Chapter 5

Apoptosis as a Therapeutic Target in Cancer and Cancer Stem Cells: Novel Strategies and Futures Perspectives

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1. Introduction

Apoptosis is an essential part of the normal development. The homeostatic balance between cell proliferation and cell death rate is critical for maintaining normal physiological processes. Aberrant regulation of apoptotic cell death mechanisms is one of the hallmarks of cancer development and progression, and many cancer cells exhibit significant resistance to apoptosis signalling [1]. Triggering of apoptosis can be achieved via the activation of two distinct molecular pathways, the extrinsic or death receptor pathway or via the intrinsic or mitochondrial apoptotic cascades. Both pathways lead to the hierarchical activation of a family of cysteine proteases called caspases [2], that cleave a series of cellular substrates which induce changes including chromatin condensation, internucleosomal DNA fragmentation, membrane blebbing and cell shrinkage [3]. Extrinsic pathway is activated from outside the cell by proapoptotic ligands that interact with specialized cell surface death receptors, including CD95 and TNF-related apoptosis-inducing ligand (TRAIL) receptors [4]. After binding to receptors apoptosis is triggered by the intracellular formation of a death-inducing signalling complex (DISC) that consists of FAS-associated death domain (FADD) and procaspase-8 and 10 [5,6]. As a result, this protein complex activates procaspase-8 and 10 inside itself, hence triggering procaspase-3 to execute the apoptosis process [7]. The mitochondria (intrinsic) pathway is activated from inside the cell by severe cell stress, such as DNA or cytoskeletal damage, inducing mitochondrial outer membrane permeabilization and transcription or post-translational activation of BH3-only proapoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family proteins [4]. This permeabilization allows the release of apoptogenic proteins, including cytochrome c and second mitochondria-derived

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activator of caspase (Smac; also known as DIABLO), from the mitochondrial intermembrane space into the cytosol [8]. Cytochrome c assembles with apoptotic protease-activating factor-1 (Apaf-1) to activate caspase 9. This caspase, in turn, activates the effector caspases 3, 6, and 7, which carry out apoptosis [4]. Smac promotes caspase activation and apoptosis by neutralization of several IAP proteins, including XIAP, c-IAP1 and c-IAP2 [9,10]

Chemotherapeutic agents act by inhibiting tumour cell proliferation and survival and most of them can kill tumour cells by activating common apoptotic pathways [11]. Therefore, apoptosis plays an important role in the treatment of cancer as it is a popular target of many treatment strategies. 5-fluorouracil (5-FU), an antimetabolite analogue of uracil employed primarily in the treatment of a variety of solid malignant tumours, leads to a wide range of biological effects which can act as triggers for apoptotic cell death [12,13]. However, resistance to the drug remains a major clinical problem. Given that many of the apoptotic regulators altered in multidrug resistant tumours have been identified, one new approach to therapy is to restore apoptotic potential through genetic or pharmacological methods [14]. Moreover, since defects in the mediators of apoptosis may account for chemo-resistance, the identification of new targets involved in drug-induced apoptosis is of main clinical interest. Recently, we have identified the ds-RNA-dependent protein kinase (PKR) as a key molecular target of 5-FU involved in apoptosis induction, in a p53 -independent manner. These results suggest the clinical importance that the PKR status could play in response to chemotherapy based on 5-FU. Moreover, the effectiveness of 5-FU cytotoxic activity induced by IFNα, especially in cancer cells expressing a mutated form or lacking p53, but with a functional PKR, might have relevant clinical application in patients [15] .

The increased knowledge of some of the molecular components of the apoptosis signalling pathways has paved the way for the generation of more specific agents that target one crucial signalling component. This has allowed a change in anticancer therapy trends, from classic cytotoxic strategies to the development of new non-harmful therapies which target the apoptosis response selectively only in tumour cells. Moreover, these strategies overcome the adverse effects associated with cytotoxic drugs and increase their anti-cancer activity. Novel antitumour drugs have been synthesised such as 5-FU *O,N*-acetals and benzo-fused seven-membered *O,N*-acetal in which the 5-FU moiety was changed for the naturally-occurring pyrimidine base uracil, which induced cell cycle-mediated apoptosis in breast and colon cancer cells [16,17,18,19]. The mechanism of action of these drugs was mainly centred on positive apoptosis regulatory pathway genes, and the repression of genes involved in carcinogenesis, proliferation and tumour invasion. In addition, these drugs were more selective against tumour cells with lower toxic effects in non-tumour cells [20,21].

As over-expression of IAP proteins frequently occurs in various human cancers and has been linked to tumour progression, chemo-resistance and poor prognosis, it is not surprising that IAP proteins are considered to be attractive targets for improve outcomes for patients with solid tumours and hematologic malignancies [22,23]. IAPs are also attractive as therapeutic targets because their inhibition does not appear to be toxic to normal adult cells [24]. Several therapeutic strategies have been designed to target IAP, including a small-

molecule approach that is based on mimicking the IAP-binding motif of the endogenous IAP antagonist Smac [25]. Other therapies involve antisense strategies and short-interfering RNA (siRNA) molecules [26,27]. There is increasing interest in therapeutic drug development targeting the IAP family.

MicroRNAs (miRNAs) are small RNA gene products that regulate the activity of messenger RNAs by antisense base pairing. They are involved in stem-cell self-renewal, cellular development, differentiation, proliferation, and apoptosis [28]. In cancer, miRNAs function as regulatory molecules, acting as oncogenes or tumour suppressors [29] and they are strongly related to the apoptosis. New insights indicate that many miRNAs are anti-apoptotic and mediate this effect by targeting pro-apoptotic mRNAs or positive regulators of pro-apoptotic mRNAs [30]. Conversely, many pro-apoptotic miRNAs target anti-apoptotic mRNAs or their positive regulators [31]. Therefore, their inhibition leads to the induction of programmed cell death, suggesting a promising miRNA treatment for cancer. Several drugs may have the ability to modulate the expression of miRNAs by targeting signalling pathways that ultimately converge on the activation of transcription factors that regulate miRNA encoding genes.

