. HTLV-1 infected MT-2 and C81, and uninfected Jurkat cell lines (1×10^6) were incubated in the presence of C7a at the indicated concentrations for 3 h at 37 °C. After staining with PE-Annexin V and 7-AAD, cells were analyzed by flow cytometry, as described in Material and Methods. A representative of two independent experiments is shown.



Nuclear effects of C7a were evaluated in human leukemia T cell lines. Nuclear condensation was observed in HL-60 cells treated with 5 μM C7a for 6 h (Figure 5A, panel 2) and chromosomal fragmentation was observed after 24 h (Figure 5A, panel 4), while few cells showed such patterns in control untreated cells (Figure 5A, panels 1 and 3). After treatment with 5 μM C7a for 18 h, HL-60 cells showed DNA internucleosomal fragmentation (Figure 5B, lane 3). Upon propidium iodide staining, fluorescence intensity in FACS-analyzed cells varies linearly with DNA content, ranging from 2 n (G0-G1 phases) to 4 n (G2 phase) with an intermediate plateau that corresponds to S phase. The sub-G1 peak in DNA histograms corresponds to the apoptotic population. Exposure of HTLV-1 infected C81 cells to 1.25 μM C7a for 48 h induced an 8-fold increase in the sub-G1 peak (Figure 5C, bottom panel), again demonstrating that C7a induces apoptosis in these cells. Taken together, these

results show that C7a induces the intrinsic apoptotic pathway in human leukemic T cell lines, with no participation of the lysosomal pathway.

Figure 4. Mitochondria are involved in C7a induced cell death. (**A**) Cytochrome c release. HTLV-1 infected MT-2 cells were incubated without (0 μM) or with 5 μM C7a for 3 h. The cells were fixed and incubated with anti-cytochrome *c* antibody (green). Mitochondria were detected by MitoTracker staining (red), and nuclei were visualized with DAPI (blue). Magnification (×40). Inserts: Single cell indicated by the arrow, magnification (×60). Rare cells treated with C7a that show colocalization of cytochrome c and mitochondria likely reflect the short 3 h exposure to C7a. The assay was repeated twice, and representative images are shown; (**B**) Caspase Activation. Cell lysates from MT-2 cells before treatment (Control), after treatment with 5 μM C7a for 3 h, or after exposure to 50 J of UV light (UV), as a positive control, were analyzed by Western blotting for cleaved caspase-3 and GAPDH as a loading control. This assay was repeated twice, and representative images are shown; (**C**) DTT inhibits C7a effect on leukemic human T cells. MT-2 (HTLV-1 infected) and uninfected HL-60 cells (10^5) were pre-incubated or not with 1 mM of DTT for 1 h. C7a was added to the culture (15 μM to MT-2, 2 μM to HL-60 cells) and incubated for 6 h. Viable cells were counted in presence of Trypan Blue in a hemocytometer. The bars represent the means and SD of three different experiments.

