

## REVIEW

# Emerging agents that target signaling pathways to eradicate colorectal cancer stem cells

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## Abstract

Colorectal cancer (CRC) represents the third most commonly diagnosed cancer and the second leading cause of cancer death worldwide. The modern concept of cancer biology indicates that cancer is formed of a small population of cells called cancer stem cells (CSCs), which present both pluripotency and

**Abbreviations:** 4-AAQB, 4-acetyl-antroquinol B; 5-FU, 5-fluorouracil; ABCG2, ATP binding cassette subfamily G member 2; ABL, ABL proto-oncogene 1, non-receptor tyrosine kinase; AC, adenylyl cyclase; AF2, activation function 2; ALDH1, aldehyde dehydrogenase 1; AMPK, adenosine monophosphate-activated protein kinase; APC, adenomatous polyposis coli; APCDD1, APC downregulated 1; ATL-1, atractylenolide I; *AXINI*, axin-1; Axin, axis inhibitor; BCR, BCR activator of RhoGEF and GTPase; BRAF, B-Raf proto-oncogene, serine/threonine kinase; cAMP, cyclic adenosine monophosphate; CAR-T, chimeric antigen receptor T cell; C-B, cucurbitacin-B; *CCND1*, cyclin D1; CD44s, CD44 standard isoform; CD44v, CD44 variant isoform; C-I, cucurbitacin-I; CK1 $\alpha$ , casein kinase 1 $\alpha$ ; Co-Smad, common partner Smad; CRC, colorectal cancer; CRC-SCs, colorectal cancer stem cells; CSCs, cancer stem cells; CSL, CBF1-suppressor of hairless-LAG1; CTLA4, cytotoxic T-lymphocyte-associated antigen 4; CXXC4, CXXC finger protein 4; DATS, diallyl trisulfide; DCLK1, doublecortin-like kinase-1; DKK3, dickkopf Wnt signaling pathway inhibitor 3; DNMT1, DNA methyltransferase; DVL, dishevelled; EGCG, epigallocatechin-3-gallate; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; ERBB2, erb-b2 receptor tyrosine kinase 2; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; FDMC, farnesyl dimethyl chromanol; FOLFIRI, 5-FU, leucovorin plus irinotecan; FOLFOX, 5-FU, folic acid plus oxaliplatin; FZD, seven-pass transmembrane receptor frizzled; GLI, GLI family zinc finger; GPI30, glycoprotein 130; GSK-3 $\beta$ , glycogen synthase kinase-3 beta; *HES1*, split-1; *HEY1*, hes related with YRPW motif-1; HH, hedgehog; HOXA5, homeobox A5;  $\text{I}\kappa\text{B}$ , inhibitor of NF- $\kappa\text{B}$ ; IR, ionizing radiation; I-Smad, inhibitory Smad; JAK, janus kinase; KLF4, Krüppel-like factor 4; LEF, lymphoid enhancer factor; LGR5, leucinerich repeat-containing G-protein-coupled receptor 5; LRP5/6, single-pass low-density lipoprotein receptor-related protein 5 or 6; LSD1, lysine-specific demethylase 1; MCM2, minichromosome maintenance complex component 2; miRNA, microRNA; MYC, MYC proto-oncogene, bHLH transcription factor; mTOR, mechanistic target of rapamycin; mTORC1, mTOR complex 1; NANOG, nanog homeobox; NF- $\kappa\text{B}$ , nuclear factor kappa B; NIK, NF- $\kappa\text{B}$ -inducing kinase; NKD1, NKD inhibitor of Wnt signaling pathway 1; OCT4, octamer-binding transcription factor 4; PD-1, programmed cell death protein 1; PEITC, phenethyl isothiocyanate; PI3K, phosphatidylinositol-3-kinases; PIK3C3, phosphatidylinositol 3-kinase catalytic subunit type 3; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PIK3R2, phosphoinositide-3-kinase regulatory subunit 2; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PKA, protein kinase A; PLGA, poly(D,L-lactic-coglycolic acid); PPAR, peroxisome proliferator-activated receptor; PTCH, patched; PTEN, phosphatase and tensin homolog; RAS, rat sarcoma virus; R-Smad, receptor-regulated Smad; RSPO, R-spondin; SHP-1, Src homology 2 domain-containing protein tyrosine phosphatase 1; SMO, smoothened; SOD2, superoxide dismutase 2; SOX2, SRY-box transcription factor 2; SOX4, SRY-box transcription factor 4; SOX9, SRY-box transcription factor 9; STAT, signal transducer and activator of transcription; TAp63 $\alpha$ , transactivating p63 isoform  $\alpha$ ; TCF, T-cell factor; TGFBR1, TGF- $\beta$  receptor type 1; TGFBR2, TGF- $\beta$  receptor type 2; TGF- $\beta$ , transforming growth factor- $\beta$ ; TNIK, TRAF2- and NCK-interacting kinase; TP53, tumor protein p53; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau tumor suppressor; Wnt, wingless

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self-renewal properties. These cells are considered responsible for the progression of the disease, recurrence and tumor resistance. Interestingly, some cell signaling pathways participate in CRC survival, proliferation, and self-renewal properties, and most of them are dysregulated in CSCs, including the Wntless (Wnt)/ $\beta$ -catenin, Notch, Hedgehog, nuclear factor kappa B (NF- $\kappa$ B), Janus kinase/signal transducer and activator of transcription (JAK/STAT), peroxisome proliferator-activated receptor (PPAR), phosphatidylinositol-3-kinase/Akt/mechanistic target of rapamycin (PI3K/Akt/mTOR), and transforming growth factor- $\beta$  (TGF- $\beta$ )/Smad pathways. In this review, we summarize the strategies for eradicating CRC stem cells by modulating these dysregulated pathways, which will contribute to the study of potential therapeutic schemes, combining conventional drugs with CSC-targeting drugs, and allowing better cure rates in anti-CRC therapy.

**KEYWORDS**

colorectal, cancer stem cells, cell signaling, Wnt/ $\beta$ -catenin pathway, Notch, Hedgehog, NF- $\kappa$ B, JAK/STAT signaling, PI3K/Akt/mTOR signaling, targeted therapy

**1 | BACKGROUND**

Colorectal cancer (CRC) represents the third most commonly diagnosed cancer and the second leading cause of cancer death worldwide, representing 1,900,000 new cases and an estimated number of 935,000 deaths in 2020 [1]. Surgical resection is the preferred treatment for earlier-stage CRC. Chemotherapy is indicated in patients at risk of cancer recurrence, as an adjuvant treatment, or when the patient has distant metastasis. A very important topic in the treatment of CRC is the response rate of anti-neoplastic drugs in patients with metastases [2–4].

5-Fluorouracil (5-FU) is still the recommended first-line drug treatment for advanced CRC and has achieved response rates of 10%-15%, which are improved to approximately 40%-50% when combined with irinotecan and oxaliplatin [3,5]. Second-line treatment for metastatic CRC includes some targeted therapies. These include inhibitors of vascular endothelial growth factor (VEGF), which reduce tumor angiogenesis. Other drugs target growth factor pathways such as epidermal growth factor receptor (EGFR) and abnormal B-Raf proto-oncogene serine/threonine kinase (BRAF) [2,3]. The immune system also plays an important role in regulating the development of CRC. The immunological pathways that have been studied include cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD-1) [6]. Drugs that promote the inhibition of CTLA4 produce a large increase in T helper cell-dependent immune responses. Inhibition of the PD-1 pathway can enhance the anti-tumor immune response since PD-1 is a cell

surface receptor that limits T cell activity [7]. Despite advances in surgical and medical therapies, the mortality from CRC has changed little in recent decades, indicating the need for research on other pathways in advanced diseases [8].

The current concept of cancer biology indicates that cancer is a heterogeneous disease derived from a small subpopulation of undifferentiated cancer cells called cancer stem cells (CSCs) or tumor-initiating cells. CSCs are defined as cells with three unique properties: the ability to renew themselves indefinitely; the ability to recreate the complete cancer cell repertoire of the original tumor; and the expression of a distinct set of surface biomarkers [9]. Some pluripotent transcription factors, such as octamer-binding transcription factor 4 (*OCT4*) [10], SRY-box transcription factor 2 (*SOX2*) [11], nanog homeobox (*NANOG*) [12], and *MYC* proto-oncogene, bHLH transcription factor (*MYC*) [13], have been associated with the regulation of these CSC properties.

Self-renewal involves the ability of CSCs to maintain their proportion through a combination of symmetric division (which produces two daughter cells with CSC properties) and asymmetric division (which produces a daughter cell with CSC properties and a daughter cell without CSC properties) [14]. In addition to the symmetric/asymmetric division theory, CSCs can differentiate into cells without CSC properties, and these cells can dedifferentiate back into CSCs through a bidirectional interconversion process [15]. This indicates that, for a good clinical outcome, cancer cells with or without properties of CSCs must be eradicated.

Although CSCs are self-renewable, they are relatively quiescent. In fact, they have longer cell cycle times than cancer cells without properties of CSCs due to the permanence of CSCs in a phase of the cell cycle similar to  $G_0$  [16]. These unique properties of CSCs can explain the failure of many anti-neoplastic drugs, which affect cells that divide rapidly, causing a reduction in the number of cancer cells, while CSCs divide slowly and are not sensitive to most cytotoxic drugs [16,17].

An important feature of CSCs in culture is that they are able to form multicellular spheroid structures when grown in non-adherent conditions without serum and supplemented with growth factors. These spheres are characterized by a well-rounded morphology, ability to persist as floating cultures, and small size [18]. Thus, spheroid cultures are useful for enriching population cells that exhibit characteristics of CSCs. Likewise, the secondary and tertiary sphere formation method, in the absence of any agent, is useful to verify the self-renewal capacity of cells in multiple passages [19,20].

Several biomarkers have been identified in CRC stem cells (CRC-SCs), including CD133 [21,22], CD44 [22,23], CD166 [23], CD29 [24], CD24 [24], aldehyde dehydrogenase 1 (ALDH1) [25], CD26 [26], and epithelial cell adhesion molecule (EpCAM) [27]. On the other hand, although these molecules are considered standard markers of CRC-SCs, some of them are also found in stem cells from normal tissues and other types of cancer with different degrees of expression [28,29]. CSCs can be identified and separated by combining specific biomarkers and separation techniques to increase the sensitivity and specificity of the assay. Normally, cells with positive/high expression of these biomarkers are considered to have stemness properties.

CD133 was the first biomarker identified, and although still contradictory, most clinical data support the hypothesis that CD133 expression is associated with a poor prognosis in CRC [30–33]. Curiously, the CD133 protein is not lost in differentiating CRC-SCs, but its AC133 epitope is [34]. Therefore, monoclonal antibodies against the AC133 epitope are most commonly used to appropriately identify CD133 in CRC-SCs. Interestingly, CRC CD133<sup>+</sup> cells, but not CD133<sup>-</sup> cells, have the ability to form solid tumors in immunodeficient NOD/SCID mice [21]. In particular, treatment with 5-FU initially decreases the tumor mass; however, upon completion of treatment, enrichment of CD133<sup>+</sup> cells in residual tumors is found, contributing to recurrence and decreasing progression-free survival in patients with CRC [35,36]. In addition, CD133 has also been used as a marker for CSCs in different types of cancer, such as brain [37], prostate [38], and liver cancers [39].

CD44 is also an important CRC-SC marker that is a target of T-cell factor (TCF)/ $\beta$ -catenin-mediated tran-

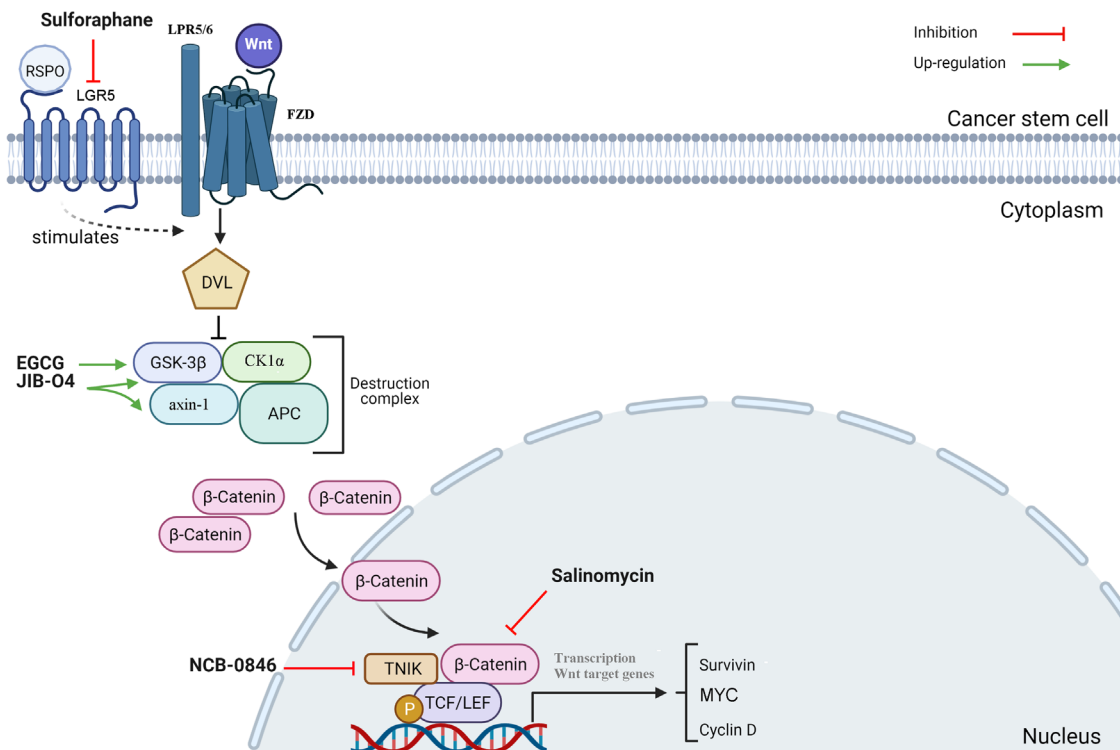
scription [40]. In colorectal carcinogenesis in  $Apc^{Min/+}$  mice, CD44<sup>-/-</sup>  $Apc^{Min/+}$  mice had fewer intestinal adenomas than CD44<sup>+/+</sup>  $Apc^{Min/+}$  mice [41]. Likewise, CD44 expression is also associated with a poor prognosis in CRC [42]. Different CD44 isoforms have been identified that are generated by alternative splicing of 10 variant exons and are known as CD44 variant isoforms (CD44v) to differ from CD44 standard isoforms (CD44s) [43]. Interestingly,  $Apc^{Min/+}$  mice expressing only CD44v4-10 could promote adenoma initiation, but not those expressing CD44s, indicating CD44v as a potential treatment target in CRC [44].