Evasion of apoptosis is one of the main mechanisms involved in tumourigenesis and drug resistance. Most cancers have a small population of tumour cells, as few as 1%, with stem cell characteristics and the capacity for self-renewal, termed cancer stem cells (CSCs). A malignant tumour can be viewed as an abnormal organ in which small populations of tumourigenic CSCs have escaped the normal limits of self-renewal, giving rise to abnormally differentiated cancer cells that contribute to tumour progression and growth [32]. These CSC express high levels of ATP-binding cassette (ABC) drug transporters, providing for a level of resistance [33]; are relatively quiescent; have higher levels of DNA repair and a lowered ability to enter apoptosis . Several cancer therapy approaches targeting ABC transporters and increasing apoptosis could be employed to selectively and more efficiently kill CSC.

The current review will focus on recent development of several therapeutic strategies, which interfere with apoptosis and are currently used or tested for treatment of cancer. They induce cancer cell death or enhance the responsiveness of cancer cells and CSCs to certain cytotoxic drugs. Some of them such as caspases activators, indirectly modulators of apoptosis or agents targeting apoptosis-related proteins are still in their preclinical or clinical trials. We also include future approaches directed to target apoptotic pathways with promising application in patients with cancer.

2. Novel apoptotic markers/targets in cancer

The main goal in cancer therapy is the abrogation of tumour cell growth and proliferation, and ultimately the complete elimination of tumour cells. It is commonly accepted that tumour cells treated with anticancer agents undergo apoptosis, and that cells resistant to apoptosis often do not respond to anticancer therapy [34]. Moreover, it is widely demonstrated that some oncogenic mutations suppressing apoptosis may lead to tumour

initiation, progression or metastasis [11]. Apoptosis plays a major control role in cell death when DNA damage is irreparable and multiple stress-inducible molecules have been implied in transmitting the apoptotic signal [35]. Because of the potential detrimental effects on cell survival in case of inappropriate activation of apoptosis programs, apoptosis pathways have to be tightly controlled. However, the concept that apoptosis represents the major mechanism by which cancer cells are eliminated may not universally apply and caspase-independent apoptosis or other modes of cell death have also to be considered as cellular response to anticancer therapy [36].

Different determinants of drug resistance exist, including loss of cell surface receptors or transporters, altered metabolism, or mutation of specific apoptotic target [37]. The apoptotic signalling pathways are regulated by numerous hub proteins such as p53, Bcl-2, NFkB and MAPKs which function in common. In the following sections, we will summarize some of the cellular proteins considered as potential apoptotic biomarkers in cancer.

1- The tumour suppressor p53 is an important pro-apoptotic factor and tumour inhibitor, and numerous anti-tumour drugs would exert their functions through targeting p53-related signalling pathways. Some clinical investigations indicated that under abnormal situations such as chemotherapy and UV or DNA damage may occur and activate the expression of p53. Activated p53 protein binds to the regulatory sequences of a number of target genes to initiate a program of cell cycle arrest, DNA repair, apoptosis, and angiogenesis [38]. If the damage cannot be repaired completely, over-activation of p53 leads to the tumour growth stagnation or even apoptosis [39,40]. Loss of p53 function is critical in tumourigenesis, and alterations to the *p53* gene (mutations, often resulting in protein over-expression) are frequent events in cancer. Associations of *p53* tumour alterations with patient prognosis and response to adjuvant chemotherapy have been widely studied, and findings are contradictory. The fluoropyrimidine 5-FU is widely used in the treatment of a range of cancers including colorectal cancer and breast cancer [41,42,43], but resistance to the drug remains a major clinical problem. P53 was the first target described for 5-FU- induced apoptosis, however, although several reports have demonstrated that 5-FU-induced apoptosis is dependent on the tumour suppressor p53 protein, apoptosis can also occur in mutant p53 cell lines [44,45,46,47]. Moreover, the relationship between p53 status and sensitivity to chemotherapeutic drugs, including 5- FU, is still controversial. In clinical studies in which adjuvant chemotherapy– treated and non-treated groups could be analyzed, stage III colorectal patients whose tumours demonstrated no *p53* alterations experienced significantly longer survival following 5-FU–based chemotherapy than patients whose tumours over-expressed p53 [48,49]. However, other studies in colon cancer patients failed to demonstrate correlations between *p53* alterations and benefit from adjuvant therapy [50,51]. The identification of new targets involved in 5-FU-induced apoptosis could contribute to clarify the controversy results obtaining in clinic.

2- Recently, we have identified the interferon-induced protein kinase PKR, as a molecular target of 5-FU with an interesting role in the apoptosis induced by this chemotherapeutic drug [15]. The double-stranded RNA (dsRNA)-dependent kinase PKR was initially

identified as an innate immune anti-viral protein approximately 35 years ago [52,53]. Since then, PKR has been linked to normal cell growth and differentiation, inflammation, cytokine signalling, and apoptosis [54]. PKR is a serine/threonine kinase, characterized by two distinct kinase activities: autophosphorylation, which represents the activation reaction, and phosphorylation of eIF-2α [55,56], which impairs eIF-2 activity, resulting in inhibition of protein synthesis [57]. In addition to its translational regulatory function, PKR has a role in signal transduction and transcriptional control through the IkB/NF-kB pathway [58,59]. PKR is activated in response to dsRNA of cellular, viral, or synthetic origin. PKR can also be activated by polyanions such as heparin, dextran sulfate, chondroitin sulfate, and poly-L-glutamine [60]. A range of cellular stresses can also activate PKR, such as arsenite, thapsigargin, H_2O_2, ethanol and ceramide [61,62,63] presumably through the PKR-associated activator (PACT)/RAX protein [64]. Moreover, it is induced by interferon type I and mediates in part, several functions of these cytokines. Altered PKR activity has been shown to play a role in neurodegenerative diseases (Alzheimer's, Creutzfeldt–Jakob, Huntington's, and Parkinson's) and cancer [65,66,67,68,69,70,71,72,73,74].