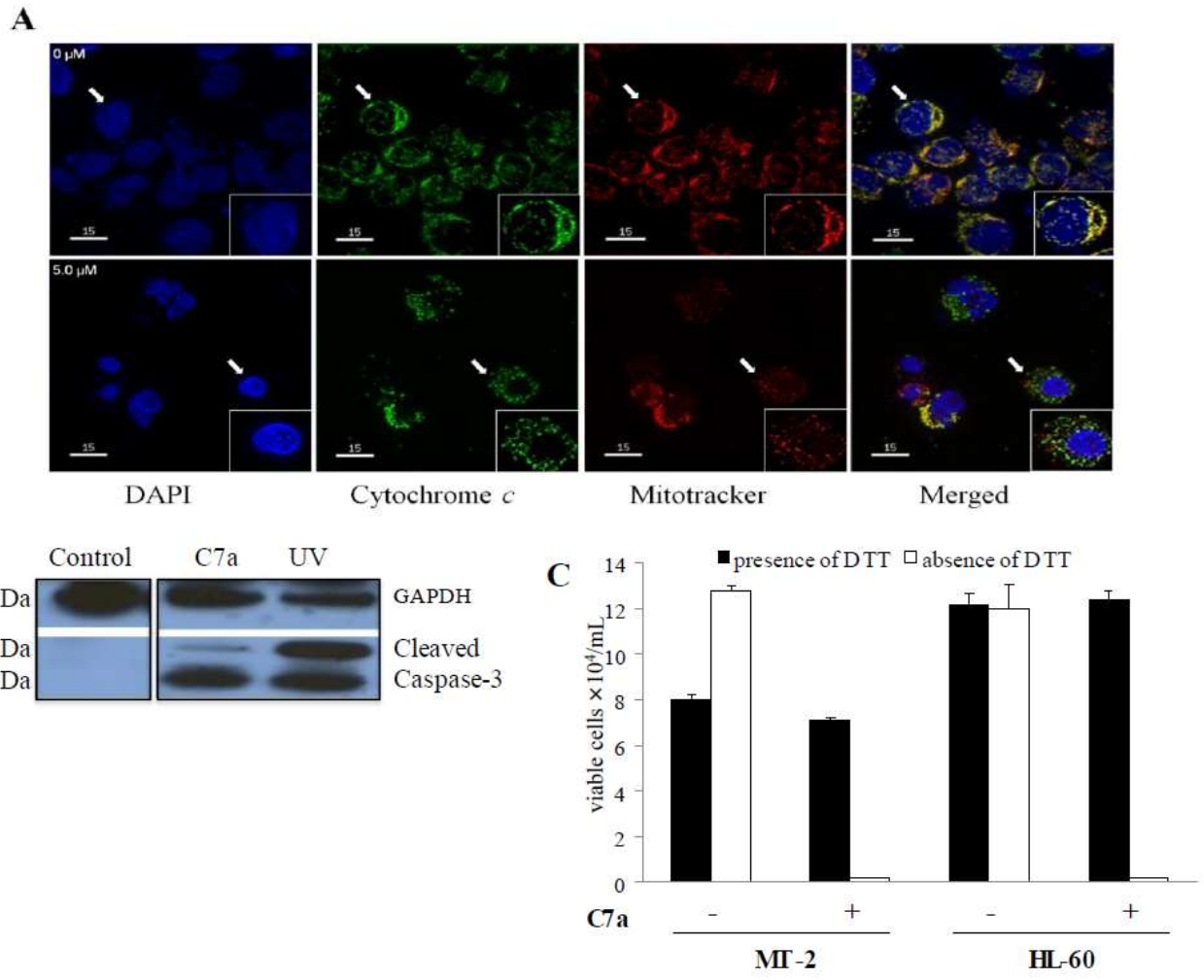
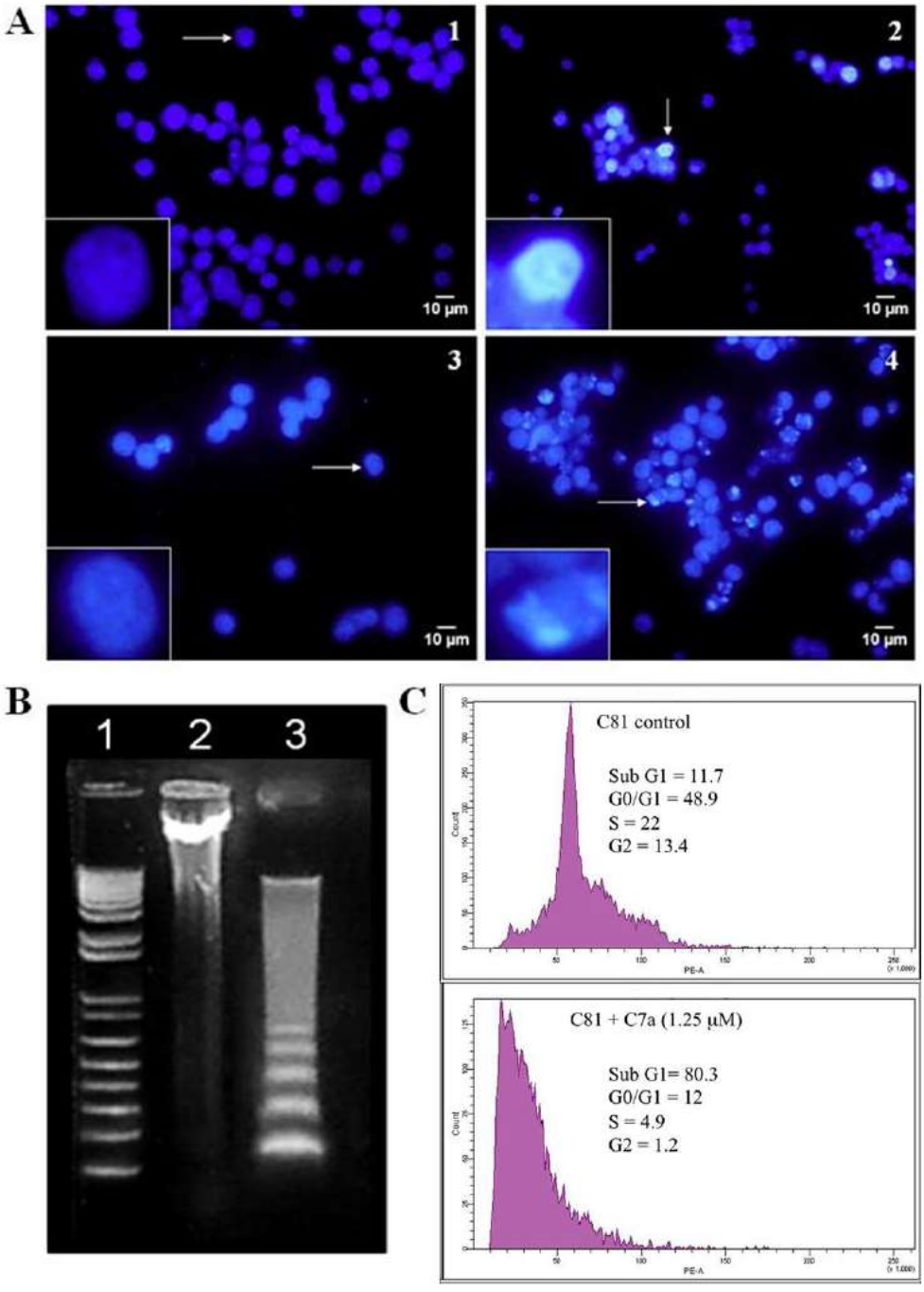