Despite advances in cancer therapy, to date, there are no clinically approved effective drugs targeting CRC-SCs. Cell signaling pathways participate in CRC survival, proliferation, and self-renewal properties, and most of them are dysregulated in CRC-SCs. From this perspective, understanding the participation of the signaling pathways activated in CRC-SCs is very promising, as their therapeutic targets can contribute to the improvement of anti-CRC therapy. In this review, the roles of the Wntless (Wnt)/ $\beta$ -catenin, Notch, Hedgehog, nuclear factor kappa B (NF- $\kappa$ B), Janus kinase/signal transducer and activator of transcription (JAK/STAT), peroxisome proliferator-activated receptor (PPAR), phosphatidylinositol-3-kinase/Akt/mechanistic target of rapamycin (PI3K/Akt/mTOR), and transforming growth factor- $\beta$  (TGF- $\beta$ )/Smad pathways in CRC-SCs were discussed as drug targets to eradicate this cell subpopulation.

## 2 | CELL SIGNALING PATHWAYS

### 2.1 | Wnt signaling pathway

The canonical and non-canonical Wnt pathways have been associated with the development and progression of cancer. The canonical Wnt pathway acts by controlling the level of  $\beta$ -catenin available to regulate gene expression (Figure 1). In the absence of Wnt signaling,  $\beta$ -catenin does not accumulate in the cytoplasm. A multimeric protein complex composed of adenomatous polyposis coli (APC), axin-1, glycogen synthase kinase-3 beta (GSK-3 $\beta$ ) and casein kinase 1 $\alpha$  (CK1 $\alpha$ ) is responsible for the degradation of  $\beta$ -catenin by ubiquitination and digestion, thereby maintaining low levels of free  $\beta$ -catenin in the cytoplasm and nucleus. On the other hand, in the presence of Wnt ligands, the destruction complex function is interrupted, since Wnt binds to the seven-pass transmembrane receptor Frizzled (FZD) and single-pass low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). The Wnt-FZD-LRP5/6 trimeric complex recruits dishevelled (DVL), resulting in dissociation of the  $\beta$ -catenin phosphorylation complex, followed by events that stabilize and accumulate



**FIGURE 1** The Wnt/ $\beta$ -catenin signaling pathway. In the absence of Wnt signaling,  $\beta$ -catenin is bound to a multimeric protein complex that contains APC, GSK-3 $\beta$ , axin-1 and CK1 $\alpha$ , leading to proteasomal degradation of  $\beta$ -catenin. In the presence of Wnt signaling, the destruction of the complex function is interrupted. Wnt binds to LRP5/6 and FZD and inhibits the activity of the multimeric protein complex, which makes  $\beta$ -catenin enter the nucleus, with subsequent translocation to the nucleus, binds to TCF/LEF to form a complex, and then recruits cofactors to initiate downstream gene expression. Abbreviations: APC, adenomatous polyposis coli; CK1 $\alpha$ , casein kinase 1 $\alpha$ ; DVL, dishevelled; EGCG, epigallocatechin gallate; FZD, seven-pass transmembrane receptor Frizzled; GSK-3 $\beta$ , glycogen synthase kinase-3 beta; LEF, lymphoid enhancer factor; LGR5, leucine-rich repeat-containing G-protein-coupled receptor 5; LRP5/6, single-pass low-density lipoprotein receptor-related protein 5 or 6; MYC, MYC proto-oncogene, bHLH transcription factor; RSPO, R-spondin; TCF, T-cell factor; TNIK, TRAF2- and NCK-interacting kinase; Wnt, wingless

$\beta$ -catenin in the cytoplasm with subsequent translocation to the nucleus. This event allows direct binding to TCF/lymphoid enhancer factor (LEF) transcription factors for activation of Wnt-responsive gene expression and subsequent transcription of several target genes of Wnt, such as *AXIN2*, *SURVIVIN*, *ALDH*, and cyclin D1 (*CCND1*) [45–47].

The canonical Wnt pathway (the Wnt/ $\beta$ -catenin signaling pathway) is essential for the normal homeostasis of intestinal stem cells, and aberrant activation or mutation is responsible for the establishment of approximately 90% of intestinal cancers through the loss of APC, leading to increased  $\beta$ -catenin function. These events drive tumor initiation and maintenance of CSCs, regulating the capacity for self-renewal, interacting with the microenvironment and immune system and promoting tumor invasion and metastasis [36,48,49]. Importantly, the CRC-SC biomarkers leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) and CD44 were identified as genes involved in Wnt signaling [50]. The Wnt pathway

is also crucial to support the tumor initiation potential of CRC-SC precursors that occur in the transition from ulcerative colitis to CRC [51].

Interestingly, Chen et al. [52] investigated the biological function of microRNAs (miRNAs), small non-coding RNAs that regulate gene expression, and miR-199a/b in cisplatin-resistant CRC-SCs. The expression of miR-199a/b in CRC tissues was associated with short patient survival. Additionally, the overexpression of miR-199a/b contributes to cisplatin resistance, upregulating ATP binding cassette subfamily G member 2 (ABCG2), an important multidrug resistance pump located downstream of the Wnt/ $\beta$ -catenin pathway. Blocking the Wnt/ $\beta$ -catenin pathway decreases ABCG2 levels in ALDH1<sup>+</sup> CRC-SCs. In addition, miR-92a promotes the properties of CRC-SCs, increases the expression levels of stem cell markers CD133, SOX2, and OCT4, and is upregulated by Wnt/ $\beta$ -catenin signaling activity via downregulation of Krüppel-like factor 4 (KLF4), GSK-3 $\beta$ , and dickkopf Wnt signaling pathway inhibitor 3 (DKK3), negative regulators of Wnt/ $\beta$ -catenin signaling [53].

The decreased expression of miR-148a, a tumor suppressor, causes increased expression of CRC-SC markers through the Wnt/ $\beta$ -catenin signaling pathway, while its hyperexpression increases cisplatin chemosensitivity, reducing cell invasion and migration [54]. Additionally, miR-30-5p expression is normally reduced in CRC cells resistant to 5-FU and in CD133<sup>+</sup> CRC cells. In contrast, its overexpression significantly reduces cell viability and the expression of stem cell markers CD133 and SOX2, since miR-30-5p attenuates the expression of *AXIN2* and *MYC*, target genes of Wnt/ $\beta$ -catenin signaling [55]. Moreover, the 5-FU-resistant CRC HT-29 cell line showed increased Wnt signaling and stemness properties [56].

Therefore, many aspects of how this pathway controls the growth and proliferation of normal cells and CSCs have been studied [51,57–59]. Moreover, new studies focusing on the anti-cancer properties of molecules that eradicate CRC-SCs via the Wnt pathway have been carried out and are described below.

### 2.1.1 | Natural products

#### *Phenethyl isothiocyanate*

Phenethyl isothiocyanate (PEITC) is a natural product present in many cruciferous vegetables with anti-cancer and chemopreventive activities in clinical trials and has been associated with the induction of oxidative stress [60]. Interestingly, PEITC has also been reported to be able to eliminate CSCs of different types of cancer, including cervical [61,62], lung [63], ovarian [64], and breast cancers [64,65]. Importantly, PEITC inhibited the properties of CRC-SCs through downregulation of the Wnt/ $\beta$ -catenin pathway, inhibition of proliferation, and induction of apoptosis in CRC DLD-1 and SW480 cell lines. PEITC decreased the percentage of CD133<sup>+</sup> cells by approximately 3–16-fold [66]. Activation of the Wnt/ $\beta$ -catenin pathway with lithium chloride, an agonist of canonical Wnt signaling, elevated CD133 expression and suppressed the effects of PEITC on CRC-SCs [66]. PEITC also suppressed the expression of pluripotency factors, including OCT4, SOX2, and NANOG, in NCCIT cells, a pluripotent stem cell line from mediastinal mixed germ cell tumors [67,68]. In vitro self-renewal capacity and clonogenicity and in vivo tumor growth and expression of pluripotency factors were also observed to be reduced after treatment with PEITC in an EpCAM-expressing CSC model derived from the CRC HCT116 cell line [67,68]. These results support that PEITC is a candidate to eradicate CRC-SCs targeting Wnt signaling.

#### *Sulforaphane*

Sulforaphane is an isothiocyanate obtained from cruciferous vegetables with anti-cancer properties. Some studies indicate that it can act on CSCs [69–72]. Interestingly, sulforaphane reduced pancreatic CSCs by blocking the sonic hedgehog-GLI family zinc finger (GLI) [70] and NF- $\kappa$ B pathways [71] and enhanced imatinib against CSCs of chronic myelogenous leukemia by inhibiting Wnt/ $\beta$ -catenin function [72]. In CRC, sulforaphane caused cell cycle arrest and apoptosis in CRC HT-29 and Caco-2 cell lines [73,74]. Recently, Chen et al. [75] demonstrated that sulforaphane inhibited CRC-SC properties by suppressing the transactivating p63 isoform  $\alpha$  (TAp63 $\alpha$ )/LGR5/ $\beta$ -catenin axis in vitro and in vivo. In addition, activation of the Wnt/ $\beta$ -catenin pathway leads to the expression of LGR5, which is also a promoter of the Wnt/ $\beta$ -catenin pathway when binding to R-spondin (RSPO), suggesting that LGR5 may be used not only as a marker but also as a target in CRC-SCs. LGR5 acts through TAp63 $\alpha$  to enhance Wnt signaling and the activation of  $\beta$ -catenin targets, promoting the maintenance of stem cells. Sulforaphane treatment led to downregulation of CRC-SC markers (CD133, CD44, NANOG, and OCT4), suppressing the formation of colonies in a spheroid cell model with CRC HCT116 and SW480 cell lines. Moreover, the upregulation of TAp63 $\alpha$  blocked the inhibitory effect of sulforaphane. These data indicated that sulforaphane is a useful drug to eliminate CRC-SCs by suppressing Wnt signaling.

#### *Salinomycin*

Salinomycin, an anti-bacterial polyether isolated from *Streptomyces albus*, selectively eliminated CD133<sup>+</sup> cells in CRC [76]. Salinomycin induced caspase activation, increased DNA damage and caused disruption of the Wnt/ $\beta$ -catenin/TCF complex and apoptosis of human CRC-SCs, and decreased tumor growth and expression of CSC-related Wnt genes, including *LGR5* [77,78]. Treatment with salinomycin alone or in combination with the FOLFOX regimen (5-FU, folic acid plus oxaliplatin) was also evaluated in primary CSCs of patients with hepatic metastases of CRC or primary CRC [79]. Salinomycin exhibited superior anti-CSC activity and tumor growth inhibition when compared to the FOLFOX protocol, and salinomycin plus FOLFOX resulted in improvement of these effects when compared to salinomycin alone. Salinomycin increased the expression of LGR5 and mitigated the expression of ALDH1. Furthermore, salinomycin also caused apoptosis related to decreased cellular ATP production and the accumulation of dysfunctional mitochondria and

reactive oxygen species [79]. Likewise, Singh et al. [80] demonstrated that salinomycin improved cell death receptors in CRC-SCs by inhibiting enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), a histone-lysine N-methyltransferase enzyme. Importantly, salinomycin has also been reported to target CSCs of various types of cancer, including melanoma [81], osteosarcoma [82], breast [83], liver [84], brain [85], ovarian [86], and oral cancers [87]. Salinomycin specifically eliminated epithelial CSCs [88].

Wang et al. [89] developed salinomycin nanocrystals to improve their oral bioavailability/solubility and reduce toxicity. Salinomycin nanocrystals exhibited greater cell uptake efficiency, cytotoxicity and Wnt-inhibiting effects three times better than regular salinomycin, indicating that salinomycin nanocrystals have great potential for the clinical treatment of CRC. The combination of SN38, the active form of irinotecan, with salinomycin in nanoformulations also diminished the adverse effects and enhanced the treatment efficacy against CRC-SCs [90]. These results suggest salinomycin as a drug capable of eliminating CRC-SCs by targeting Wnt signaling and improving conventional chemotherapeutic effects, including the FOLFOX protocol.

#### *Epigallocatechin gallate*

Epigallocatechin gallate (EGCG), also known as epigallocatechin-3-gallate, is one of the catechins found in green tea. It has been widely studied as an anti-CSC agent against different types of cancer, including lung [91], bladder [92], pancreas [93], and breast cancers [94]. In combination with cisplatin or oxaliplatin, EGCG increased the therapeutic effect on human CRC cells [95]. Furthermore, EGCG was also effective in suppressing CRC-SCs by downregulating CD133, CD44, NANOG, OCT4, ALDH1, and Notch1 expression, inhibiting the ability to form spheres of CRC cells, and upregulating the expression of GSK-3 $\beta$ , a negative regulator of Wnt/ $\beta$ -catenin [96,97]. In addition, EGCG induced the sensitivity of CRC-SCs to 5-FU [97].

In a pilot study with 125 patients, green tea extract reduced the recurrence of colorectal adenoma in approximately 50% of patients [98]. Moreover, in a randomized controlled clinical trial, 10 volunteers received 800 mg EGCG, and 11 volunteers received the same amount of EGCG plus Brazil nuts for 6 weeks. DNA methyltransferase (DNMT1) (DNA methylation is a molecular marker in CRC) and NF- $\kappa$ B expression were decreased in rectal biopsies of both groups [99]. In a clinical trial with 223 healthy Japanese individuals, *Escherichia coli* containing polyketide synthase (tumorigenic bacteria for CRC) in the gut microbiota was downregulated by ingestion of green tea [100].

Taken together, these preclinical results support that EGCG reduces the population of CRC-SCs and enhances the effect of cisplatin and oxaliplatin. Furthermore, clinical data suggest EGCG and/or green tea extract as a beneficial strategy in CRC therapy/prevention.

#### *Farnesyl dimethyl chromanol*

Farnesyl dimethyl chromanol (FDMC), a chemical structure that is part of the tocopherol and tocotrienol molecules (vitamin E), exhibits antioxidant properties [101]. Recently, Husain et al. [102] demonstrated that FDMC decreased the viability and self-renewal of CRC-SCs by downregulating Wnt/ $\beta$ -catenin signaling in vitro and in vivo. FDMC reduced spheroid and organoid formation and downregulated the expression of the pluripotent transcription factors NANOG, OCT4 and SOX2 in CD24<sup>+</sup>/CD44<sup>+</sup>/LGR5<sup>+</sup> CRC cells. Induction of DNA fragmentation, exposure to phosphatidylserine, cleaved caspase 3 and cleaved PARP and reduced migration, invasion, VEGF and NF- $\kappa$ B and Wnt signaling were also observed in CRC-SCs treated with FDMC in vitro. In an orthotopic xenograft metastasis model, FDMC inhibited tumor growth and liver metastasis and suppressed angiogenesis and NF- $\kappa$ B and  $\beta$ -catenin signaling. This indicates that FDMC is effective in eradicating CRC-SCs.

#### *4-Acetyl-antroquinonol B*

4-Acetyl-antroquinonol B (4-AAQB), a mycelial from *Antrodia camphorate*, has been reported as a cytotoxic agent [103,104]. In CRC, 4-AAQB reduced migration, invasion and clonogenicity in CRC DLD1 and HCT116 cell lines, with less effect on the viability and proliferation of normal colon cells. Downregulation of N-cadherin, vimentin, MYC and Bcl-xL and upregulation of E-cadherin and BAX proteins were found in 4-AAQB-treated CRC cells. 4-AAQB also reduced the superoxide dismutase 2 (SOD2)-mediated CSC phenotype by hsa-miR-324. In vivo, 4-AAQB reduced tumor growth and enhanced the FOLFOX protocol by inhibiting SOD2 and increasing hsa-miR-324-5p expression [103]. In addition, 4-AAQB also downregulated the LGR5/Wnt/ $\beta$ -catenin, JAK/STAT, and non-transmembrane receptor tyrosine kinase signaling pathways and stemness-related factors in CRC cell lines. A reduction in tumor size was also observed after 4-AAQB treatment in CRC animal models [104]. These results suggest that 4-AAQB is useful to reduce the stemness properties of CRC cells and may improve the effect of conventional chemotherapeutics such as the FOLFOX protocol.