Over-expression or continued activation of PKR leads to apoptosis [75,76,77]. PKR mediates the apoptosis induced by several viruses and cellular stresses [78] by activation of intrinsic and extrinsic apoptosis pathways through the FADD/caspase 8 and mitochondrial APAF/caspase 9 activation pathways [79,80]. Recently, PKR has been shown to play an important role in apoptotic cancer cell death induced by 5-FU, doxorubicin and etoposide [15,81,82] and the antitumour activity of tumour suppressors like p53 and PTEN [81,83]. Preclinical studies in mice have shown than in tumours which do not express sufficient levels of PKR are more resistant to doxorubicin and etoposide that tumour expressing higher PKR levels. We have demonstrated that PKR is up-regulated and activated in colon and breast cancer cell lines by inducing apoptotic cell death in response to 5-FU treatment. In addition, cancer cell lines deficient in PKR expression were more resistant to the cytotoxic effect of 5-FU with an IC_{50} being 2-3 fold higher than cells expressing an active PKR protein. Moreover, apoptosis mediated by PKR in response to 5-FU occurred independently on p53 status highlighted the importance that both p53 and PKR play in the 5-FU-induced cancer cell death, and the relevance acquired by PKR in tumour cells where p53 is mutated. Such results raise the importance of determining PKR status in tumours from patients treated with 5-FU-based chemotherapy [15].

3- One pathway being targeted for antineoplastic therapy is the Bcl-2 family of proteins (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl1/A-1, and Bcl-B) that bind to and inactivate BH3-domain pro-apoptotic proteins. It is controversial whether some BH3-domain proteins (Bim or tBid) directly activate multidomain pro-apoptotic proteins (e.g., Bax and Bak) or act via inhibition of those anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl1/A-1, and Bcl-B) that stabilize pro-apoptotic proteins [84]. Since the anti-apoptotic properties of Bcl-2 were discovered, the over-expression of Bcl-2 conferring chemoresistance was reported, and the 3-D protein structure of Bcl-XL was determined. These properties have contributed to the development of protein inhibitors. The first agent targeting Bcl-2 that entered clinical trials was a Bcl-2 antisense (oblimersen sodium), which showed chemosensitizing effects when

combined with conventional chemotherapy drugs in chronic lymphocytic leukemia (CLL) patients, leading to improved survival [85,86]. More recent advances include the discovery of small molecule inhibitors of the Bcl-2 family proteins. They are designed to bind the hydrophobic groove of anti-apoptotic Bcl-2 proteins in place of BH3-only proteins (i.e., BH3-mimetics). They can oligomerize Bax or Bak, which can subsequently depolarize mitochondrial membrane potential to release cytochrome *c*. To date, one Bcl-2 antisense and three small molecule Bcl-2 protein inhibitors are being tested in clinical trials. Preclinical studies seem promising, especially in combination with additional chemotherapy agents. Ongoing and planned phase II clinical trials to define the activity of single agents and drug combinations will determine the direction of future clinical development of the Bcl-2 inhibitors [84].

4- PUMA (p53 upregulated modulator of apoptosis) is a BH3-only Bcl-2 family member and a critical mediator of p53-dependent and -independent apoptosis induced by a wide variety of stimuli, including genotoxic stress, deregulated oncogene expression, toxins, altered redox status, growth factor/cytokine withdrawal and infection. It serves as a proximal signalling molecule whose expression is regulated by transcription factors in response to these stimuli. PUMA transduces death signals primarily to the mitochondria and acts indirectly on the Bcl-2 family members Bax and/or Bak by relieving the inhibition imposed by anti-apoptotic members. It directly binds and antagonizes all known anti-apoptotic Bcl-2 family members to induce mitochondrial dysfunction and caspase activation [87]. Several lines of evidence suggest that the function of PUMA is compromised in cancer cells. PUMA expression was found to be reduced in malignant cutaneous melanoma, and PUMA expression appears to be an independent predictor of poor prognosis in patients [88]. In addition, approximately 40% of primary human Burkitt's lymphomas do not express detectable levels of PUMA, which is attributable, in part, to DNA methylation [89]. Evidence of PUMA induction by therapeutic agents in patients has just begun to emerge. Analysis of tissue biopsies from breast cancer patients showed that *PUMA* mRNA was induced within 6 h of chemotherapy [90]. Increased expression of *PUMA* and *Bim* is associated with better prognosis in patients receiving 5-FU-based therapy in stage II and III colon cancer, and is an independent prognostic marker for overall and disease-free survival [91]. PUMA ablation or inhibition leads to apoptosis deficiency underlying increased risks for cancer development and therapeutic resistance. Although elevated PUMA expression elicits profound chemo- and radio-sensitization in cancer cells, inhibition of PUMA expression may be useful for curbing excessive cell death associated with tissue injury and degenerative diseases. Therefore, PUMA is a general sensor of cell death stimuli and a promising drug target for cancer.

5- The apoptosome, a complex of cytochrome-c and APAF-1, recruits and activates the initiator pro-caspase 9, leading to the activation of the effector caspases, in particular pro-caspase 3, culminating in those biochemical and morphological changes associated with apoptotic cell death [92]. This pathway is further regulated by the inhibitor of caspase protein XIAP, which works through the direct inhibition of active caspases 9 and 3 [93] and is also implicated in the ubiquitination of caspases, targeting them for proteasomal degradation [94]. Moreover there is a direct interaction between XIAP and its antagonist Smac [95].

Because of the importance of this pathway in cancer progression and chemotherapy-induced cell death, apoptosome-associated proteins may be important markers for colorectal cancer prognosis and chemotherapy response. Several studies have examined the immunohistochemical expression of individual proteins associated with apoptosis execution in colorectal cancer [96]. Increased expression of APAF-1 has been shown to be associated with longer patient survival in rectal cancer patients [97] and loss of APAF-1 has been implicated in tumour progression and more aggressive disease [98]. Similarly, longer overall survival has been associated with increased Smac [99] and caspase 9 [100]. The anti-apoptotic XIAP has also been implicated as a potential prognostic marker for colorectal cancer, with increased expression correlating with poor patient outcome [101]. However, no study to date has provided a comprehensive analysis of these key regulatory proteins as markers for colorectal cancer prognosis or chemotherapy response. Recently, the pro-caspase 3 expression in colorectal tumours has been associated with better recurrence-free and overall survival, and serves as an independent prognostic marker in localised Stage II disease [102]. This result is in agreement with previous studies that demonstrated as caspase 3 expression is a positive prognostic marker in hepatocellular carcinoma [103] and diffuse large B-cell lymphoma [104].