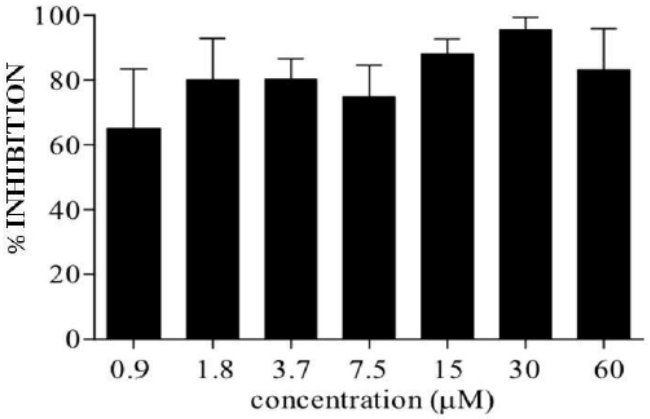
Figure 5. C7a induces nuclear alterations characteristic of apoptosis. (**A**) Nuclear condensation. Uninfected HL-60 cells were untreated (1) or treated with 5 μM C7a (2) for 6 h. Alternatively, HL-60 cells were untreated (3) or treated with 2.5 μM C7a (4) for 24 h. After incubation with C7a, cell nuclei were stained with Hoechst 33342. Inserts: Single cell indicated by the arrow, ×25 magnification. The assay was repeated three times, and representative images are shown; (**B**) DNA fragmentation. HL-60 cells (3×10^6) were treated (lane 3) with 5 μM of C7a or not (lane 2) for 18 h. Lane 1: MW standard. The assay was repeated three times, and a representative gel is shown; (**C**) Cell cycle analysis. HTLV-1 infected C81 cells were cultured with 1.25 μM C7a (bottom panel) or not (top panel) for 48 h. Cells were stained with propidium iodide (50 μg/mL) and cell cycle analysis was performed by flow cytometry. Percentage of cells in each phase of the cycle is shown.