#### *Diallyl trisulfide*

Diallyl trisulfide (DATS) is a garlic-derived organosulfur compound with anti-tumor properties. Zhang et al. [105] reported that DATS reduced the size and number of

spheroid forms of the CRC SW480 and DLD-1 cell lines and decreased the expression of CD133, CD44, ALDH1, OCT-4 and NANOG. The characteristics of cell death by apoptosis were found to be increased, including decreased Bcl-2 levels and increased BAX, caspase-3, -8 and -9. This effect was associated with the suppression of the activity of the Wnt/ $\beta$ -catenin pathway, observed by the increased expression of GSK-3 $\beta$  and decreased expression of  $\beta$ -catenin, MYC and cyclin D1. Pretreatment of the culture with lithium chloride blocked the effects of DATS. These results indicate that DATS can inhibit proliferation and induce apoptosis of CRC-SCs by suppressing Wnt signaling.

### Zerumbone

Zerumbone, a sesquiterpene from *Zingiber zerumbone*, has anti-tumor effects in various types of cancer. In the CRC HCT-116 and SW-48 cell lines, zerumbone suppressed epithelial-mesenchymal transition (EMT), as observed by the increased expression of E-cadherin and reduced expression of vimentin, ZEB1 and N-cadherin [106]. A reduction in cell invasion, migration and spheroid formation was also found, as well as in the expression of CSC markers, including CD133, CD44, BMI1 and ALDH1. Zerumbone also reduced  $\beta$ -catenin expression. Interestingly, silencing of miR-200c inhibited these zerumbone effects, indicating that the inhibition of the  $\beta$ -catenin pathway through miR-200c may be a target of the anti-CRC-SC properties of zerumbone [106].

## 2.1.2 | Small synthetic molecules

### Retinoids

Homeobox A5 (HOXA5), a regulator of gene expression, morphogenesis, and differentiation, is downregulated in CRC. It inhibits Wnt signaling to reinforce differentiation through antagonists of APC downregulated 1 (APCDD1), CXXC finger protein 4 (CXXC4), and NKD inhibitor of Wnt signaling pathway 1 (NKD1) pathways [58]. All-*trans* retinoic acid and 9-*cis* retinoic acid upregulated HOXA5 levels, leading to tumor regression in the ALDH1<sup>+</sup> HT-29 cell line. In this context, tumor regression via HOXA5 induction by retinoids suppressed Wnt signaling, leading to the differentiation and elimination of CRC-SCs, preventing tumor development, and inhibiting metastases [58]. All-*trans* retinoic acid also eliminated CSCs of head and neck cancer by inhibiting the Wnt/ $\beta$ -catenin pathway [107].

### JIB-04

JIB-04 is an epigenetic modulator that acts as a pan-selective inhibitor of histone demethylases [108]. JIB-04 decreased the viability of HCT116 cells and reduced CD133

mRNA expression, CD133 protein expression levels, and clonogenic potential, indicating that JIB-04 resulted in a reduction in the CRC-SC population and self-renewal to form colonies [109]. Beyond that, JIB-04 mitigated invasion, migration, and growth/recurrence in an in vitro model. The expression level of E-cadherin mRNA (an epithelial marker) was increased, while those of vimentin and N-cadherin (mesenchymal markers) were decreased in cells treated with JIB-04. It also reduced the tumorigenic activity of CRC-SCs in vivo. These data were associated with increases in the expression of *AXIN1* (axin-1) and *GSK3 $\beta$* , genes involved in the inhibition of  $\beta$ -catenin signaling, and decreases in the expression of  $\beta$ -catenin target genes, including *ALDH1* and SRY-box transcription factor 4 (*SOX4*) [109]. Importantly, JIB-04 had less effect on the viability of normal cells [110]. These results suggest that JIB-04 can be used to specifically target CRC-SCs.

### IC-2

IC-2, a derivative of ICG-001 (a Wnt modulator), efficiently represses Wnt/ $\beta$ -catenin signaling [111]. Interestingly, IC-2 reduced the transcriptional activity of Wnt/ $\beta$ -catenin in CRC cells more effectively than 5-FU. IC-2 reduced the expression levels of CSC marker proteins, such as CD44, CD133, OCT3/4, NANOG, and LGR5. Then, CD44<sup>high</sup> and CD44<sup>low</sup> DLD-1 cells were isolated by cell sorting, and CD44<sup>high</sup> sphere numbers were selectively reduced by IC-2, indicating that IC-2 preferentially suppresses CRC-SCs. Nevertheless, IC-2 did not affect viability at concentrations below 10  $\mu$ mol/L. IC-2 also improved the cytotoxic effect of 5-FU, suggesting that IC-2 sensitizes CRC cells to 5-FU through the suppression of CRC-SCs [111]. Interestingly, IC-2 has also been reported as an anti-CSC agent against liver [112] and oral cancers [113].

### NCB-0846

NCB-0846 is the first TRAF2- and NCK-interacting kinase (TNIK) inhibitor available orally with high activity against Wnt signaling by binding to TNIK, an essential activator of Wnt target genes. The anti-tumor and anti-CRC-SC activities of NCB-0846 were demonstrated [57,114]. Pharmaceutical companies have developed other TNIK inhibitors that are in clinical trials, including ON108600 (Onconova Therapeutics, Newtown, PA, USA), which was efficient in acting on CSCs [115]. Mebendazole, an anthelmintic agent, has also been identified as a selective TNIK inhibitor [116]. The combination of mebendazole and sulindac, a non-steroidal anti-inflammatory agent, reduced tumor initiation in CRC in a preclinical model [117].

### 36-077

36-077, an inhibitor of phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3), was synthesized by Kumar

et al. [118] as a potent autophagy inhibitor. In particular, 36-077 improved the efficacy of 5-FU in CRC Caco-2 and HCT116 cell lines grown in monolayers. Treatment of Caco-2 cells growing in spheroids or colon tumors derived from *Apc<sup>Min/+</sup>* mice with 5-FU plus 36-077 also reduced spheroid growth more than 5-FU or 36-077 alone. This effect was associated with the inhibition of the CSC population through GSK-3 $\beta$ /Wnt/ $\beta$ -catenin signaling. These data indicate that 36-077 can inhibit CRC-SCs by suppressing Wnt/ $\beta$ -catenin signaling and enhances 5-FU treatment.

### *CBB1003*

CBB1003, a lysine-specific demethylase 1 (LSD1) inhibitor, has anti-tumor properties. Treatment with CBB1003 in HCT116 cells inhibited proliferation and colony formation and decreased the levels of the CSC marker LGR5 [119]. Moreover, the overexpression of LGR5 reduced cell death caused by CBB1003. Inactivation of  $\beta$ -catenin/TCF signaling was observed after treatment with CBB1003. The expression of LSD1, LGR5,  $\beta$ -catenin and MYC was found to be lower in adjacent normal tissues than in CRC tissues as well as in CRC cell lines [119]. These data indicate LSD1 as a target to affect CRC-SCs, inhibiting Wnt signaling. CBB1003 may be a useful anti-CRC-SC drug, acting by inhibiting LSD1.

### *Rimonabant*

Rimonabant is an antagonist/inverse agonist of cannabinoid receptor 1 that can induce  $\beta$ -catenin degradation in CRC cell lines. The target genes Wnt/ $\beta$ -catenin, cyclin D1, MYC and cyclooxygenase-2 were reduced after treatment with rimonabant [120]. Non-canonical Wnt signaling was activated in CRC cells treated with rimonabant, as observed by the induction of Wnt5A and activation of calcium/calmodulin-dependent protein kinase II. Rimonabant also inhibited tumor growth and  $\beta$ -catenin transfer to cell nuclei in mice xenografted with HCT116 cells [120]. Fiore et al. [121] reported that rimonabant reduced primary CRC-SCs in culture through downregulation of  $\beta$ -catenin without affecting healthy colon epithelial cells. These results suggest rimonabant as an effective drug to eliminate CRC-SCs.

### *FH535*

FH535 is a dual inhibitor of  $\beta$ -catenin/TCF/LEF and PPARs with anti-tumor action [122]. It reduced the migration and invasion of SW480 cells and downregulated the expression of matrix metalloproteinases 7 and 9, vimentin, and snail. FH535 also inhibited the expression of the CSC markers CD24, CD44 and CD133 in HT29 cells by inhibiting the Wnt/ $\beta$ -catenin pathway. FH535 also reduced tumor growth in mice xenografted with HT-29 [123]. This suggests that

FH535 is able to eradicate CRC-SCs by targeting Wnt signaling.

## 2.1.3 | Monoclonal antibody

### *HZ8CV2*

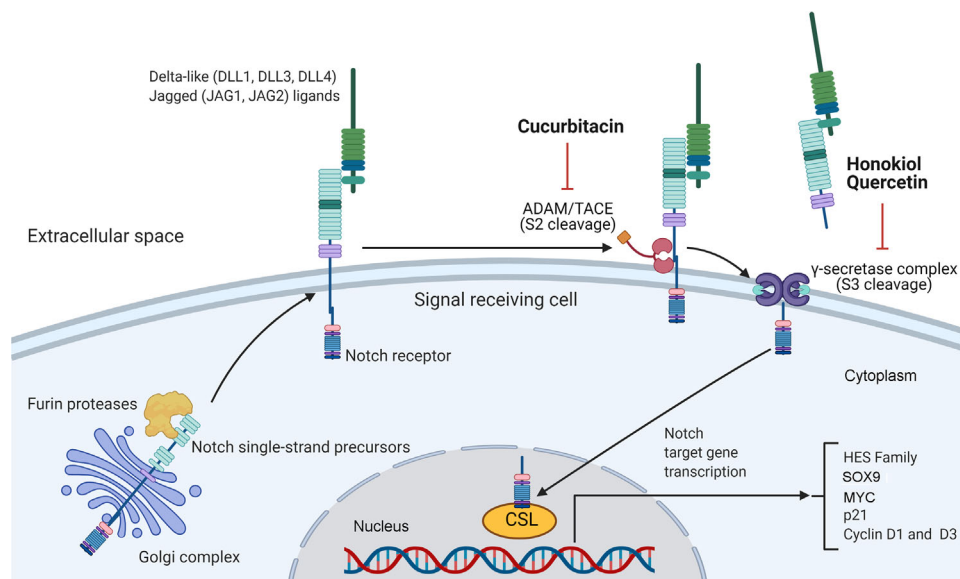
Progastrin is overexpressed and detectable in the supernatant of CRC cell lines and patient-derived CRC cells and stimulates the survival of CSCs [124]. Downregulation of progastrin decreases Wnt/ $\beta$ -catenin activity and Wnt-induced tumorigenesis [125]. Prieur et al. [126] created a humanized anti-progastrin monoclonal antibody (HZ8CV2) that was effective against CRC with a KRAS mutation. Treatment of SW480 cells with HZ8CV2 for 96 h decreased the expression of survivin, a key component of the Wnt/ $\beta$ -catenin signaling pathway, by 40.7%. Beyond that, combination chemotherapy with the HZ8CV2 antibody increased chemosensitivity and delayed recurrence. HZ8CV2 in combination with 5-FU decreased 57.9% of the CSC population in KRAS-mutated T84 cells. In the xenograft model, the combination of HZ8CV2 with irinotecan decreased the frequency of CSCs by 55.9% and 70.1% in T84 and SW620 tumors, respectively, inhibiting the migratory and invasive properties of these CRC cells. These data corroborate that progastrin is a target to eliminate CRC-SCs and that HZ8CV2 is an effective anti-progastrin monoclonal antibody that inhibits CRC-SCs and improves 5-FU and irinotecan treatments.

These data indicate that the Wnt/ $\beta$ -catenin signaling pathway is a critical pathway to eradicate CRC-SCs. On the other hand, crosstalk among pathways can lead to resistance to single-pathway inhibitors and maintenance of the CSC phenotype [127,128]. Therefore, multiple cellular pathways must be targeted to effectively eradicate CRC-SCs.

## 2.2 | Notch signaling pathway

Notch signaling is involved in the regulation of cell differentiation, proliferation, and tumorigenesis [129,130]. Notch is a transmembrane receptor activated by series cleavage (Figure 2), and four different Notch receptors (Notch1, Notch2, Notch3, and Notch4) are known. While the non-canonical pathway works independently of Notch receptors, the interaction between the ligands of the delta family (DLL1, DLL2, DLL3 and DLL4) or jagged-4 family (JAG1/JAG2) and the receptors occurs in canonical pathway activation. Canonical Notch single-strand precursors are cleaved by furin proteases in the Golgi complex to form a large fragment containing the extracellular domain and a small fragment containing transmembrane and intracellular regions, which combine to form a mature





**FIGURE 2** The Notch signaling pathway. The Notch single-stranded pathways undergo proteolytic processing in the Golgi complex, which is furin protease-mediated (S1 cleavage). The receptor is transported to the cell surface membrane. The extracellular domain of the Notch receptor in the signaling cell binds to the Notch ligands (Delta and Jagged) expressed by the adjacent cell. This induces the second proteolytic step by ADAM/TACE metalloproteases and  $\gamma$ -secretase, which releases the intracellular Notch domain that translocates to the nucleus and then binds to the CSL transcription factor and activates the expression of Notch target genes. Abbreviations: ADAM, a disintegrin and metallopeptidase; CSL, CBF1 suppressor of hairless LAG1; MYC, MYC proto-oncogene, bHLH transcription factor; SOX9, SRY-box transcription factor 9; TACE, tumor necrosis factor- $\alpha$  converting enzyme

receptor. The results of this interaction expose the extracellular metalloprotease site that becomes susceptible to cleavage by a disintegrin and metallopeptidase/tumor necrosis factor- $\alpha$  converting enzyme (ADAM/TACE) transmembrane family to release the extracellular fragment. The residual transmembrane Notch fragment acts as a substrate for proteolysis by the  $\gamma$ -secretase complex, resulting in the release of the intracellular domain of the receptor that translocates to the nucleus and then binds to the CBF1 suppressor of hairless LAG1 (CSL) transcription factor and activates the expression of Notch target genes [129–131].

The activation of Notch signaling was associated with short survival, CSC phenotype, and EMT, resulting in tumor progression in CRC [132]. Huang et al. [133] demonstrated that the CRC cell line HCT116 was resistant to 5-FU or oxaliplatin and that parental HCT116 cells cultured in spheroids showed a greater number of CD133<sup>+</sup> and CD44<sup>+</sup> cells than parental HCT116 cells cultivated in monolayers. These effects were associated with enhanced Notch signaling. Moreover, inhibition of the Notch pathway reduced the growth of HCT116 cells resistant to 5-FU or oxaliplatin and parental HCT116 cells cultured in spheroids in vitro and in vivo models. In this context, several compounds that act on CRC-SCs by inhibiting the Notch signaling pathway have been studied and point to promising strategies for the treatment of CRC.