6- The extrinsic apoptosis pathway is triggered by the binding of death ligands of the tumour necrosis factor (TNF) family to their appropriate death receptors (DRs) on the cell surface. One TNF family member, TRAIL or Apo2L, seems to preferentially cause apoptosis of transformed cells and can be systemically administered in the absence of severe toxicity. Therefore, there has been enthusiasm for the use of TRAIL or agonist antibodies to the TRAIL DR4 and DR5 in cancer therapy. Nonetheless, many cancer cells are very resistant to TRAIL apoptosis in vitro. Therefore, there is much interest in identifying compounds that can be combined with TRAIL to amplify its apoptotic effects [105]. The combination of TRIAL DR agonists with numerous conventional and investigational anticancer drugs has been reported. Synergy has been described for the combination of TRAIL with a variety of cytotoxic agents including irinotecan, camptothecin, 5-FU, carboplatin, paclitaxel, doxorubicin, and gemcitabine in diverse preclinical models [106,107]. Many human and mouse cancer cells lines can be sensitized by proteasome inhibitors such as bortezomib (VELCADE) to the apoptotic effects of TRAIL DR agonists. Interestingly, non-transformed cells seem to be much more resistant to the apoptotic effects of bortezomib and TRAIL than are cancer cells. This suggests that a therapeutic window may exist in vivo where this combination may have a therapeutic benefit in the absence of accompanying toxicity. However, the molecular mechanism(s) of action whereby proteasome inhibition in cancer cells results in sensitization to TRAIL apoptosis remains unclear [108].

3. Selective antitumour-drug inducers of apoptosis

Improved clinical response may be obtained by identifying therapies that are particularly effective in activating apoptosis and determining how those therapies may be modified to effect maximum apoptosis induction. The cell cycle apparatus and apoptosis have attracted the attention of researchers intent on developing new types of anticancer therapy [109,110].

We will concentrate in this part of the review on the evolution of the chemical structures and on the biological properties, whilst the chemical syntheses will be referred to through the corresponding original references.

3.1. Benzo-fused seven-membered derivatives linked to 5-fluorouracil (5-FU)

We have reported the synthesis and anticancer activities of compounds **1**-**5** [16] and *trans*-**6** [17] (Figure 1). In all cases, the linkage between the 5-FU moiety and the seven-membered ring was carried out through its *N*-1 atom. The structural nature of **5** implies that this compound cannot be considered as a 5-FU prodrug and it was suspected that the remaining compounds (**1**-**6**) would not be 5-FU prodrugs.

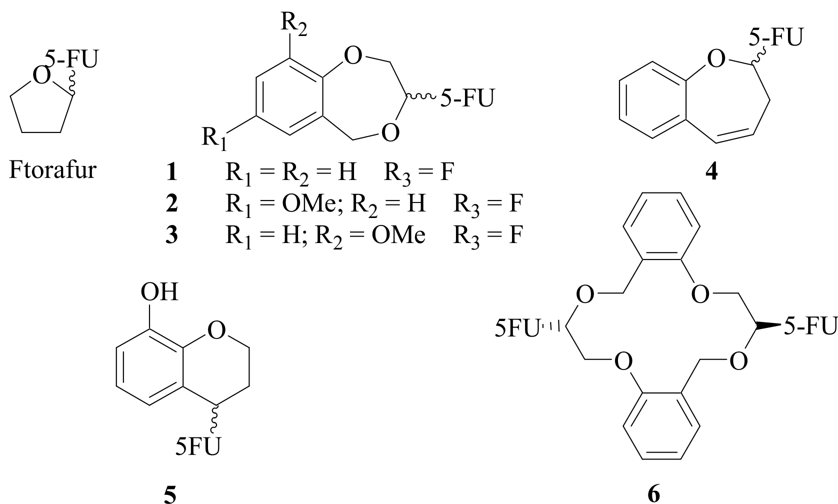


Figure 1. Several 5-FU derivatives showing interesting anti-tumour activities.

The IC_{50} values of the 5-FU cyclic *O,N*-acetals are shown in Table 1 (entries 4-9). The most active compounds are **1**, **2** and **6** (entries 4, 5 and 9). On comparing structures **4** and **1**, it is worth emphasizing that the bioisosteric change of carbon for oxygen and the saturation of the double bond in compound **1** increases the anti-proliferative activity two fold in (IC_{50} = 7.00 ± 0.61 μM, entry 4). The introduction of a methoxy group into the benzene ring of **1** provokes different influences on the anti-proliferative activities. Thus, the C-7 substitution produces an increase of the anti-proliferative activity (**2**, IC_{50} = 4.50 ± 0.33 μM, entry 5), whilst if C-9 is the substituted position it gives rise to a decrease in the anti-proliferative activity of **3** (IC_{50} = 22.0 ± 0.93 μM, entry 6).

Apoptosis has been studied in terms of cancer development and treatment with attempts made to identify its role in chemotherapeutic agent-induced cytotoxicity. Cytotoxic agents often induce only a fraction of the cells to become apoptotic. To fully exploit apoptosis as a mechanism of anti-neoplastic agent response, a larger proportion of cells needs to be recruited into apoptosis. Paclitaxel (Taxol®), cyclophosphamide and cytosine arabinoside

are the only commonly used cytotoxic agents shown to elicit apoptosis in breast cancer cells [111,112]. Quantitation of apoptotic cells was done by monitoring the binding of fluorescein isothiocyanate (FITC)-labelled annexin V (a phosphatidylserine-binding protein) to cells in response to our title compounds as described [113]. The apoptosis study shows that **3**, **4** and **5**, at their IC_{50} concentrations, provoke early apoptosis in the cells treated for 24 and 48 h. It is worth pointing out that **3** (entry 6) induces greater apoptosis at 48 h (46.73%) than at 24 h (40.08%). The compounds that show the most important apoptotic indexes at 24 h are **4** (57.33%, entry 7) and **5** (54.33%, entry 8), whereas at 48 h is **4** (51.37%, entry 7). These compounds are more potent as apoptosis inductors against the MCF-7 human breast cancer cells than paclitaxel (Taxol®), which induced programmed cell death of up to 43% of the cell population [114]. Accordingly, the early apoptotic inductions and the low IC_{50} values give rise to a significant anti-tumour activity.