2.4. C7a Treatment Reduces Spontaneous Proliferation of PBMC from HTLV-1-Infected Patients

C7a does not have a cytotoxic effect on PBMC from healthy HTLV-1-infected individuals (Figure 1A). However, C7a was able to reduce the spontaneous *in vitro* proliferation of PBMC from HTLV-1-infected individuals (Figure 6). Spontaneous proliferation of HTLV-1-infected PBMCs was reduced to 60% after 24 h incubation with the lowest C7a concentration evaluated (0.9 μM) and reached 90% with 30 and 60 μM concentrations of C7a.

Figure 6. *In vitro* inhibition of spontaneous proliferation of PBMC from HTLV-1-infected patients by C7a. Isolated PBMC from HTLV-1-infected individuals were cultured in the presence or absence of C7a compound for 24 h. Bars represent average inhibition and standard deviation from four patients, compared to cells from the same patient cultivated in the absence of C7a.



3. Discussion

Recently, a number of new compounds and therapies have been shown to specifically induce apoptosis in HTLV-1 infected cells and in ATLL leukemic cells [13]. Here we have demonstrated that RV-ATL engrafted mice treated with C7a, a biphosphinic cyclopalladated compound, demonstrated up to 80% survival over the course of the study, with significantly greater protection against tumor-mediated death than untreated mice. In addition, we demonstrated that C7a effectively induced mitochondria-dependent apoptosis in human HTLV-1-infected T cells. Interestingly, C7a also induced the same effects in human transformed cell lines that are not HTLV-1-infected, such as acute T-lymphoblastic leukemia HL-60, CEM and Jurkat.

Although important progress has been made in the treatment of ATLL such as combination therapy with AZT and IFN-α [14], immunotherapy with anti-IL-2 receptor or anti-CC chemokine receptor 4 monoclonal antibodies [15], allogeneic bone marrow or stem cell transplantation [16] and apoptosis induction [17–21], ATLL remains highly intractable to conventional therapeutics. Testing of novel compounds against cancer *in vivo* requires the use of preclinical animal models. Establishment of newer strains of immunocompromised mice capable of supporting efficient levels of engraftment of ATLL cells from patients has accelerated the testing of drugs and targeted therapy against ATLL [22]. To test the antitumor effects of C7a in an *in vivo* model of ATLL, we used an established ATLL patient-derived xenograft model in immunocompromised (NSG) mice [2,3]. NSG mice injected

intraperitoneally with RV-ATL cells develop lymphoma associated with the mesenteric and pancreatic lymph nodes, liver and spleen. Significantly, mice treated with low dose C7a (20 μM) for the duration of the study had a 35% greater survival than untreated mice. Interestingly, mice that were untreated for the first half of the study and started on high dose (80 μM) C7a at day 22 (only two days prior to the average death of untreated mice) survived longer than untreated mice. Forty percent of these mice survived for the duration of the study suggesting that C7a is also effective against established tumors.

Over the last 15 years studies showed that arsenic trioxide (As_2O_3), alone or in combination with other pro-apoptotic stimuli, induces apoptosis in HTLV-1-infected cells lines [23,24] and have been examined as possible treatments for ATLL. As_2O_3 induces apoptosis leading to cytochrome c release and caspase activation [25]. Previous studies demonstrated that a combination of As_2O_3 and IFN-γ, known to trigger Tax proteolysis, cures Tax-driven ATLL in mice [26]. However clinical use of arsenic is problematic as there are differences in sensitivity to As_2O_3, and arsenic itself is toxic at high doses [13]. C7a and the *R*(+) enantiomer complex have been described as potent inducers of apoptosis through targeting of the mitochondria by interacting with thiol-groups in the mitochondrial membrane of murine melanoma cells [10], and in rat isolated mitochondria [12], respectively. Treatment of isolated rat liver mitochondria with the *R*(+) enantiomer of C7a, {Pd_2 [$R_{(+)}C^2$, N-dmpa]$_2$ (μ-dppe)Cl_2} results in mitochondrial permeabilization as indicated by Ca^{+2}- and ROS-independent mitochondrial swelling, release of cytochrome *c*, dissipation of the mitochondrial transmembrane potential, uncoupling of oxidative phosphorylation, and mitochondrial calcium release [12]. Additionally, an ionic cyclopalladated compound with a ferrocene ligand {[Pd(C2,*N*-(S(-) dmpa)(dppf)] Cl}] (termed BPC) induces apoptosis in acute leukemia cells through a novel lysosomal pathway with cathepsin B acting as the death mediator [27]. The C7a complex used in this study induces mitochondrial-dependent, lysosomal-independent, apoptosis in HTLV-1-infected and uninfected transformed cell lines as characterized by the cleavage of caspase 3 and release of cytochrome c. Thus, while cyclopalladated compounds have been clearly demonstrated to induce apoptosis by activating a variety of cellular pathways, C7a is the only compound that has demonstrated antitumor effects *in vivo* [9].