### 2.2.1 | Natural products

#### *Honokiol*

Honokiol, a naturally active biphenolic compound from *Magnolia species* used in traditional Chinese medicine, has anti-tumor properties against various types of cancer [134]. Honokiol is a potent inhibitor of melanoma stem cells that suppresses Notch signaling [135]. It also inhibits tumor progression and stem cells in glioma [136], breast cancer [137], and oral carcinoma cells [138]. In CRC, the combination of honokiol with ionizing radiation (IR) inhibited the levels of expression of CSC proteins, such as doublecortin-like kinase-1 (DCLK1), SRY-box transcription factor 9 (SOX9), CD133, CD44, Notch signaling-related proteins, and proteins of the  $\gamma$ -secretase complex in vitro, using HCT116 and SW480 cell lines, and in xenograft tissues using the HCT116 cell line [139]. Honokiol also slowed the growth of the tumor xenograft, inhibited proliferation, and promoted apoptosis. Furthermore, ectopic expression of the Notch intracellular domain partially rescues the honokiol effect, indicating that this compound is an anti-CRC-SC agent targeting Notch signaling [139].

Honokiol also suppressed the Hippo signaling pathway in CRC cells [140]. In this pathway, YAP1 is translocated to the nucleus to induce gene expression, which is inhibited by phosphorylation at Ser127. Honokiol inhibited YAP1 phosphorylation and caused apoptosis in HCT116

and SW480 cells. Inhibition of the expression of the CSC marker DCLK1 was also observed. In addition, honokiol prevented colitis-associated cancer growth in a mouse model using azoxymethane and dextran sulfate sodium [140].

#### *Cucurbitacins*

Cucurbitacin-B (C-B) and cucurbitacin-I (C-I) are phytochemicals present in various fruits and vegetables, such as bitter melon [141]. C-B and C-I inhibited cell proliferation and induced cell cycle arrest and apoptosis in CRC HCT116 and SW480 cell lines by downregulating ADAM9, which participates in the cleavage of the Notch receptor at the extracellular domain [142]. In addition, C-B and C-I reduced the spheroid and colony formation of CRC cells and inhibited the expression of the CRC-SC markers CD44, DCLK1, and LGR5. C-B and C-I also reduced tumor growth in mice xenografted with HCT116, as such reduced the expression of CSC markers and Notch signaling components in tumor tissues [142]. Together, these data suggest that cucurbitacins inhibit CRC, including CRC-SCs, by downregulating the Notch signaling pathway. Additionally, C-B was also able to eliminate liver [143] and gastric CSCs [144]. C-I also eliminated CSCs of the lung [145], brain [146], and head and neck [147] by blocking the JAK2/STAT3 signaling pathway.

#### *Evodiamine*

Evodiamine, a derivative of the traditional herbal medicine *Evodia rutaecarpa*, has an antiproliferative effect in the CRC LoVo cell line, leading to the induction of caspase-dependent apoptosis and S-phase arrest [148,149]. The surviving cells after treatment with evodiamine were plated using serial dilutions and under conditions for the enrichment of CSCs by spheroids or implanted subcutaneously in immunodeficient SCID mice [150]. The results showed that evodiamine decreased the self-renewal of surviving cells in vitro and in vivo. Beyond that, the expression of the genes related to Wnt/ $\beta$ -catenin and Notch signaling was suppressed by treatment with evodiamine, indicating the elimination of CRC-SCs [150]. This indicates that evodiamine is an effective drug to eradicate CRC-SCs, suppressing Wnt and Notch signaling.

#### *Quercetin*

Quercetin, a natural flavonoid, is a compound that enhances radiosensitivity [151]. Quercetin plus IR eliminated CRC-SCs by inhibiting Notch1 signaling. Reductions in CSC markers (DCLK1, CD24, LGR5, CD29, CD44, and CD133) were observed in in vitro and in vivo studies. Quercetin plus IR suppressed the growth of both primary and secondary colonospheres and inhibited the Notch signaling pathway by downregulating  $\gamma$ -

secretase complex proteins, Jagged-1, and Notch target genes hairy and enhancer of split-1 (*HES1*) and Hes-related with YRPW motif-1 (*HEY1*) [151]. This indicates that quercetin can induce radiosensitivity and eliminate CRC-SCs.

#### *Portulaca oleracea extract*

*Portulaca oleracea* is a grassy plant with succulent leaves and presents anti-tumor activities [152]. *Portulaca oleracea* extract inhibited the proliferation of CSCs (CD133<sup>+</sup> and CD44<sup>+</sup>) by inducing apoptosis and downregulating the expression of Notch1 and  $\beta$ -catenin in CRC-SCs [153]. This indicates that *Portulaca oleracea* extract is a source of anti-CRC-SC agents.

#### *$\alpha$ -Mangostine*

$\alpha$ -Mangostine is a natural xanthone from *Garcinia mangostana*. It has antioxidant, anti-microbial, anti-cancer and anti-inflammatory properties [154].  $\alpha$ -Mangostine encapsulated in poly(D,L-lactic-coglycolic acid) (PLGA) nanoparticles inhibited self-renewal capacity, the expression of CSC markers, including CD133, CD44, Musashi, and LGR5, and pluripotency maintenance factors, such as OCT4, SOX2, KLF4, MYC, and NANOG. These effects were associated with inhibition of Notch signaling, suppression of the expression of Notch receptors (Notch1 and Notch2), their ligands (Jagged 1 and DLL4), protein of the  $\gamma$ -secretase complex (Nicastrin), and downstream target (Hes-1) in CRC HT-29 and HCT116 cell lines [155]. These results indicate  $\alpha$ -mangostine as a drug with anti-CRC-SC properties.

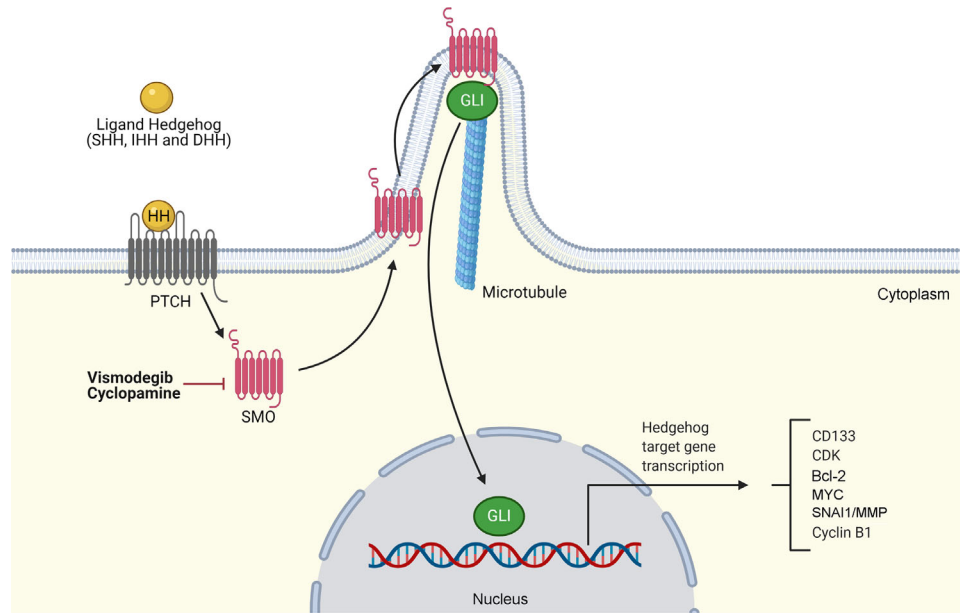
#### *Pien Tze Huang formula*

Pien Tze Huang is a traditional Chinese herbal formula with anti-tumor properties. It inhibited CRC-SCs in the SW480 cell line [156]. In addition, Pien Tze Huang formula also decreased Notch1 and Hes1 expression in CRC-SCs [156]. This indicates that Pien Tze Huang formula is able to eliminate CRC-SCs by suppressing the Notch signaling pathway.

## 2.2.2 | Monoclonal Antibodies

#### *Anti-DLL4 antibodies*

Anti-human and anti-mouse DLL4 antibodies have been developed as anti-tumor agents [157]. Moreover, anti-DLL4 antibodies were effective against both KRAS wild-type and mutant CRC cells. In combination with irinotecan, anti-DLL4 antibodies also increased CRC growth inhibition in a patient-derived xenograft model. They were also found to reduce the CRC-SC population [158]. These data indicate that these antibodies against the Notch



**FIGURE 3** The Hedgehog signaling pathway. In canonical HH signaling, HH ligands (SHH, IHH or DHH) bind to the PTCH transmembrane receptor, which relieves the inhibition of the transmembrane protein SMO and induces the GLI family of transcription factors (GLI1, GLI2, and GLI3) to enter the nucleus to regulate downstream gene transcription. Abbreviations: CDK, cyclin-dependent kinase; DHH, desert hedgehog; HH, hedgehog; IHH, Indian hedgehog; MMP, matrix metalloproteinase; MYC, MYC proto-oncogene, bHLH transcription factor; PTCH, patched; SHH, sonic hedgehog; SMO, smoothened; SNAIL, snail family transcriptional repressor 1

DDL4 ligand are effective in eradicating CRC-SCs and in potentiating the effects of irinotecan. Notch signaling can interact and influence a large number of cancer-relevant pathways, including Wnt. Furthermore, crosstalk between the Wnt and Hedgehog pathways can also determine the general effect of Notch signaling [159,160]. In this context, all cross-signaling networks in CSCs must be considered in a context-dependent manner. Clinical trials with Notch pathway inhibitors are still limited in the treatment of CRC. As observed above, these targeted agents have already been shown to be effective in preclinical studies and should also be tested in combination with classical chemotherapy drugs or other inhibitors of the signaling pathway in clinical trials to better understand their role in CRC therapy.

### 2.3 | Hedgehog signaling pathway

The Hedgehog (HH) signaling pathway is related to embryonic development, self-renewal, migration, and stem cell population maintenance. Hyperactivation of this pathway is associated with CSCs, contributing to tumorigenesis and tumor progression. Conserved HH transmits the signal from the cell membrane to the nucleus [161,162]. Abnormal dysfunction or activation of HH signaling has been implicated in the pathogenesis of several cancers, including CRC [163].

HH signaling can be classified as canonical or non-canonical. In canonical HH signaling, HH ligands (sonic HH, Indian HH, or desert HH) bind to the patched (PTCH) transmembrane receptor, which leads to the release of smoothened (SMO) and consequent activation of a downstream cascade [161,164]. This event culminates in the activation of the GLI family of transcription factors (GLI1, GLI2, and GLI3), which is responsible for the regulation of several target genes. In the absence of ligands, the PTCH receptor inhibits SMO, deactivating the pathway (Figure 3) [161,164]. Non-canonical HH signaling refers to all cellular and tissue responses to any of the HH isoforms that are independent of transcriptional changes mediated by GLI activation [165].

The crosstalk between Wnt and HH signaling has been shown to be important in the development and progression of CRC. Some studies report that GLI-dependent canonical HH signaling is a negative regulator of Wnt signaling in normal intestine and intestinal cancers [166,167]. On the other hand, non-canonical HH signaling in CSCs acts as a positive regulator of Wnt signaling to regulate the survival of CRC-SCs [168]. HH signaling in CRC-SCs is autocrine and sonic Hedgehog- and PTCH1-dependent [168].

In primary cultures and CRC cell lines, HH signaling is critical for *in vitro* cell proliferation and survival, since CRC-SCs require active HH signaling for self-renewal and expansion in advanced cancers [169]. HH signaling is also necessary for the growth of CRC xenografts, recurrence,

and induction of EMT necessary for metastases [169]. In contrast, arsenic trioxide, an anti-leukemic drug known to inhibit the HH signaling pathway by targeting GLI1 [170], increased CRC-SCs from patient samples [171].

### 2.3.1 | Natural product

#### *Cyclopamine*

Cyclopamine, a naturally occurring alkaloid, is a classic inhibitor of the HH signaling pathway through the inhibition of SMO [172]. HCT-116 cells grown in serum-free non-adherent spheroids were treated with cyclopamine and showed reduced mRNA levels of CSC markers and genes related to HH signaling, including *PTCH1*, *SMO*, and *GLI1* [173].

Zhou et al. [174], using bioinformatics analysis, selected a series of non-coding RNAs related to stemness in CRC-SCs and identified long non-coding RNAs (lncRNA-cCSC1) that are highly expressed in CRC and CRC-SCs and indicated a poor prognosis. After characterization, they observed that the depletion of lncRNA-cCSC1 levels was proportional to the increased sensitivity of CRC cells to 5-FU and to the inhibition of CRC-SCs' self-renewal capacity via the HH pathway. The overexpression of lncRNA-cCSC1 increased the protein levels of factors related to stemness, such as CD133, CD44, and NANOG, and effector proteins of the HH signaling pathway, SMO and GLI1. In contrast, the use of cyclopamine caused a reduction in the levels of biomarkers related to stemness. These data indicate the potential action of the anti-SMO drug cyclopamine in eliminating CRC-SCs.

### 2.3.2 | Small synthetic molecule

#### *Vismodegib*

Vismodegib (also known as GDC-0449) is a potent, orally bioavailable, specific SMO receptor antagonist. It is used to treat basal cell skin cancer in clinical practice approved by the United States-Food and Drug Administration [175]. Although still controversial, vismodegib has been reported as a drug able to inhibit CRC-SCs, since Wu et al. [176] showed that vismodegib inhibited cell proliferation and triggered apoptotic cell death with downregulation of Bcl-2 in CRC cells. In addition, it reduced the expression of the CRC-SC markers CD44 and ALDH by approximately 70%.

On the other hand, Chen et al. [177] demonstrated that vismodegib did not activate apoptosis in human CRC cells. Moreover, in a clinical trial of 199 patients with CRC treated with the FOLFOX or FOLFIRI (5-FU, leucovorin plus irinotecan) protocol combined with vismodegib, no benefit associated with the combination was observed [178].

Although some negative results have been found with HH signaling inhibitors, new inhibitors must be evaluated in CRC-SCs, especially compounds capable of inhibiting non-canonical HH signaling. These new data can help understand the potential of HH signaling inhibitors in CRC therapy.