Since the synthesized compounds induce very important apoptosis, we have carried out studies of the expression of some of the genes that intervene in this phenomenon, among which p53 and the family bcl-2 are outstanding. The tumour suppressor gene p53 protects the integrity of the genome so that if the DNA of the cell is damaged by an agent, an over-expression of it is produced inducing the stopping in G_1 for the repair of the damage, or if this is not possible, enter apoptosis [115]. On the other hand, the members of the family of proteins Bcl-2 work as regulators of apoptosis, Bcl-2 and Bcl-XL protecting against apoptosis. Bax, Bak and Bad induce such a phenomenon [116]. The treatment of the MCF-7 cells (wild-type p53) with these compounds provoked in general an increase in the protein expression of p53, mainly for 5-FU and **4**, and a marked decrease of the levels of bcl-2 for all of them. These data show that p53 activity is restored with the compounds, allowing the entrance of the tumour cells in apoptosis, which permits their elimination by this mechanism. In the same way bcl-2 inhibition facilitates the entrance of cells into the programmed cell death.

Entry	Compound	IC_{50} (μM)[a]	Cell Cycle (48 h)[b]			Apoptosis[c]	
			G_0/G_1	G_2/M	S	24 h	48 h
1	Control		68.39	12.04	19.57	1.24	1.24
2	**5-FU**	2.75	58.07	2.10	39.38	56.75	52.81
3	**Ftorafur**	3.00 ± 0.11	45.62	0.00	54.38	52.20	58.06
4	**1**	7.00 ± 0.61	74.41	15.77	9.82	8.45	12.17
5	**2**	4.50 ± 0.33	73.41	13.15	13.44	1.50	3.50
6	**3**	22.0 ± 0.93	71.76	10.08	18.16	40.08	46.73
7	**4**	14.0 ± 1.02	86.14	1.60	12.26	57.33	51.37
8	**5**	69.0 ± 2.31	68.61	9.60	21.79	54.33	35.49
9	**6**	5.50 ± 0.58	82.48	5.13	12.40	14.37	19.05

[a]See [117]. [b]Determined by flow cytometry: see [16]. [c]Apoptosis was determined using an annexin V-based assay [113]. The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations.

Table 1. Anti-proliferative activitiy, cell cycle dysregulation, and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 24 and 48 h for the compounds.

3.2. 1-[2-(2-hydroxymethylphenoxy)-1-methoxyethyl]-5-fluorouracils 7a-f: antiproliferative activity, cell cycle dysregulation and apoptotic induction against breast cancer cells

Acyclic 5-FU *O,N*-acetals **7a-f** were previously reported by us (Figure 2) [17]. The aminalic bond is established through *N*-1 of the 5-FU moiety.

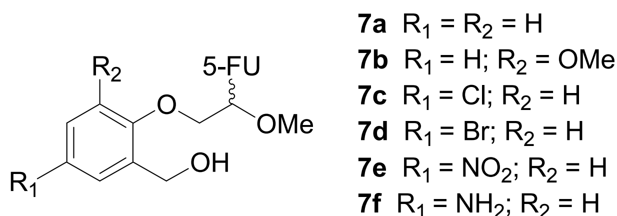
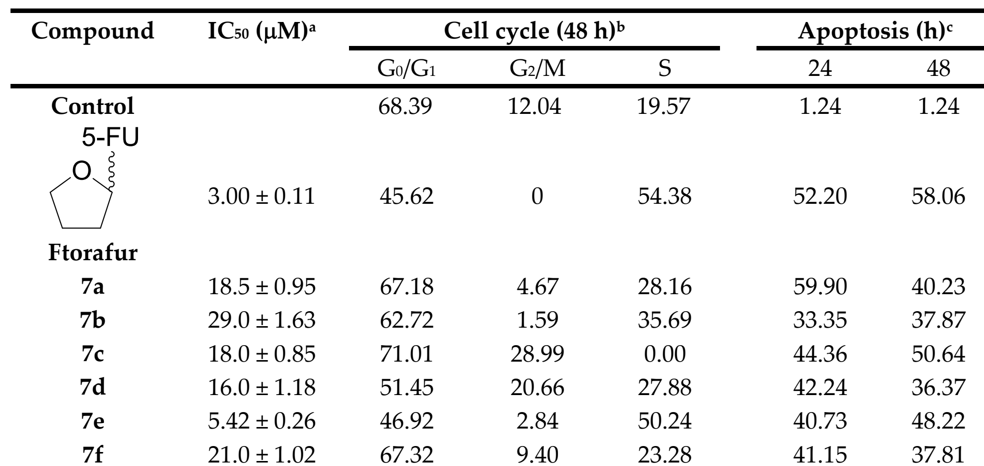


Figure 2. Several acyclic 5-FU *O,N*-acetals previously reported by us [17].