A hallmark of HTLV-1-infection is the spontaneous proliferation of peripheral blood mononuclear cells (PBMC) that is observed in approximately 50% of HTLV-1-infected individuals [28]. We found that C7a could inhibit more than 80% of the spontaneous proliferation of PBMC from HTLV-1-infected individuals, the majority diagnosed with HAM/TSP disease. The mechanisms involved in the inhibition of spontaneous proliferation by C7a remains unclear and further studies are necessary to elucidate the mechanisms of action of this drug on cell proliferation.

Since C7a is capable of inducing mitochondrial-dependent apoptosis in acute leukemia cells (HL-60), and peripheral T-cell lymphomas have a low response rate to anthracyclin-based combination chemotherapy and high relapse rate [29], C7a offers an exciting alternative for treating other peripheral lymphomas. Further studies to test the efficacy of C7a in *in vivo* leukemia models are required.

4. Experimental Section

4.1. Culture Conditions for Human Leukemia Cell Lineages and PBMC Isolation

Peripheral blood mononuclear cells (PBMC) from heparinized venous blood of three healthy controls were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). PBMC were *in vitro* stimulated for 3 days with 2 μg/mL phytohaemagglutinin (PHA) and subsequently cultured with 10 U/mL human recombinant IL-2 (both from Sigma-Aldrich, St. Louis, MO, USA) for 2 more days. PBMC from one asymptomatic HTLV-infected individual and seven patients diagnosed with HAM/TSP were obtained from heparinized venous blood samples by SepCell density gradient centrifugation (LGCBiotechnology, São Paulo, Brazil). Samples were screened for HTLV-1/2 antibodies by an enzyme-linked immunosorbent assay (ELISA) (Ab-Capture ELISA test system; Ortho-Clinical Diagnostics, Inc., Raritan, New Jersey, USA), and results were confirmed by Western blotting assay (HTLV Blot 2.4; Genelabs Technologies, Singapore). All patients presented high proviral load (>5000 copies/10^6 PBMCs). The Ethical Board of Oswaldo Cruz Foundation (FIOCRUZ) approved this study, and informed consent was obtained from all enrolled patients. Immortalized human cell lines used in this study were HTLV-infected T cell lines (C81, HUT 102 and MT-2) and non-infected T cell leukemia lineages (CEM, HL-60 and Jurkat). Cell lines as well as PBMC were cultured *in vitro* in complete RPMI medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate, 100U/ml penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum, all provided by Sigma-Aldrich). PBMC from HTLV-infected patients were cultured *in vitro* in RPMI 1640 medium supplemented with 5% human group AB serum.

RV-ATL cells were isolated from an ATL patient and expanded in SCID mice as previously described [2,3]. The RV-ATL cell line was further modified by the addition of a luciferase cassette [30]. The full phenotype of the RV-ATL cell line is human $CD45^+$, $CD4^+$, $CD8^{low}$, $CD25^{low}$ [2]. This tumor line was originally derived from PBL from a patient with acute leukemia. The phenotype of the RV-ATL cell line used in this study is human $CD45^+$, $CD4^+$, $CD25^{low}$, which is comparable to both the primary derived RV-ATL cell line and to the malignant lymphocytes from the original patient. $CD25^{low}$ expression is a reflection of the specific patient from which these cells were derived. This RV-ATL cell line is the same malignant clone derived from the original patient sample as molecularly determined by Southern blot analysis of HTLV integration. RV-ATL cells were cultivated for short term *in vitro* experiments in IMDM medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin/glutamine.