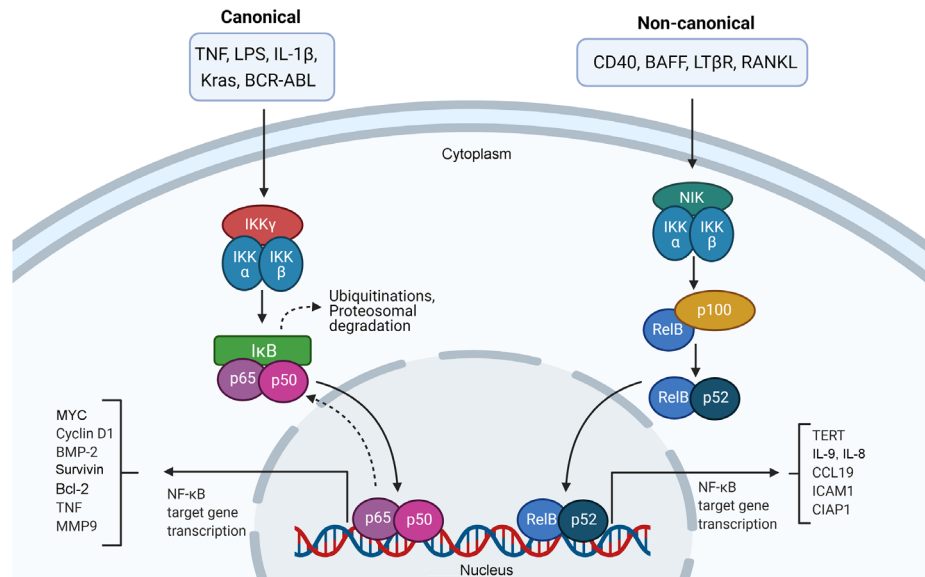
### 2.4 | NF- $\kappa$ B signaling pathway

NF- $\kappa$ B is a transcription factor that plays a crucial regulatory role in the innate immune system and in the inflammatory response. NF- $\kappa$ B signaling occurs canonically or non-canonically. In the canonical pathway, an inhibitor of NF- $\kappa$ B (I $\kappa$ B) prevents the translocation of the p65/p50 and c-Rel/p50 dimers to the cell nucleus [179,180]. The ubiquitination of I $\kappa$ B and its subsequent degradation release these proteins to be translocated to the cell nucleus, leading to the activation of the target genes. In the non-canonical pathway, NF- $\kappa$ B-inducing kinase (NIK) induces the ubiquitination of p100 and its subsequent processing by proteasomes in p52. Then, RelB/p52 is translocated to the cell nucleus to activate the target genes (Figure 4) [179,180].

Several studies have shown that the NF- $\kappa$ B pathway is largely associated with the biology of cancer and is upregulated in most hematological malignancies and solid cancers [181,182]. The NF- $\kappa$ B pathway can be activated in the loss of tumor suppressor genes, such as tumor protein p53 (*TP53*), von Hippel-Lindau tumor suppressor (*VHL*), and phosphatase and tensin homolog (*PTEN*), and by the expression of some oncogenes, such as the rat sarcoma virus (*RAS*) family and BCR activator of RhoGEF and GTPase (*BCR*)/ABL proto-oncogene 1, non-receptor tyrosine kinase (*ABL*). Moreover, when NF- $\kappa$ B is inhibited in cancer cells, apoptosis is increased, corroborating the pro-survival and anti-apoptotic role of NF- $\kappa$ B in cancer cells [182–185].

The activity of NF- $\kappa$ B subunits arises from a prolonged chronic inflammatory microenvironment or from various oncogenic mutations [186]. In the tumor microenvironment, vascular disorganization causes tissue damage due to hypoxia, and inflammatory pathways are activated with increased NF- $\kappa$ B activity [187]. Increased NF- $\kappa$ B activity promotes the tumorigenic microenvironment of CRC [187].

The NF- $\kappa$ B pathway participates in several processes in CRC-SCs, such as stimulation of cell proliferation, prevention of apoptosis, EMT, angiogenesis, invasiveness, and metastasis [186]. The activation of NF- $\kappa$ B can regulate the maintenance of CRC-SCs by reducing the expression of miR-195-5p and miR-497-5p [188]. The elevation of miR-195-5p and miR-497-5p levels by a specific mimic inhibited the effects of NF- $\kappa$ B on CRC-SCs in vitro and in vivo. Minichromosome maintenance complex component 2 (*MCM2*) has been identified as the target gene for



**FIGURE 4** The NF- $\kappa$ B signaling pathway. In the canonical pathway, the inhibitory protein I $\kappa$ B inhibits the translocation of the p65/p50 and c-Rel/p52 dimers to the cell nucleus. The ubiquitination of I $\kappa$ B and its subsequent degradation release these proteins to be translocated to the cell nucleus, leading to the activation of the target genes. In the non-canonical pathway, NIK induces the ubiquitination of p100 and its subsequent processing by proteasomes in p52. Then, RelB/p52 is translocated to the cell nucleus to activate the target genes. Abbreviations: ABL, ABL proto-oncogene 1, non-receptor tyrosine kinase; BAFF, B cell-activating factor; BCR, BCR activator of RhoGEF and GTPase; BMP-2, bone morphogenetic protein-2; CCL19, C-C motif chemokine ligand 19; CIAP1, cellular inhibitor of apoptosis protein 1; ICAM1, intercellular adhesion molecule 1; IKK, inhibitor of NF- $\kappa$ B kinase; I $\kappa$ B, inhibitor of NF- $\kappa$ B; Kras, Kirsten rat sarcoma viral oncogene homolog; LPS, lipopolysaccharide; LT $\beta$ R, lymphotoxin beta receptor; MMP9, matrix metalloproteinase 9; MYC, MYC proto-oncogene, bHLH transcription factor; NF- $\kappa$ B, nuclear factor kappa B; NIK, NF- $\kappa$ B-inducing kinase; RANK, receptor activator of NF- $\kappa$ B; TERT, telomerase reverse transcriptase; TNF, tumor necrosis factor

miR-195-5p and miR-497-5p in CRC-SC culture. Overexpression of MCM2 restored the stem cell profile of CRC cells in the presence of miR-195-5p and miR-497-5p, suggesting that miR-195-5p and miR-497-5p may impair CRC-SCs by inhibiting MCM2 transcription [188].

Even with only a few data on the role of NF- $\kappa$ B in CRC-SCs, these data suggest promising potential for NF- $\kappa$ B inhibitors in CRC therapy.

## 2.5 | JAK/STAT signaling pathway

JAK/STAT signaling is a fundamental signaling pathway for the development of various types of cancer and is directly involved in growth, progression, and metastasis and indirectly involved in immune surveillance modulation [189]. Activation of this cell signaling occurs when the JAK protein is recruited and activated by cytokine receptors. Subsequently, JAK catalyzes the tyrosine phosphorylation of the receptor, allowing the recruitment of STAT proteins. After phosphorylation of STAT, its dimers are translocated to the cell nucleus to bind to DNA, resulting in the transcription of target genes (Figure 5) [190,191].

Activation of JAK/STAT signaling in cancer cells can occur by several mechanisms, the best known being

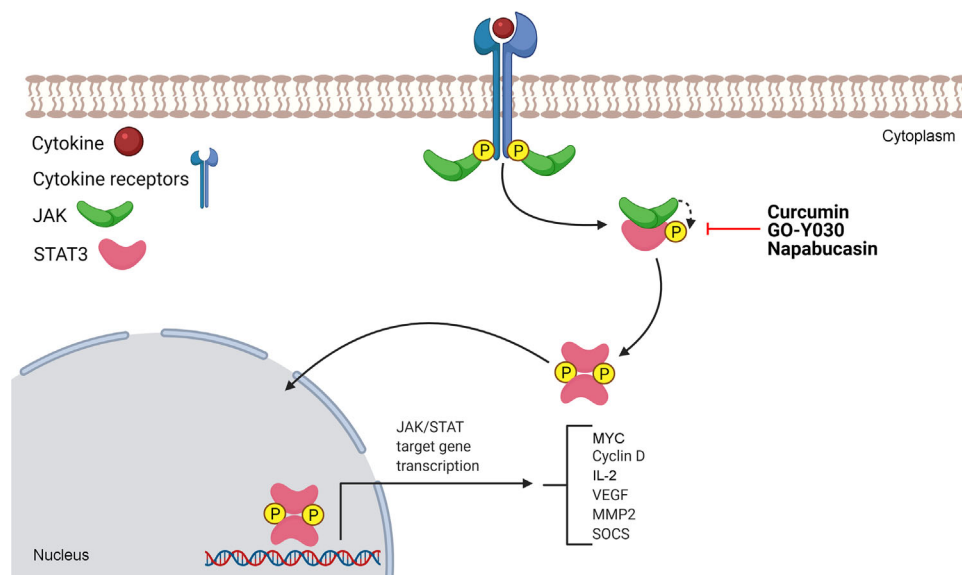
mutations in STAT3 or glycoprotein 130 (GP130, also known as IL6-beta), which promote activation independent of the STAT3 ligand in liver cancers, and the elevated expression of cytokines such as IL-6 [192]. In CSCs, this pathway induces increased tumorigenic capacity, metastasis, and chemoresistance in cancer via increased EMT [193].

High STAT3 activity was found in CRC-SCs and tumor-infiltrating lymphocytes but not in non-cancerous colon epithelia [194]. In addition, a relationship was observed between the JAK/STAT pathway and the tumor microenvironment. CRC-derived cells lost STAT3 activity in culture; however, when these CRC cells were implanted in mice, STAT3 activity was restored. Furthermore, blocking STAT3 activation in CRC-derived xenograft tumors reduced tumor development, corroborating the results that STAT3 participates directly in CRC growth [194].

### 2.5.1 | Natural products

#### *Curcumin and GO-Y030*

Curcumin is a polyphenol from *Curcuma longa*, and GO-Y030 is a novel curcumin analog. ALDH<sup>+</sup>/CD133<sup>+</sup>



**FIGURE 5** The JAK/STAT signaling pathway. JAK/STAT signaling starts with the interaction of cytokines or growth factors with their receptors, inducing the dimerization/oligomerization of these receptors and consequent activation. Activated JAKs autophosphorylate and phosphorylate their associated receptors. Therefore, cytoplasmic STATs bind to phosphorylated receptors and undergo homodimerization or heterodimerization after their phosphorylation, and they are able to translocate to the nucleus and activate the transcription of target genes. Abbreviations: JAK, Janus kinase; MMP2, matrix metalloproteinase 2; MYC, MYC proto-oncogene, bHLH transcription factor; SOCS, suppressors of cytokine signaling; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor

subpopulations from CRC express high levels of phosphorylated STAT3, which enhances the proliferation and survival of CRC-SC lines [195]. Interestingly, curcumin and GO-Y030 inhibited STAT3 phosphorylation and induced apoptosis, as observed by increased expression of cleaved PARP and cleaved caspase-3 in CRC-SCs. In addition, curcumin induced cell cycle arrest, inhibited cell viability, decreased the ability to form spheroids, and suppressed tumor growth in a xenograft model. The effects of GO-Y030 were more potent than those of curcumin [195]. These data indicate curcumin and its analog GO-Y030 as drug candidates to eliminate CRC-SCs by suppressing STAT3 activity.

## 2.5.2 | Small synthetic molecule

### *Napabucasin*

Napabucasin (also known as BBI608) is an orally administered STAT3 inhibitor with anti-cancer activity against various types of cancer. It killed CRC-SC SW480 cells isolated by Hoechst-33342 dye exclusion sorting [196]. In a phase I clinical trial with four patients with metastatic CRC, napabucasin (240 mg twice daily) plus FOLFIRI and bevacizumab showed a safety profile in Japanese patients with metastatic CRC [197]. All four patients had diarrhea, two patients reported decreased appetite, and decreased neutrophil counts were observed in three patients. Deaths

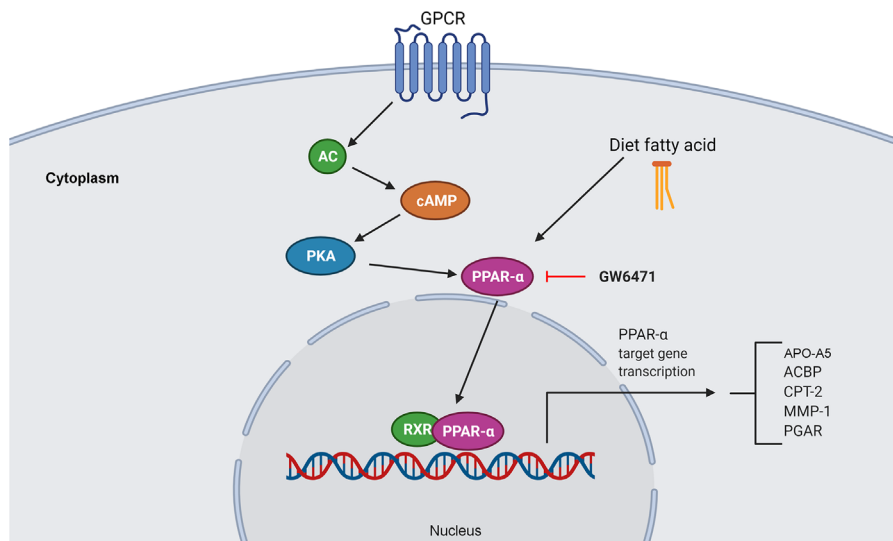
or serious adverse effects were not reported. A phase III clinical trial with patients with advanced p-STAT3<sup>+</sup> CRC treated with napabucasin showed a significant survival benefit compared to placebo (median overall survival of 5.1 vs. 3 months, hazard ratio = 0.41,  $P = 0.0025$ ) [198].

These preclinical and clinical data indicate that napabucasin is a promising anti-CSC drug in CRC therapy. It is the most advanced drug in development that targets cell signaling pathways to eradicate CRC-SCs and the only one with published results from phase III clinical trials. Napabucasin may be the first anti-CRC drug approved for clinical use targeting CSCs.

### *SC-43 and SC-78*

SC-43 and SC-78, STAT3 inhibitors that stimulate Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) to inactivate STAT3, suppressed the activation of STAT3 in CRC HCT-116 and HT-29 cell lines [199]. They also reduced the ability to form spheroids in an assay with cells grown on non-adherent spheroids without serum and supplemented with growth factors and to form colonies in a soft agar colony formation assay. A decrease in the CD133<sup>+</sup>/CD44<sup>+</sup> subpopulation was also observed. Moreover, they synergize with oxaliplatin and/or irinotecan to inhibit sphere formation of these cells.

These data corroborate that STAT3 inhibitors may be useful to improve anti-CRC therapy targeting CRC-SCs.



**FIGURE 6** The PPAR signaling pathway. This signaling pathway begins through the interaction of a ligand with a GPCR, which triggers a series of signal conductions passed by AC, cAMP, and PKA until culminating in the activation and translocation of PPAR $\alpha$  to the nucleus to modulate the expression of target genes. Abbreviations: AC, adenylyl cyclase; ACBP, acyl-CoA-binding protein; APO-A5, apolipoprotein A5; cAMP, cyclic adenosine monophosphate; CPT-2, carnitine palmitoyltransferase 2; GPCR, G protein-coupled receptor; MMP1, matrix metalloproteinase 1; PGAR, PPAR gamma angiopoietin-related gene; PKA protein kinase A; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor

## 2.6 | PPAR signaling pathway

PPARs are nuclear receptor proteins that, when activated, act as transcription factors and play a critical role in the regulation of some diseases, including cancer (Figure 6). This pathway is activated by the interaction of a ligand with a G protein-coupled receptor, which triggers a series of signal conductions, including adenylyl cyclase (AC), cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) proteins, until culminating in the activation and translocation of PPAR for the nucleus to modulate the expression of the target genes [200–202]. There are three variants with different functions for these receptors: PPAR $\alpha$ , PPAR $\delta$ , and PPAR $\gamma$ . In tumor development, PPARs are involved in the regulation of cell proliferation, survival, apoptosis, and tumor growth and can inhibit or promote tumor development [200–202]. PPARs are also involved in the initiation and regulation of CSC functions and in the modulation of the EMT process [203]. They also regulate lipid droplets, which are organelles that store neutral lipids as an energy source in some CSCs [204].