The IC_{50} values of compounds are shown in Table 2. The most active compound is **7e** (5.42 ± 0.26 μM), with an anti-proliferative activitiy in the same order as that of Ftorafur (3.00 ± 0.11 μM). Cell cycle regulation has attracted a great deal of attention as a promising target for cancer research and treatment. The use of cell-cycle-specific treatments in cancer therapy has greatly benefited from the major advances that have been recently made in the identification of the molecular actors regulating the cell cycle and from the better understanding of the connections between cell cycle and apoptosis. As more and more 'cell cycle drugs' are being discovered, their use as anticancer drugs is being extensively investigated [118]. To study the mechanisms of the anti-tumour and anti-proliferative activities of the compounds, the effects on the cell cycle distribution were analyzed by flow cytometry. DMSO-treated cell cultures contained 68.39% G_0/G_1-phase cells, 12.04% G_2/M-phase cells and 19.57% S-phase cells. In contrast, MCF-7 cells treated during 48 h with the IC_{50} concentrations of **7a–f** showed important differences in cell cycle progression compared with DMSO-treated control cells. The treatment with Ftorafur showed a decrease of the G_0/G_1-phase cells and a corresponding accumulation of S-phase cells (45.62% G_0/G_1-phase cells and 54.38% S-phase cells). Moreover, there was an almost total disappearance in the G_2/M population of the cells treated with this drug. In general the cell cycle regulatory activities for the newly synthesized compounds can be divided into the following three groups: (a) **7c** and **7d** accumulated the cancerous cells in the G_2/M-phase, in the former compound at the expense of the S-phase cells, and (b) **7e** induced a S-phase cell cycle arrest (50.24%) in a similar percentage to that caused by Ftorafur (54.28%, Table 2). Therefore, it can be affirmed that the nitro derivative (**7e**) may act as a 5-FU prodrug. Nevertheless, this hypothesis needs to be corroborated by further assays. In response to **40a**, the percentage of apoptotic cells increased, from 1.24% in control cells to a maximum of 59.9% apoptotic cells (24 h) at a concentration equal to its IC_{50} against the MCF-7 cell line. This is a remarkable property because the demonstration of apoptosis in MCF-7 breast cancer cells by known apoptosis-inducing agents has proved to be difficult and only few cytotoxic agents act preferentially through an apoptotic mechanism in human breast cancer cells [113,114]. Finally, a fact worth

emphasizing is that **7e** (the only compound tested) induced neither toxicity nor death in mice after one-month treatment when administered intravenously twice a week, with a 50 mg/kg dose each time.



Compound	IC_{50} (μM)[a]	Cell cycle (48 h)[b]			Apoptosis (h)[c]	
		G_0/G_1	G_2/M	S	24	48
Control		68.39	12.04	19.57	1.24	1.24
5-FU **Ftorafur**	3.00 ± 0.11	45.62	0	54.38	52.20	58.06
7a	18.5 ± 0.95	67.18	4.67	28.16	59.90	40.23
7b	29.0 ± 1.63	62.72	1.59	35.69	33.35	37.87
7c	18.0 ± 0.85	71.01	28.99	0.00	44.36	50.64
7d	16.0 ± 1.18	51.45	20.66	27.88	42.24	36.37
7e	5.42 ± 0.26	46.92	2.84	50.24	40.73	48.22
7f	21.0 ± 1.02	67.32	9.40	23.28	41.15	37.81

[a]See [117]. [b]Determined by flow cytometry: see [16]. [c]Apoptosis was determined using an annexin V-based assay [113]. The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations.

Table 2. Anti-proliferative activitiy, cell cycle dysregulation, and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 24 and 48 h for the compounds.

3.3. Benzo-Fused Seven-Membered Derivatives Linked To Purines

Compounds **8-10** (Figure 3) were synthesized as reported.

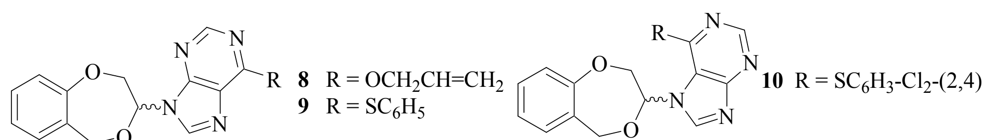


Figure 3. Several cyclic *O,N*-acetals reported by us [119].

The anti-tumour potential of the target molecules is stated against the MCF-7 human breast cancer cell line (Table 3). The most active compound (**8**), that presents an allyloxy group as substituent at position 6 of the purine ring, shows an IC_{50} = 5.04 ± 1.68 μM nearly equipotent as 5-FU. Compounds, **9** and **10**, present bulky substituents as the phenylthio and 2,4-diclorophenylthio ones, respectively.

To study the mechanisms of the anti-tumour and anti-proliferative activities **8-10**, the effects on the cell cycle distribution were analyzed by flow cytometry (Table 3). DMSO-treated cell cultures contained a 58.62 ± 0.74 of the G_0/G_1-phase cells, a 33.82 ± 0.72 of the S-phase cells and a 7.55 ± 1.34 of the G_2/M-phase cells. In contrast, MCF-7 cells treated during 48 h with

the IC_{50} concentrations of **8**, **9** and **10** showed important differences in cell cycle progression compared with DMSO-treated control cells. The cell cycle regulatory activities can be divided into the following two groups: (a) the breast cancer cells showed an accumulation in the S-phase, up to 37.00 ± 2.00 of the cells, mainly at the expense of the G_0/G_1-phase population that decreased to a percentage of 55.63 ± 1.57 of the cells; (b) compounds **9** and **10** accumulated the cancerous cells in the G_2/M-phase (11.08 ± 1.01 and 19.16 ± 0.56, respectively) at the expense of the S-phase cells (26.82 ± 1.26 and 22.73 ± 0.37, respectively). In response to **9** (and **10**), the percentage of apoptotic cells increased, from 0.22 ± 0.31 in control cells to a maximum of 73.37 ± 0.12 (and 65.28 ± 1.92) apoptotic cells at a concentration equal to their IC_{50} against the MCF-7 cell line. This is a remarkable property because the demonstration of apoptosis in MCF-7 breast cancer cells by known apoptosis-inducing agents has proved to be difficult.