Cyclopalladated compound. The cyclopalladated complex C7a was synthesized from *N,N*-dimethyl-1-phenethylamide (dmpa), complexed to 1,2 ethanebis (diphenylphosphine, dppe) ligand, as previously described in [9]. The compound was diluted to a final concentration of 10 mM in DMSO (cell culture tested, Sigma Aldrich), and for *in vivo* and *in vitro* assays diluted to the final concentration in media or PBS.

In vitro *cellular toxicity assay.* To evaluate the cytotoxic effects of C7a on PBMC (HTLV-1 positive and HTLV-1 negative individuals) and in malignant human T cell lineages, 1 or 5 × 10^5 cells were

seeded in 96-well plates in triplicate, serial dilutions of C7a (ranging from 0.9 to 60 μM) were added and plates were incubated for 48 h. Cellular viability was measured by Trypan Blue exclusion dye using the automated cell counter Vi-Cell XR Viability Analyzer (Beckman Coulter®). Control cells were incubated in the absence of C7a, and considered to be 100% viable. RV-ATL cells were expanded by intraperitoneal inoculation of SCID mice for 3 weeks and freshly harvested cells were plated at 10^5 cells per well in a 24-well plate. For additional studies, RV-ATL cells were thawed from frozen stocks, plated in IMDM with 10% FBS and 1% P/S/G for 6 h at 37 °C prior to treatment with C7a. All RV-ATL cells were incubated in the presence of C7a at concentrations between 0 and 80 μM, diluted in complete culture IMDM media at 37 °C for 48 h. Cytotoxicity was determined by Trypan blue method in a hemocytometer. C7a compound was diluted in complete culture media, and plates were incubated at 37 °C in a 5% CO_2 humidified atmosphere. To verify the inhibitory effect of dithiothreitol (DTT) or a lysosomal cathepsin B inhibitor (CA-074) on C7a cytotoxicity *in vitro*, 10^5 MT-2 or HL-60 cells were pre-incubated with 1 mM DTT for 1 hour or with 10 mM CA-074 [*N*-(L-3-*trans*-propylcarbamoyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline] for 2 h (both from Sigma Aldrich). C7a was added to MT-2 (15 μM) and HL-60 cells (2 μM) and incubated for 6 (DTT) or 18 h (CA-074). Viable cells were counted by Trypan Blue exclusion in a hemocytometer. Assays were done in triplicate, and repeated three times.

RV-ATL SCID Mouse Studies. The animal research described in this manuscript was performed according to the guidelines for the Committee for Humane Use of Animals at SUNY Upstate Medical University in accordance with all federal, state and local guidelines. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were obtained from our own breeding colony (breeder mice originally from Jackson Laboratories). All mice were housed under specific pathogen-free conditions. Prior to all manipulations, mice were anesthetized with isofluorane. RV-ATL cells from frozen stocks were expanded intraperitoneally in NSG mice prior to injection of study mice. Age-matched (22.5 to 23 week old) female NSG mice were intraperitoneally (IP) injected with 10^7 RV-ATL cells on day 0. Mice were treated with 100 μL of PBS (with or without C7a) by IP injection every other day from day 4 through 42. Drug concentration per gram of mouse body weight was not statistically different based on weekly weight measurements (data not shown). Tumor cells were removed by peritoneal lavage using PBS at two-week intervals (study days 14, 28 and 42). Mice were euthanized by carbon dioxide exposure as necessary due to tumor burden or severe clinical symptoms (usually dehydration). Remaining mice were euthanized on day 42 upon termination of the experiment.