### 2.6.1 | Small synthetic molecule

#### GW6471

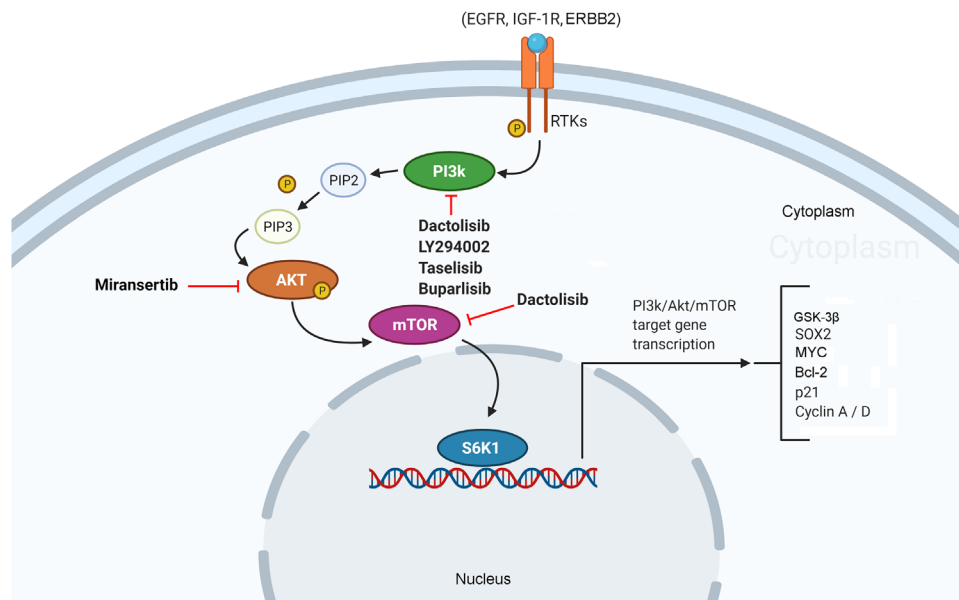
GW6471, a PPAR $\alpha$  antagonist, interacts directly with PPAR $\alpha$  activation function 2 (AF2) and prevents it from

assuming an active conformation [204]. A CRC study using SW620, HT-29, WiDr, and SW480 cell lines showed that the PPAR $\alpha$  pathway is active in CRC-SCs. Interestingly, GW6471 reduced the expression of CSC markers (SOX2, NANOG, and OCT4) and the ability of CRC cells to form spheroids [204]. These results suggest that GW6471 is a drug able to eliminate CRC-SCs. The relationship between the PPAR pathway and CRC-SCs is consistent, although more robust studies are needed to confirm the importance of this pathway in CRC-SCs.

## 2.7 | PI3K/Akt/mTOR signaling pathway

The PI3K/Akt/mTOR signaling pathway plays a key role in the cell cycle, metabolism, quiescence, and proliferation and is considered a master regulator of cancer [205–207]. The conversion of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2) into the membrane begins after activation of the growth factor receptor protein tyrosine kinase, providing coupling sites to signal proteins, such as Akt. In the final activation of Akt, the mTORC2 complex phosphorylates Akt at Ser473. Akt activates mTOR complex 1 (mTORC1), improving the synthesis of target proteins (Figure 7).

Some studies have shown the association between the PI3K/Akt/mTOR signaling pathway and the biology



**FIGURE 7** The PI3K/Akt/mTOR signaling pathway. In the pathway cascade, after growth factors bind to their RTKs, the PI3K signaling pathway is activated, converting PIP2 into PIP3 until Akt activation is reached. This process is downregulated by PTEN. Akt, in turn, stimulates mTOR to phosphorylate target proteins to modulate gene expression. Abbreviations: EGFR, epidermal growth factor receptor; ERBB2, erb-b2 receptor tyrosine kinase 2; GSK-3 $\beta$ , glycogen synthase kinase-3 beta; IGF-1R, insulin-like growth factor 1 receptor; mTOR, mechanistic target of rapamycin; MYC, MYC proto-oncogene, bHLH transcription factor; PI3K, phosphatidylinositol-3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog; RTKs, receptor tyrosine kinases; S6K1, ribosomal protein S6 kinase 1; SOX2, SRY-box transcription factor 2

of CRC-SCs [208]. Chen et al. [209] demonstrated that human CRC xenografts overexpressed various components of the PI3K/Akt/mTOR signaling pathway, including phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2, a PI3K regulatory subunit), in CRC-SCs. Through a collection of primary cell cultures growing on spheroids obtained from 60 CRC specimens, Mangiapane et al. [210] showed that the high expression of erb-b2 receptor tyrosine kinase 2 (ERBB2) in CRC-SCs is associated with the activation of the PI3K/Akt pathway, promoting acetylation in the regulatory elements of the ERBB2 gene. mTOR expression was also related to poor prognosis in patients with stage II CRC [211]. Moreover, a transcriptome of CRC-SC primary cultures and their normal stem cell counterparts revealed enrichment of genes central to PI3K/Akt and Wnt signaling in CRC-SCs [212].

### 2.7.1 | Natural products

#### *Piplartine/piperlongumine*

Piplartine, also known as piperlongumine, is an alkaloid amide isolated from peppers (the *Piperaceae* family). It presents multiple pharmacological activities, including cytotoxicity and in vivo anti-tumor effects against different types of cancers. The mechanism of action of piplartine has been associated with its capacity to induce oxida-

tive stress by inhibiting the antioxidant system, including depletion of glutathione [213–217]. In particular, piplartine inhibited the Ras/PI3K/Akt/mTOR pathway and reduced tumor cell growth in a dimethylhydrazine/dextran sulfate sodium-induced colon carcinogenesis animal model [218].

Auranofin is a clinically approved synthetic drug for the treatment of rheumatoid arthritis. Anticancer properties have been reported for this gold molecule, where its main mechanism of action is inhibition of cellular antioxidant enzymes, such as thioredoxin reductase, causing oxidative stress and cell death in cancer cells [219]. Auranofin demonstrated selective capacity to improve the inhibition of CT26 colon tumor growth in a mouse model of abdominal irradiation without enhancing radiation toxicity in the normal intestine [220]. Auranofin also sensitized HCT116 and SW620 cells to 5Z-7-oxozeaenol, an inhibitor of TGF- $\beta$ -activated kinase 1 [221].

Moreover, Tanaka et al. [222] demonstrated that piplartine plus auranofin decreased the expression level of the CD44v9 surface marker and reduced CRC growth in a patient-derived xenograft model. Piplartine also showed lower cytotoxicity to fibroblast cells than cancer cells growing in spheroids. This indicates that piplartine plus auranofin is able to eliminate CRC-SCs. Interestingly, piplartine also inhibited stemness properties in leukemia [223] and oral cancer [224] and had less effect on the viability of normal cells [213, 214].



### *Atractylenolide I*

Atractylenolide I (ATL-1) is a plant-based compound found in the rhizome of *Atractylodes macrocephala* used in traditional Chinese medicine. In a study using CRC COLO205 and HCT116 cell lines, ATL-1 treatment induced apoptosis, inhibited invasion, downregulated the Akt/mTOR pathway by reducing the phosphorylation of pathway-related proteins, and reduced the stem cell phenotype [225]. In an *in vivo* experiment, ATL-1 reduced tumor weight and reduced CRC-SCs. This suggests that ATL-1 can inhibit CRC-SCs *in vitro* and *in vivo*.

### *Rapamycin*

Rapamycin, a macrolide from *Streptomyces hygroscopicus*, is clinically used due to its immunosuppressant functions. It is a known mTOR inhibitor. Interestingly, rapamycin treatment decreased the spheroid-forming ability and ALDH1 activity in CRC Caco-2 and SW480 cell lines [211]. These effects were also observed in co-treatment with 5-FU and oxaliplatin. Importantly, 5-FU and oxaliplatin generally increased the CRC-SC subpopulation, but co-treatment with rapamycin reduced this cell subpopulation. These data indicate that rapamycin can inhibit CRC-SCs and potentiate the effect of 5-FU and oxaliplatin.

## 2.7.2 | Small synthetic molecules

### *Dactolisib*

Dactolisib (also known as BEZ235), a synthetic imidazoquinoline derivative, is a dual PI3K/mTOR inhibitor that selectively inhibits PI3K class I ( $p110\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ ), mTORC1, and mTORC2, inhibiting their catalytic activities and cell signaling [226]. Dactolisib suppressed the proliferation of CRC-SCs and reduced the expression of CD133 and LGR5 in HCT116 cells. In particular, insulin, a positive regulator of the PI3K/Akt/mTOR pathway, reduced the effect of treatment with dactolisib. This result suggests that dactolisib is a drug with the ability to eliminate CRC-SCs.

### *LY294002*

LY294002, a synthetic compound developed based on the flavonoid quercetin, acts as a PI3K inhibitor. Treatment with LY294002 resulted in decreased proliferation, spheroid formation, and self-renewal properties, together with reduced Akt phosphorylation and cyclin D1 expression in CRC-SCs *in vitro* [209]. In an *in vivo* experiment, LY294002 reduced tumorigenicity, increased the detection of cleaved caspase 3, and increased the expression of the inflammatory chemokine IL-8. These data indicate that LY294002 is capable of eliminating CRC-SCs in preclinical models.

### *Taselisib, miransertib, and buparlisib*

Taselisib, an inhibitor of PI3K class I, selectively inhibits phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) and its mutant forms in the PI3K/Akt/mTOR pathway. Treatment of CRC-SCs from 60 samples from CRC patients with trastuzumab (an ERBB2 inhibitor), dabrafenib (a MEK inhibitor) or taselisib induced *in vitro* cell death and regression of tumor xenografts, including those with KRAS and PIK3CA mutations [210]. Furthermore, taselisib alone was also able to kill CRC-SCs and inhibited the formation of liver metastases in immunocompromised mice after injection of CRC sphere cells. Buparlisib (also known as BKM120), another PI3K inhibitor, and miransertib, an Akt inhibitor, also reduced the viability of CD44v6<sup>+</sup> cells *in vitro* [210].

In a phase Ib clinical trial in patients with KRAS wild-type advanced CRC, buparlisib (60 mg given 5 out of 7 days per week) plus panitumumab (6 mg/kg injected intravenously every 2 weeks) was recommended for a phase II clinical trial [227].

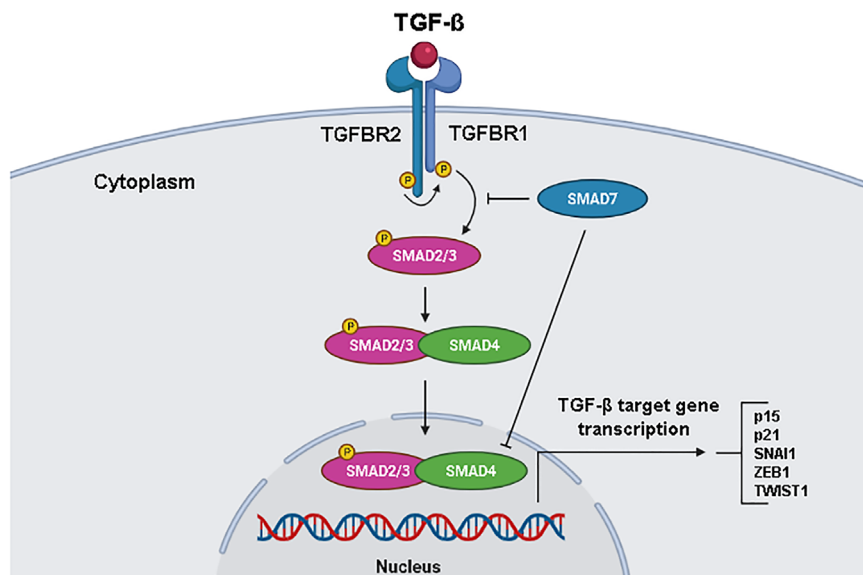
### *Metformin*

Metformin, an oral anti-diabetic drug, has also been reported as a cytotoxic agent with the ability to reduce the CSC population in different types of cancer [228–230]. In both flow cytometric analysis (CD44<sup>+</sup>/CD133<sup>+</sup>) and tumor spheroid growth assays, CSCs decreased after treatment with metformin in CRC HT-29 and DLD-1 cell lines [231]. Augmentation of the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and reduction of mTOR (p-S6) were also observed after treatment with metformin. In a xenograft mouse model using HT-29 cells, tumor growth and CSC populations were decreased by metformin administration. Metformin effects were prevented by mevalonate treatment [231]. Interestingly, metformin also reduced CSCs in patients with colorectal and other gastrointestinal cancers in a pilot clinical trial [232].

These preclinical and clinical results indicate metformin as a promising anti-CRC-SC drug, but more clinical data are needed to use it in CRC therapy.

### *Torin 1, Torkinib and MK-2206*

Torin 1, a selective inhibitor of mTOR, has anti-tumor properties. Torin 1 was able to induce cell death in CRC-SCs *in vitro* and *in vivo* without affecting the proliferation of normal colon stem cells *in vivo* [233]. Torkinib (PP242) is an ATP-competitive second-generation inhibitor of mTOR. It reduced CRC-SCs in *in vitro* models when it was used alone or in combination with 5-FU and oxaliplatin [211]. MK-2206 is an allosteric Akt inhibitor. It inhibited CRC-SCs, as observed by the reduction of the capacity to



**FIGURE 8** The TGF- $\beta$ /Smad signaling pathway. During activation, TGF- $\beta$  ligands bind to TGFBR2, which phosphorylates TGFBR1. TGFBR1 then phosphorylates receptor-regulated Smads (Smad2/3) that bind to Smad4. This complex is translocated to the cell nucleus and acts as a transcription factor to regulate target gene expression. Abbreviations: SNAI1, snail family transcriptional repressor 1; TGFBR1, TGF- $\beta$  receptor type 1; TGFBR2, TGF- $\beta$  receptor type 2; TGF- $\beta$ , transforming growth factor- $\beta$ ; TWIST1, twist family BHLH transcription factor 1; ZEB1, zinc finger e-box binding homeobox 1

form spheres in vitro and to initiate tumor formation in vivo using the CRC SW480 cell line [212]. MK-2206 was also effective in reducing patient-derived spheroid formation.

These data indicate the importance of the PI3K/Akt/mTOR pathway in the control of CRC-SCs, showing that this pathway may be a target for therapeutic agents and can improve CRC therapy in the future.