Compound		**Cell cycle**[a]			**Apoptosis**[b]
	IC_{50} (μM)	**G_0/G_1**	**S**	**G_2/M**	
Control		58.62 ± 0.74	33.82 ± 0.72	7.55 ± 1.34	0.22 ± 0.16
8	5.04 ± 1.68	55.63 ± 1.57	37.00 ± 2.00	7.37 ± 0.43	44.47 ± 2.98
9	7.12 ± 0.46	59.10 ± 1.28	26.82 ± 1.26	11.08 ± 0.01	73.37 ± 0.12
10	8.40 ± 0.91	58.10 ± 0.19	22.73 ± 0.37	19.16 ± 0.56	65.28 ± 1.92

[a] Determined by flow cytometry.[3] [b] Apoptosis was determined using an Annexin V-based assay [120]. The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations.

Table 3. Anti-proliferative activity, cell cycle distribution and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h for the three most active compounds.

3.4. Anti-cancer activity of 9-(2,3-Dihydro-1,4-benzoxathiin-3-ylMethyl)-9H-purines

Compounds **1-6** may be considered as drugs with their own entity and anti-tumour activity independent of that of 5-FU. If the previously described compounds are not prodrugs, it is not necessary to maintain the *O,N*-acetalic characteristic with the corresponding weakness of the *O,N*-acetalic bond. Therefore, molecules are being designed in which both structural entities (such as the benzo-heterocyclic ring and the purine base) are linked by a heteroatom-C-C-base-N-atom bond. The design, synthesis and biological evaluation of a series of 2- and 6-disubstituted 9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purine derivatives **11-13** were described (Figure 4, Table 4) [121].

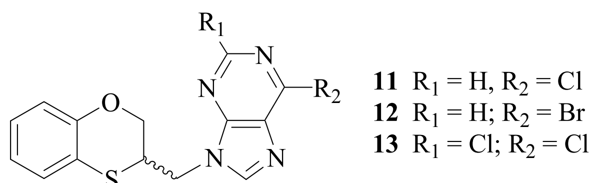


Figure 4. Several non-acetalic purine derivatives reported by us [121].

Compounds **11-13** were subjected to cell cycle and apoptosis studies on the MCF-7 human breast cancer cell line (Table 4). The following two consequences can be stated: (a) in contrast to 5-FU, the six-membered compounds **11-13** provoked a G_0/G_1-phase cell cycle arrest when the MCF-7 cells were treated during 48 h with the IC_{50} of the compounds, mainly at the expense of the S-phase populations. The fact that at similar doses the novel derivatives exhibit different sequences of cell cycle perturbations in comparison with 5-FU indicates that these compounds act by different pathways [12]. In the case of **12** it is worth pointing out that, moreover, there is an increase in the G_2/M-phase of the cancerous cells; and (b) the apoptotic indices of the target compounds are very important, especially for **13** (58.29% for **11**, 63.05% for **12**, and 76.22% for **13**). Up to now and according to our knowledge, compound **13** is the most important apoptotic inducer against the MCF-7 human breast cancer cell line so far reported.

Compound		Cell cycle[a]			Apoptosis[b]
	IC_{50} (μM)	G_0/G_1	S	G_2/M	
Control		58.62 ± 0.74	33.82 ± 0.72	7.55 ± 1.34	0.22 ± 0.16
5-FU[c]	4.32 ± 0.02	58.07 ± 0.11	39.38 ± 0.98	2.10 ± 0.12	52.81 ± 1.05
11	10.6 ± 0.66	69.71 ± 1.50	23.73 ± 1.65	6.56 ± 0.17	58.29 ± 0.75
12	6.18 ± 1.70	62.85 ± 0.87	26.71 ± 1.25	10.43 ± 0.38	63.05 ± 0.26
13	8.97 ± 0.83	70.30 ± 0.32	23.67 ± 2.40	6.06 ± 2.72	76.22 ± 2.02

[a]Determined by flow cytometry [12]. [b]Apoptosis was determined using an Annexin V-based assay [12]. The data indicate the percentage of cells undergoing apoptosis in each sample. [c]Data were taken from [117]. All experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations.

Table 4. Anti-proliferative activity, cell cycle distribution and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h for the three most active compounds as anti-proliferative agents.

3.5. Anti-cancer activity of cyclic and acyclic O,N-acetals derived from purines and 5-FU

We have recently published two *O,N*-acetals with outstanding anti-proliferative activities. The most potent antiproliferative agent against the MCF-7 adenocarcinoma cell line belongs to the benzoxazepine *O,N*-acetalic family is 9-[1-(9*H*-fluorenyl-9-methoxycarbonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-yl]-6-chloro-9*H*-purine (**14**, IC_{50} = 0.67 ± 0.18 M), whilst 7-{2-(*N*-hydroxymethylphenyl)-2-nitrobenzenesulfonamido]-1-methoxyethyl} -6-chloro-7*H*-purine (**15**) shows the lowest IC_{50} value among the family of acyclic *O,N*-acetals (IC_{50} = 3.25 ± 0.23 M) (Figure 5). The global apoptotic cells caused by **14** and **15** against MCF-7 were 80.08% and 54.85% of cell population after 48 h, respectively. cDNA microarray technology reveals potential drug targets, which are mainly centred on positive apoptosis regulatory pathway genes, and the repression of genes involved in carcinogenesis, proliferation and tumour invasion [21]. We demonstrated later on that, when the anthranilic alcohol-derived acyclic 5-fluorouracil *O,N*-acetal **16** was administered to human breast cancer cells MCF-7, had no activity against classic pro-apoptotic genes such as *p53*, and even induced the down-regulation

of anti-apoptotic genes such as *Bcl-2*. In contrast, several pro-apoptotic genes related with the endoplasmic reticulum (ER)-stress-induced apoptosis, such as *BBC3* and *Noxa*, appeared up-regulated. These results seem to show that the mechanism of action and selectivity of **16** was via the activation of the ER-stress-induced apoptosis. The linkage between the 5-FU moiety and the chain of **16** is through the *N*-1 atom of the pyrimidine [20] (Figure 5).

14 **15** **16**, R = SO_2-C_6H_4-*o*NH_2

Figure 5. Several *O,N*-acetalic purine derivatives reported by us [20,21]. Fmoc is the fluorenylmethyloxycarbonyl group; *o*Ns is the -SO_2-C_6H_4-*o*NO_2 group.