Transmission Electron Microscopy analysis. For transmission electron microscopy (TEM) analysis, HL-60 cells (5×10^6) were cultured in presence or absence of 2 μM C7a for 6 h in complete RPMI medium. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and post-fixation was performed using 1% osmium tetroxide, 0.08% potassium ferricyanide, 5 mM calcium chloride in the same buffer for 60 min in the dark (all reagents from Sigma-Aldrich). Cells were dehydrated using an acetone series and infiltrated in polybed epoxy resin (Polysciences, PA, USA). Ultrathin sections obtained by ultramicrotomy were collected on 300 mesh grids and stained with uranyl acetate and lead citrate for contrast. The ultrastructure analysis was done in a transmission electron microscope (Zeiss EM-900).

Cytochrome c release assay. MT-2 cells, cultured in round coverslips (24 mm), were preincubated for 30 min with 62.5 nM MitoTracker (Molecular Probes, Eugene, OR) and then treated with 5 μM of C7a for 3 h. The cells were washed once with PEM buffer [80 mM piperazine-*N,N'*-bis(2-ethanesulfonic) acid pH 6.8, 5 mM EGTA pH 7.0, 2 mM $MgCl_2$], and fixed for 30 min at 4 °C with 5% formaldehyde diluted in PEM buffer. Cells were washed three times in PEM buffer, and permeabilized for 30 min at room temperature in PEM buffer containing 0.5% Triton X-100. Anti-cytochrome c antibody (1:800, Cell Signaling, MA, USA) diluted in 5% BSA and 0.1% Tween 20-containing TBS (TBS-T) was incubated overnight at 4 °C. After washing three times in TBS-T, cells were incubated for 2 h at room temperature in the dark with a fluorescein isothiocyanate-conjugated anti-rabbit IgG Alexa 488 (1:400, Sigma-Aldrich, MO USA) diluted in 5% BSA-containing TBS-T. Coverslips were washed three times with TBS-T, cells were counter-stained with DAPI (Sigma-Aldrich) and mounted on slides using Slow-Fade Anti-fade Mounting Media (Molecular Probes). Deconvolution images were taken using Olympus IX71 with 60X oil objective (NA = 1.42). All images were deconvolved using Delta Vision software (Applied Precision) using Ratio (conservative) method, with medium noise filtering for 6 cycles and correction applied. Single staining images were processed by the ACDsee software (ACD Systems, Canada).

Detection of caspase-3 activation. MT-2 cells were treated or not with 5 μM of C7a for 1, 2 or 3 h. Alternatively, cells were exposed to 50J of UV light for 5 seconds as a positive control. Cell extracts were prepared as described in [31] and separated by electrophoresis in 0.1% SDS, 12% polyacrylamide gels. Proteins were blotted onto nitrocellulose membranes, and subsequently blocked for 1h at room temperature with 5% BSA in TBS-T. Membranes were incubated with an antibody against cleaved caspase-3 (CellSignaling, MA, USA), and GAPDH as a load control (Sigma-Aldrich MO, USA), diluted 1:1000 in TBS-T, 5% skim milk), overnight at 4 °C. After washing with TBS-T, the membranes were incubated with anti-rabbit Ig conjugated to horseradish peroxidase (Sigma Aldrich, diluted 1: 40000 in TBS-T, 5% skim milk). After 1 h incubation at room temperature, the membranes were washed extensively with TBS-T, and developed with ECL (GE Healthcare) according to the manufacturer's instructions.

4.2. Analysis of Nuclear Alterations

Hoescht 33342 staining: HL-60 cells (1×10^6) were plated in 12 wells plate and treated or not (control) with 2.5 μM C7a for 6 h or 5 μM for 24 h. Cells were then collected, washed 3 times in PBS and collected on round coverslips by centrifugation at 3000 rpm for 10 min. Cells were fixed for 15 min in 2% formaldehyde and 30 min in absolute methanol, washed and stained with Hoescht 33342 (Sigma-Aldrich MO, USA) for 15 min. Coverslips were mounted on glass slides with Vectashield (4 μL), sealed with nail polish and immediately analyzed in a fluorescent Olympus BX61 microscope (magnification 400X) at 360 nm. The images were acquired using Cell^M Software.