## 2.8 | TGF- $\beta$ /Smad signaling pathway

The TGF- $\beta$ /Smad signaling pathway plays an important role in tissue maintenance and is dysregulated in various cancers, including CRC [234,235]. TGF- $\beta$  superfamily ligands include TGF- $\beta$ , activin A, and nodal. There are three distinct subtypes of Smads: receptor-regulated Smads (R-Smads, e.g., Smad2 and Smad3), common partner Smads (Co-Smads, e.g., Smad4), and inhibitory Smads (I-Smads, e.g., Smad7). Activation begins when TGF- $\beta$  ligands bind to TGF- $\beta$  receptor type 2 (TGFBR2) and then phosphorylate TGF- $\beta$  receptor type 1 (TGFBR1). This type I receptor phosphorylates R-Smads that bind Co-Smads. This complex is translocated to the cell nucleus and acts as a transcription factor to regulate target gene expression (Figure 8) [234–236].

In CRC, mutations in TGFBR2, affected by DNA repair deficiency with microsatellite instability, increase the cell survival rate. Furthermore, when Smad4 undergoes mutations and loses its function, there is an increase in tumor progression [237,238]. When Smad4 loss occurs together with mutations in the APC gene in intestinal epithelial cells, a malignant invasive phenotype arises in mouse mod-

els. The absence of Smad4 is found in approximately 20%–40% of CRC patients [239]. Although the loss of heterozygosity on chromosome 18 may be the main cause of Smad4 loss in CRC, there are other post-transcriptional and post-translational mechanisms that can contribute to its loss or dysfunction, such as ubiquitylation, sumoylation, and microRNA interference [240]. In addition, TGF- $\beta$  signaling also interacts with the Wnt pathway, where the loss of Smad4 promotes  $\beta$ -catenin expression [241]. Therefore, Wnt activation in the intestinal epithelium triggers the acquisition of stem cell properties and leads to dedifferentiation and rapid adenoma formation in the differentiated intestinal epithelium of a mouse model.

A study using miR-4666-3p and miR-329 demonstrated that TGF- $\beta$ 1 expression decreased with the weakening of stemness properties and that the activation of the TGF- $\beta$ 1/Smad pathway could function as a tumor suppressor gene for inhibiting the stemness of CRC. The authors later tested both miR-4666-3p and miR-329 in 73 tumor samples and paired normal tissues and revealed that these two miRNAs are related to poor prognosis and advanced tumor stage of CRC. This study confirms the importance of the TGF- $\beta$ /SMAD pathway for CRC-SCs and provides a new epigenetic regulation mechanism in CRC-SCs, which can be used in new therapeutic strategies for cancer treatment [242].

### 2.8.1 | Natural product

#### Baicalin

Baicalin is a monomeric flavonoid from the root of *Scutellaria baicalensis*, a traditional Chinese herb. Baicalin has

**TABLE 1** Drugs that target cell signaling pathways in CRC-SCs

Drugs	Mechanism of action	Reference
36-077	Suppresses GSK-3 $\beta$ /Wnt/ $\beta$ -catenin signaling	[118]
4-Acetyl-antroquinonol B	Suppresses LGR5/Wnt/ $\beta$ -catenin and JAK-STAT signaling	[104]
Anti-DLL4 antibodies	Suppresses Notch signaling	[158]
Atractylenolide I	Suppresses Akt/mTOR signaling	[225]
Baicalin	Suppresses TGF- $\beta$ /Smad signaling	[244]
Buparlisib (BKM120)	Suppresses PI3K/Akt/mTOR signaling by inhibition of PI3K	[210]
CBB1003	Suppresses $\beta$ -catenin/TCF signaling	[119]
Cucurbitacins	Suppresses Notch signaling by downregulation of the expression of ADAM9	[142]
Curcumin	Suppresses JAK/STAT signaling by inhibition of STAT3	[195]
Cyclopamine	Suppresses HH signaling by inhibition of SMO	[171]
Dactolisib	Suppresses PI3K/Akt/mTOR signaling by inhibition of PI3K	[226]
Diallyl trisulfide	Suppresses Wnt/ $\beta$ -catenin signaling	[105]
Epigallocatechin-3-gallate	Suppresses Wnt signaling by upregulation of GSK-3 $\beta$	[96]
Evodiamine	Suppresses Wnt and Notch signaling	[150]
Farnesyl dimethyl chromanol	Suppresses Wnt/ $\beta$ -catenin signaling	[102]
FH535	Suppresses Wnt/ $\beta$ -catenin signaling	[123]
GO-Y030	Suppresses JAK/STAT signaling by inhibition of STAT3	[195]
GW6471	Suppresses PPAR signaling (PPAR $\alpha$ antagonist)	[204]
Honokiol	Suppresses Notch signaling by inhibition of the levels of the $\gamma$ -secretase complex	[139]
HZ8CV2	Suppresses Wnt signaling	[126]
IC-2	Suppresses Wnt signaling	[111]
JIB-04	Suppresses Wnt signaling by increasing the expression of <i>AXIN1</i> and <i>GSK3<math>\beta</math></i>	[109]
LY294002	Suppresses PI3K/Akt/mTOR signaling by inhibition of PI3K	[209]
$\alpha$ -Mangostine	Suppresses Notch signaling	[155]
Metformin	Suppresses PI3K/Akt/mTOR signaling by inhibition of mTOR	[231]
Miransertib	Suppresses PI3K/Akt/mTOR signaling by inhibition of Akt	[210]
MK-2206	Akt inhibitor	[212]
Napabucasin (BBI-608)	Suppresses JAK/STAT signaling by inhibition of STAT3	[196]
NCB-0846	Suppresses Wnt signaling by block TNIK	[57,114]
Phenethyl isothiocyanate	Downregulates Wnt/ $\beta$ -catenin	[66]
Pien Tze Huang formula	Suppresses Notch signaling	[156]
Piplartine/piperlongumine	Suppresses PI3K/Akt/mTOR signaling	[218,222]
<i>Portulaca oleracea</i> extract	Downregulates the expression of Notch1 and $\beta$ -catenin	[153]
Quercetin	Suppresses Notch signaling by suppression of the $\gamma$ -secretase complex	[151]
Rapamycin	mTOR inhibitor	[211]
Retinoids	Suppresses Wnt signaling via differentiation therapy by HOXA5 induction	[58]
Rimonabant	Suppresses $\beta$ -catenin	[121]
Salinomycin	Disruption of the Wnt/ $\beta$ -catenin/TCF complex	[78,89]
SC-43 and SC-78	Suppresses STAT3 signaling	[199]
Sulforaphane	Suppresses TAp63 $\alpha$ /LGR5/ $\beta$ -catenin axis	[75]
Taselisib	Suppresses PI3K/Akt/mTOR signaling by inhibition of PI3K	[210]
Torin 1	mTOR inhibitor	[233]
Torkinib	mTOR inhibitor	[211]
Vismodegib	Suppresses HH signaling by inhibition of SMO	[176]
Zerubone	Suppresses $\beta$ -catenin signaling	[106]

Abbreviations: CRC-SCs, colorectal cancer stem cells; *AXIN1*, axin-1; GSK-3 $\beta$ , glycogen synthase kinase-3 beta; HH, hedgehog; HOXA5, homeobox A5; JAK/STAT, Janus kinase/signal transducer and activator of transcription; LGR5, leucinerich repeat-containing G-protein-coupled receptor 5; NF- $\kappa$ B, nuclear factor kappa B; PI3K/AKT/mTOR, phosphatidylinositol-3-kinases/akt/mechanistic target of rapamycin; PPAR, peroxisome proliferator-activated receptor; SMO, smoothened; TAp63 $\alpha$ , transactivating p63 isoform  $\alpha$ ; TCF, T-cell factor; TNIK, TRAF2- and NCK-interacting kinase; Wnt, wingless.

**TABLE 2** Drugs that target cell pathways in CRC-SCs and are tested in clinical trials as anti-CRC agents\*

ID	Study Title	Conditions	Interventions	Phase	First Posted	Current Status
NCT01304602	A trial of irinotecan and BKM120 in previously treated advanced colorectal cancer	Colorectal cancer	Drug: Irinotecan Drug: BKM120	Phase 1	February 25, 2011	Completed
NCT01591421	PI3Kinase inhibitor BKM120 in combination with panitumumab in metastatic/advanced RAS-wild type colorectal cancer	Metastatic colorectal cancer	Drug: BKM120 Drug: Panitumumab	Phase 1 Phase 2	May 4, 2012	Completed
NCT01571024	BKM120 + mFOLFOX6 in advanced solid tumors with expansion cohort pancreatic cancer	Advanced solid tumors Metastatic colorectal cancer Metastatic pancreatic cancer	Drug: BKM120 Drug: mFOLFOX6	Phase 1	April 4, 2012	Completed
NCT01501604	BKM120 in cancers with PIK3CA activating mutations	Lung cancer Breast cancer Colorectal cancer Cholangiocarcinoma Solid tumors	Drug: BKM120	Phase 2	December 29, 2011	Withdrawn (the study has been closed due to lack of accrual)
NCT01576666	Phase Ib, dose-escalation study of oral LDE225 in combination with BKM120 in patients with advanced solid tumors	Dose escalation Safety Preliminary efficacy Advanced solid tumors, including metastatic colorectal cancer	Drug: LDE225 Drug: BKM120	Phase 1	April 12, 2012	Completed
NCT02439385	Avastin/FOLFIRI in combination with curcumin in colorectal cancer patients with unresectable metastasis	Colorectal cancer	Drug: Avastin/FOLFIRI Dietary supplement: Curcumin	Phase 2	May 8, 2015	Completed
NCT00973869	Curcumin in preventing colorectal cancer in patients undergoing colorectal endoscopy or colorectal surgery	Colorectal cancer	Dietary supplement: curcumin	Phase 1	September 9, 2009	Unknown
NCT01859858	Effect of curcumin on dose-limiting toxicity and pharmacokinetics of irinotecan in patients with solid tumors	Advanced colorectal cancer	Dietary supplement: curcumin Drug: irinotecan	Phase 1	May 22, 2013	Completed

(Continues)

TABLE 2 (Continued)

ID	Study Title	Conditions	Interventions	Phase	First Posted	Current Status
NCT01333917	Curcumin biomarkers	Colorectal cancer	Drug: Curcumin C3 tablet	Phase 1	April 12, 2011	Completed
NCT01490996	Combining curcumin with FOLFOX chemotherapy in patients with inoperable colorectal cancer	Colonic cancer metastasis	Drug: Oral complex C3 curcumin + chemotherapy Drug: Chemotherapy only	Phase 1 Phase 2	December 13, 2011	Completed
NCT00027495	Curcumin for the prevention of colon cancer	Colorectal cancer	Dietary supplement: curcumin	Phase 1	January 27, 2003	Completed
NCT00003365	Sulindac and plant compounds in preventing colon cancer	Colorectal cancer	Dietary supplement: curcumin Dietary Supplement: rutin Drug: quercetin Drug: sulindac	Not applicable	May 21, 2004	Terminated (study completed)
NCT00118989	Curcumin for the chemoprevention of colorectal cancer	Adenomatous polyps	Dietary Supplement: curcuminoids	Not applicable	July 12, 2005	Terminated (could not be completed due to technical problems and cost constraints)
NCT00003365	Sulindac and plant compounds in preventing colon cancer	Colorectal cancer	Dietary supplement: curcumin Dietary supplement: rutin Drug: quercetin Drug: sulindac	Not applicable	May 21, 2004	Terminated (study completed)
NCT02891538	Chemopreventive effects of epigallocatechin gallate in colorectal cancer patients	Colon cancer	Drug: epigallocatechin gallate	Early phase 1	September 7, 2016	Recruiting
NCT02321969	Green tea extracts for the prevention of colorectal adenomas and colorectal cancer	Neoplasms, colorectal	Dietary supplement: green tea extract	Not applicable	December 22, 2014	Completed
NCT01941953	Metformin and 5-fluorouracil for refractory colorectal cancer	Metastatic colorectal cancer	Drug: Metformin and 5-Fluorouracil	Phase 2	September 13, 2013	Completed

(Continues)

TABLE 2 (Continued)

ID	Study Title	Conditions	Interventions	Phase	First Posted	Current Status
NCT02614339	Effect of adjunctive metformin on recurrence of non-DM colorectal cancer stage II high-risk/III colorectal cancer	Non-DM stage II high-risk colorectal cancer Non-DM stage III colorectal cancer	Drug: Metformin Drug: Control	Phase 3	November 25, 2015	Recruiting
NCT01816659	An open-labeled pilot study of biomarker response following short-term exposure to metformin	Colorectal carcinoma	Metformin extended release	Phase 1	March 22, 2013	Terminated (slow accrual)
NCT01312467	Trial of metformin for colorectal cancer risk reduction for a history of colorectal adenomas and elevated BMI	Adenomatous polyp Colorectal cancer Obesity	Drug: Metformin hydrochloride	Phase 2	June 25, 2015	Completed
NCT01926769	A phase II study to determine the safety and efficacy of second-line treatment with metformin and chemotherapy (FOLFOX6 or FOFIRI) in the second line treatment of advanced colorectal cancer	Previously treated advanced colorectal cancer	Drug: Metformin	Phase 2	August 21, 2013	Terminated (slow accrual rate)
NCT01523639	A randomized, placebo-controlled, double-blind phase II study evaluating if glucophage can avoid liver injury due to chemotherapy-associated steatosis	Colorectal cancer steatohepatitis	Drug: Metformin/Placebo	Phase 2	February 1, 2012	Terminated (prematurely due to slow recruitment)
NCT03800602	Nivolumab and metformin in patients with treatment-refractory MSS colorectal cancer	Colorectal neoplasms	Drug: Metformin Biological: Nivolumab	Phase 2	January 11, 2019	Active, not recruiting
NCT01930864	Metformin plus irinotecan for refractory colorectal cancer	Colorectal Neoplasms Adenocarcinoma	Drug: Metformin Drug: Irinotecan	Phase 2	August 29, 2013	Unknown

(Continues)

TABLE 2 (Continued)

ID	Study Title	Conditions	Interventions	Phase	First Posted	Current Status
NCT03047837	A randomized, 2x2 factorial design biomarker prevention trial of low-dose aspirin and metformin in stage I-III colorectal cancer patients	Tertiary prevention in colon cancer	Drug: Aspirin + Metformin Drug: Aspirin Drug: Metformin Drug: Placebos	Phase 2	February 9, 2017	Unknown
NCT01440127	Impact of pretreatment with metformin on colorectal cancer stem cells and related pharmacodynamic markers	Colon cancer	Drug: Metformin	Phase 1	September 26, 2011	Terminated (unable to accrue all planned subjects in a timely fashion, but data collected will be analyzed)
NCT01340300	Exercise and metformin in colorectal and breast cancer survivors	Colorectal cancer Breast cancer	Behavioral: Exercise training Drug: Metformin Other: Educational information	Phase 2	September 18, 2017	Completed
NCT04033107	High dose vitamin C combined with metformin in the treatment of malignant tumors	Hepatocellular cancer Pancreatic cancer Gastric cancer Colorectal cancer	Drug: Vitamin C Drug: Metformin	Phase 2	July 25, 2019	Recruiting
NCT01632020	Effect of metformin on biomarkers of colorectal tumor cell growth	Colorectal neoplasms	Drug: Placebo Drug: Metformin	Phase 2	June 29, 2012	Terminated (inadequate accrual)
NCT01333475	MK-2206 and AZD6244 in patients with advanced colorectal carcinoma	Colorectal neoplasms	Drug: MK-2206 + AZD6244	Phase 2	April 12, 2011	Completed
NCT0186705	Clinical and translational study of MK-2206 in patients with metastatic KRAS-wild-type, PIK3CA-mutated, colorectal cancer	Colon cancer Rectal cancer	Drug: MK-2206	Phase 2	August 23, 2010	Terminated (lack of accrual)