DNA degradation assay: HL-60 cells (3×10^6) were incubated with 5 μM C7a at 37 °C for 18 h in 6 well-plates. Cells were recovered from the culture supernatant by centrifugation, lysed in TELT buffer (50 mM Tris-HCl pH 8.0, Triton X-100 0.4%, 2.5 mM EDTA pH 9.0 and 2.5M LiCl), and centrifuged for 15 min at 12000 g/4 °C. DNA was extracted by phenol-chloroform and precipitated

with 0.1 volume of 3 M sodium acetate, pH 7.0, and 2.5 volumes of absolute ethanol, following incubation at −80 °C for 20 min. All reagents were obtained from Sigma-Aldrich. Precipitated DNA was centrifuged, diluted in RNase-free water (25 μg/mL), separated by electrophoresis on a 1% agarose gel (100V) and photographed with a digital camera (Kodak, EDAS DC290).

4.3. Flow Cytometry Analysis

Cell-cycle analysis. C81, Jurkat, HL-60 and MT-2 cells (1×10^6) were cultured in the absence or presence of 2.5 μM C7a for 48 h in 24 well-plates at 37 °C in a 5% CO_2 humidified atmosphere. Cells were then collected, fixed in chilled methanol, and suspended in solution containing RNase A (100 U/mL; Sigma Aldrich) before staining with 50 μg/mL of propidium iodide. A minimum of 1×10^5 cells were acquired by flow cytometry in a FACSCanto and data was analyzed using the FACSDiva software (Becton Dickinson, Mountain View, CA).

Assessment of tumor burden in RV-ATL engrafted mice. Tumor burden in RV-ATL engrafted mice was assessed by flow cytometric analysis of peritoneal lavage cells. Engrafted cells were harvested by peritoneal lavage, washed with PBS and blocked in PBS containing 5% each human and murine serum for at least 20 min at 4 °C. RV-ATL cells were identified by staining with antibodies for human CD45, human CD4 and human CD25 compared with murine CD45 (all antibodies from Biolegend) for at least 30 min at 4 °C. Cells were washed twice with PBS and fixed for 15 min at 4 °C in 2% formalin in water then acquired on an LSRII flow cytometer (Becton Dickinson) and analyzed by FlowJo software (TreeStar, Ashland, CA).

Detection of phosphatidylserine translocation by PE-Annexin V/7-AAD. C81, Jurkat and MT-2 cells were incubated in the presence of C7a at concentrations ranging from 0 to 15 μM at 37 °C for 3 h. Untreated and treated cells were then stained with PE-Annexin V and 7-AAD (7-Amino-actinomycin D), using the Apoptosis Detection Kit I (BD Pharmingen, NJ, USA) according to the manufacturer's instructions. The cells were acquired by flow cytometry (LSRFortessa, Becton Dickinson) and analyzed by FACSDiva software.

In vitro *HTLV-1-infected PBMC proliferation assay.* To evaluate the effects of C7a on spontaneous *in vitro* proliferation of HTLV-1-infected PBMC, PBMC from one asymptomatic HTLV-infected individual and six patients diagnosed with HAM/TSP were cultured separately in 96-well U-bottom culture plates (Costar, Cambridge, MA, 1×10^5 cells/well), in triplicate, at 37 °C in a 5% CO_2 humidified atmosphere. Serial dilutions (0.9 to 60 μM) of C7a and 5 μM CarboxyFluorescein diacetate Succinimidyl Ester (CFSE, Invitrogen, Eugene, USA) were added to the cells, and after 24 h incubation, cells were harvested, washed two times in 2 mL of PBS containing 1% bovine serum albumin, and fixed in PBS containing 4% paraformaldehyde. Analyses were performed using a FACSAria (Becton Dickinson) and FlowJo software. At least 10^5 events were analyzed per sample. Proliferation intensity was determined by the percentage of divided cells and the division index. The cut-off values were >0.06 for the division index and >5.8% for the percentage of divided cells (3X the division index mean and 3X the percentage of divided cells in PBMC controls.).

5. Conclusions

C7a suppresses proliferation of HTLV-1-infected patient PBMCs, induces apoptosis in transformed cell lines and significantly enhances the survival of ATLL tumor bearing mice. C7a is a potential novel therapeutic treatment for malignant T-cell diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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