(Continues)

TABLE 2 (Continued)

ID	Study Title	Conditions	Interventions	Phase	First Posted	Current Status
NCT01802320	Akt inhibitor MK-2206 in treating patients with previously treated colon or rectal cancer that is metastatic or locally advanced and cannot be removed by surgery	Colorectal neoplasms	Drug: Akt Inhibitor MK-2206 Other: Laboratory Biomarker Analysis Other: Pharmacological Study	Phase 2	March 1, 2013	Completed
NCT01243762	A study of dalotuzumab + MK-2206, dalotuzumab + MK-0752, and dalotuzumab + ridaforolimus combination therapies in participants with advanced cancer (MK-0646-027)	Neoplasms malignant	Drug: dalotuzumab Drug: MK-0752 Drug: ridaforolimus Drug: MK-2206	Phase 1	November 19, 2010	Terminated (the study was terminated for business reasons and not due to any safety or efficacy concerns related to dalotuzumab)
NCT03522649	A Phase III clinical study of napabucasin (GB201) Plus FOLFIRI in adult patients with metastatic colorectal cancer	Previously treated metastatic colorectal cancer	Drug: Napabucasin Drug: Fluorouracil Drug: Leucovorin Drug: Irinotecan	Phase 3	May 11, 2018	Recruiting
NCT02753127	A study of napabucasin (BB1-608) in combination with FOLFIRI in adult patients with previously treated metastatic colorectal cancer (CanStem303C)	Colorectal cancer	Drug: Napabucasin Drug: Fluorouracil Drug: Leucovorin Drug: Irinotecan Drug: Bevacizumab	Phase 3	April 27, 2016	Completed
NCT01776307	A study of BBI608 in adult patients with advanced colorectal cancer	Colorectal cancer	Drug: BBI608 Drug: Panitumumab Drug: Capecitabine Drug: Cetuximab	Phase 2	January 28, 2013	Completed
NCT01830621	BB1608 and best supportive care vs placebo and best supportive care in pretreated advanced colorectal carcinoma	Colorectal carcinoma	Drug: BBI608 Drug: Placebo Other: Best supportive care	Phase 3	April 12, 2013	Completed

(Continues)



TABLE 2 (Continued)

ID	Study Title	Conditions	Interventions	Phase	First Posted	Current Status
NCT02641873	A study of BBI608 administered with FOLFIRI + bevacizumab in adult patients with metastatic colorectal cancer	Metastatic colorectal cancer	Drug: BBI608 Drug: 5-FU Drug: Irinotecan Drug: Levofolinate Drug: Bevacizumab	Phase 1	December 30, 2015	Completed
NCT03647839	Modulation of the tumor microenvironment using either vascular disrupting agents or STAT3 inhibition in order to synergize with PDI inhibition in microsatellite stable, refractory colorectal cancer (MODULATE)	Colorectal cancer metastatic	Drug: Nivolumab 10 MG/ML Drug: BNC 105 Drug: BBI608	Phase 2	August 27, 2018	Completed
NCT02851004	A special combination of BBI608 and pembrolizumab	Metastatic colorectal cancer	Drug: BBI608 (Napabucasin) Drug: Pembrolizumab	Phase 1 Phase 2	August 1, 2016	Terminated (on the request of the investigational drug provider)
NCT01606124	Polyphenon E in treating patients with high-risk of colorectal cancer	Advanced colorectal adenomas adenocarcinoma of the colon stage I colon cancer stage II colon cancer stage III colon cancer	Drug: defined green tea catechin extract Other: placebo Other: questionnaire administration Other: laboratory biomarker analysis	Phase 2	May 25, 2012	Terminated (pending expiration of the supply of study agent)
NCT03439462	ABI-009 (Nab-rapamycin) in combination with FOLFOX and Bevacizumab as first-line therapy in patients with advanced or metastatic colorectal cancer	Colorectal cancer metastatic	Drug: ABI-009; nab-rapamycin; albumin-bound rapamycin	Phase 1 Phase 2	February 20, 2018	Recruiting
NCT00409994	Safety study of rapamycin administered before and during radiotherapy to treat rectum cancer	Rectum cancer	Drug: Rapamycin	Phase 1 Phase 2	December 12, 2006	Completed
NCT03190174	Nivolumab (Opdivo®) plus ABI-009 (Nab-rapamycin) for advanced sarcoma and certain cancers	Colorectal cancer and other cancers	Drug: Nab-Rapamycin Biological: Nivolumab	Phase 1 Phase 2	June 16, 2017	Recruiting

(Continues)

TABLE 2 (Continued)

ID	Study Title	Conditions	Interventions	Phase	First Posted	Current Status
NCT01522820	Vaccine therapy with or without sirolimus in treating patients with NY-ESO-1 expressing solid tumors	Recurrent colorectal carcinoma Others cancers	Biological: DEC-205/NY-ESO-1 Fusion Protein CDX-1401 Other: Laboratory Biomarker Analysis Other: Pharmacological Study Drug: Sirolimus	Phase 1	February 1, 2012	Completed
NCT00375245	Rapamycin with grapefruit juice for advanced malignancies	Tumors Neoplasm metastasis	Drug: rapamycin (sirolimus) Other: grapefruit juice	Phase 1	September 12, 2006	Completed
NCT02465060	Targeted therapy directed by genetic testing in treating patients with advanced refractory solid tumors, lymphomas, or multiple myeloma (the match screening trial)	Many tumors, including colorectal cancer and recurrent colorectal cancer	Many targeted drugs, including taselesib and vismodegib	Phase 2	June 8, 2015	Recruiting
NCT00636610	A study of vismodegib (GDC-0449, hedgehog pathway inhibitor) with concurrent chemotherapy and bevacizumab as first-line therapy for metastatic colorectal cancer	Metastatic colorectal cancer	Drug: Vismodegib 150 mg Drug: Placebo to vismodegib Drug: Bevacizumab Drug: Modified FOLFOX Drug: FOLFIRI	Phase 2	March 14, 2008	Completed
NCT00959647	A study of vismodegib (GDC-0449) in patients treated with vismodegib in a previous Genentech-sponsored phase I or II cancer study	Ovarian cancer Basal cell carcinoma Metastatic colorectal cancer	Drug: Vismodegib Drug: FOLFOX Drug: FOLFIRI Drug: Bevacizumab	Phase 2	August 14, 2009	Completed

\*All studies were accessed at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) on August 28, 2021 by using the search term "CRC" and the drugs that are presented in Table 1.

Abbreviations: CRC-SCs, colorectal cancer stem cells; BMI, body mass index; DM, diabetes mellitus; FOLFIRI, 5-fluorouracil, leucovorin plus irinotecan; FOLFOX, 5-fluorouracil, folic acid plus oxaliplatin; FOLFOX6, 5-fluorouracil, leucovorin plus oxaliplatin; MSS, microsatellite stable; PI3Kinase, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

TABLE 3 Other molecules/therapies that target CRC-SCs

Drug/Therapy	Pharmacological/ Chemical class	Action	Reference
5-aminosalicylic acid	Anti-inflammatory modulator drug	Suppress stemness features in many CRC cell lines	[256]
Antibody dual-antigen-binding specificity to CD133 and CD3	Antibody	Reduces CD133 <sup>+</sup> CRC cells in vitro and in vivo	[257]
ASR352	Chk1 inhibitor	Inhibits CRC-SCs in preclinical models	[258]
CD133-directed CAR T cells	CAR-T cells	Eliminates CD133 <sup>+</sup> cells and reduced tumor growth in a phase I clinical trial with patients with advanced metastasis colorectal, liver and pancreatic cancers	[259]
CD133-targeted oncolytic adenovirus	Oncolytic adenovirus	Eliminates CD133 <sup>+</sup> CRC in vitro and in vivo	[260]
Dabrafenib	BRAF inhibitor	Synergies with cetuximab (EGFR inhibitor) to decrease stem cell in BRAF(V600E)-mutant CRC cells	[261]
Ellagic acid	Ellagitannin metabolites	Mixed with urolithins (gut microbiota-derived) reduces CRC-SCs of Caco-2 cells and primary tumor cells from a patient with CRC	[262]
G2.2	Sulfated nonsaccharide glycosaminoglycan mimetic	Reduces CRC-SCs in cell lines via p38 MAPK activation	[263]
Gambogic acid	Xanthonoid from <i>Garcinia hanburyi</i>	Inhibits CRC-SCs by upregulation of ZFP36	[264,265]
Ginsenoside	Major active component of ginseng	Reduced growth and stemness of CRC cells in vitro and in vivo	[266]
Heparan sulfate hexasaccharide	Non-anticoagulant heparin derivative	Inhibits CRC-SCs by activation of p38 MAPK	[267]
Mithramycin A	Polyketide antibiotic	Reduces CRC-SCs in different cell lines	[268]
Nigericin	Antibiotic from <i>Streptomyces hygroscopicus</i>	Reduces CRC-SCs in HT-29 and SW116 cell lines	[269]
NSC30049	Chk1 inhibitor	Inhibits CRC-SCs in preclinical models	[270]
Parthenolide	Sesquiterpene lacton from <i>Tanacetum parthenium</i>	Eliminates CRC-SCs in preclinical models	[271]
Polydatin	Glycoside of resveratrol found in <i>Polygonum cuspidatum</i>	Combination with radiation caused apoptosis of LGR5 <sup>+</sup> CRC cells	[272]
Silibinin	Flavonolignan from <i>Silybum marianum</i>	Inhibits the growth kinetics of CRC-SCs in different cell lines	[273]
Thiostrepton	Thiazole antibiotic from <i>Streptomyces sp.</i>	Induces cell death in CRC-SCs in HCT-15 and HT-29 and synergizes with oxaliplatin	[274]
UCN-01	Staurosporin derivative	Inhibits CRC-SCs growth and increases irinotecan action in vitro and in vivo	[275]

Abbreviations: CRC-SCs, colorectal cancer stem cells; BRAF, B-Raf proto-oncogene, serine/threonine kinase; CAR-T, chimeric antigen receptor T cells; Chk1, checkpoint kinase 1; EGFR, epidermal growth factor receptor; LGR5, leucine-rich repeat-containing G-protein-coupled receptor 5; MAPK, mitogen activated protein kinases; ZFP36, ZFP36 ring finger protein.

several pharmacological effects, including anti-cancer, anti-inflammatory, antioxidant and neuroprotective effects [243]. In CRC RKO and HCT116 cell lines, baicalin caused cell cycle arrest in the G<sub>1</sub> phase, p53-independent cell apoptosis, and EMT inhibition by inhibiting the TGF- $\beta$ /Smad pathway [244]. Baicalin also inhibited the

CRC-SC subpopulation in in vitro and in vivo models using the CRC HCT116 cell line.

Altogether, the TGF- $\beta$ /Smad signaling pathway also plays an important role in CRC-SC maintenance, and novel selective inhibitors must be screened for CRC-targeting stemness properties.

### 3 | PERSPECTIVES

Tumors have high complexity and are composed of multiple morphologically and phenotypically distinct subpopulations of cancer cells. The CSC subpopulation plays a critical role in the recurrence, progression, and metastasis of CRC and consequent cell resistance to current treatments since this subpopulation appears to be more resistant to chemotherapy and radiotherapy. In this context, exploring new methods to inhibit CSCs seems essential for achieving therapeutic success in CRC. In this review, we highlight fundamental pathways for sustaining CRC-SCs. Table 1 summarizes the reported drugs that eradicate CRC-SCs by inhibiting these pathways. Among them, buparlisib, curcumin, epigallocatechin-3-gallate, metformin, MK-2206, napabucasin, quercetin, rapamycin, tselisib, and vismodegib have been tested in clinical trials as anti-CRC agents (Table 2). In particular, napabucasin, a STAT3 inhibitor, is the only agent that has been completed phase III clinical trials. It improved survival compared to placebo (median overall survival of 5.1 vs. 3 months) in advanced p-STAT3<sup>+</sup> CRC patients [198]. Clinical data with these CRC-SC inhibitors are still limited. Currently, there is no clinically effective therapy to suppress CSCs.

The Wnt canonical pathway seems to be the most relevant for the growth of CRC-SCs, also counting on the contribution of the Notch, HH, NF- $\kappa$ B, JAK/STAT, PPAR, PI3K/Akt/mTOR, and TGF- $\beta$ /Smad pathways, which needs further study. It is important to note that these signaling pathways do not operate separately but function as a network. It is necessary to suppress more than one pathway to inhibit possible crosstalk with other pathways. Interestingly, these signaling pathways are aberrantly expressed in bulk CRC and CRC-SCs, which allows the use of their protein expression levels as therapeutic biomarkers to guide the treatment of CRC patients with appropriate inhibitors.

On the other hand, many studies have reported that many of these pathways also regulate the maintenance of normal stem cell homeostasis [245–248], indicating that inhibitors of these pathways may lead to toxicity to normal tissues and limit the use of anti-CRC therapy. Although intestine and blood stem cells are in constant proliferation, most stem cells in the mammary glands, skin, and brain are usually quiescent [249–252]. Thus, more data are needed on the toxicity of inhibitors of these signaling pathways.

Complementally, CAR-T (chimeric antigen receptor T) cells, antibodies, and other molecules with different targets have also been developed to specifically target CSCs. Some of them are summarized in Table 3.

Additionally, extracellular factors affecting the tumor microenvironment, such as vascular niches, hypoxia,

tumor-associated macrophages, cancer-associated fibroblasts, and extracellular matrix, have emerged as an important topic in CSC regulation, and many strategies targeting the microenvironment of CSCs have been revealed [253–255]. Accordingly, effective treatment strategies for CRC-SCs must emphasize not only intracellular signaling pathways but also reciprocal interactions between CRC-SCs and extracellular factors.

### 4 | CONCLUSIONS

This review highlights therapeutic targets for drugs that act on the signaling pathways activated in CRC-SCs. Drugs under preclinical and clinical studies are summarized to direct future research. This knowledge will contribute to the study of potential therapeutic schemes, combining conventional drugs with CSC-targeting drugs and allowing better cure rates in anti-CRC therapy.

#### DECLARATIONS

#### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

#### CONSENT FOR PUBLICATION

Not applicable.

#### AVAILABILITY OF DATA AND MATERIALS

Not applicable.

#### CONFLICTS OF INTEREST

The authors have declared that there are no conflicts of interest.


#### AUTHORS' CONTRIBUTIONS

VRS drafted the content of Wnt/ $\beta$ -catenin, Notch and Hedgehog pathways; LSS drafted the content of NF- $\kappa$ B, JAK/STAT, PPAR, PI3K/Akt/mTOR and TGF- $\beta$ /Smad pathways; CAQ drafted the content of clinical approaches for CRC; RBD and DPB planned this review and reviewed the whole text. All authors read and approved the final manuscript.

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