4. Inhibitor of apoptosis protein (IAP) family as therapeutic target

In the apoptotic pathway, the balance between pro and anti-apoptotic proteins is tightly regulated, so an imbalance directed to the anti-apoptotic regulation involves the apparition of survival advantages onto the initiating cancerous cells. In this way, the role of the IAPs family proteins is important. This family was first identified in baculovirus and the main characteristic is the presence of a baculovirus IAP repeat (BIR) domain, that mediates interactions with a number of proteins, including caspases and also other structural domains such as a RING or caspase activation recruitment domain (CARD) [122] (Figure 6).

In the IAP family we can find proteins involved in the inhibition of apoptosis through inhibition of caspases or modulation of the nuclear factor kB (NF-kB) signalling pathways. This family includes eight members in mammals (BIRC1– BIRC8), also known as neuronal apoptosis inhibitory protein (NAIP), cellular IAP1 (cIAP1) and IAP2 (cIAP2), Xchromosome linked IAP (XIAP), survivin, Apollon (BRUCE), livin (ML-IAP) and ILP2 [123] (Figure 6).

4.1. Role of the IAPs in apoptosis

As long as apoptosis pathway must be tightly regulated, the IAPs are the only known regulatory proteins that control the activity of both initiator and effectors caspases in the process. In this way, there are studies where XIAP was found to prevent caspase-3 processing in response to caspase-8 activation. So it is suggested that XIAP is able to inhibit this extrinsic apoptotic signaling by blocking the downstream effectors caspases, avoiding the direct interference of the caspase-8 activation [124].

On the other hand, other IAPs proteins are not potent inhibitors of caspases, such as c-IAP proteins. They are able to bind with Smac having high affinities to prevent it from blocking XIAP-mediated inhibition of caspases. These IAPs also show a E3 ubiquitin ligase activity,

allowing the regulation of several cell death effectors and modulators, playing a key role in cellular survival. c-IAP1 and c-IAP2 are able to interact with TNFα-receptor-associated factors -1 and -2 (TRAFs) trough their association in a complex with TNFα receptor 2. The TNFα receptor can mediate survival and death cell signals. In this case, c-IAP1 and c-IAP2 have been proposed to reduce the level of caspase-8 activation and protect cells from apoptosis in a TNFα relationship [10].

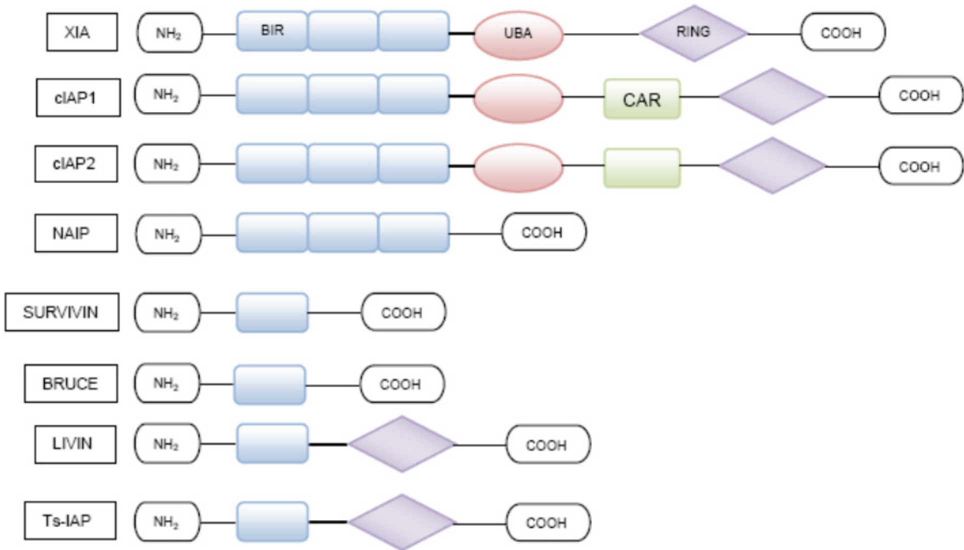


Figure 6. Identifying some of the structural motifs of IAPs. The baculoviral IAP repeat (BIR) domains; 70–80-amino-acid cysteine- and histidine-rich domains that chelate zinc ions. The presence of at least one BIR domain is the defining characteristic of the IAP family. The number of BIR domains in a given IAP varies from one to three. Another motif is the really interesting new gene (RING) zinc finger, a caspase recruitment domain and ubiquitin-associated (UBA) domains are found in individual IAPs. Finally, the CARD motif (Caspase Recruitment Domain) is a protein–protein interaction domain that mediates oligomerization with other CARD-containing proteins and is found in a number of proteins involved in the regulation of cell death.

There is another family member of the IAPs, called Livin, which are expressed in high levels in melanoma and colon cancer, in embryonic tissues and transformed cells. The over-expression of Livin isoforms α and β blocks apoptosis induced via the extrinsic death receptor pathway [125].

Survivin is involved in the control of cell proliferation and cell death and gene expression is regulated in a cell cycle dependent manner in mitosis. The spliced transcription of the survivin gene gives rise to wild-type survivin, survivin-2α, survivin-2B, survivin-ΔEx-3 and survivin-3B. This protein is stabilized by phosphorylation thanks to a p34cdc2–cyclin B1 complex during mitosis. The anti-apoptotic function of survivin has been linked with its interaction with Smac/DIABLO, with the stabilization of XIAP protein through its binding to

XIAP and with the inhibition of mitochondrial and apoptosis-inducing factor-dependent apoptotic pathways [126].

Other IAPs is Bruce/Apollon which is a membrane bounded protein involved in protein ubiquination-mediated degradation by its ability to target proteins thanks to the presence of a C-terminus E2 motif. It is also shown that Bruce is able to bind to caspase -3, -7 and -9 [127]. In Bruce regulation are involved the E2 UbcH5 and the E3 Nrdp1, which ubiquitinate the epidermal growth factor receptor family member, ErbB3. It has been shown that a decrease in Bruce content by Nrdp1 over-expression induces apoptosis in different cell lines. These studies suggest that this IAP protein play a critical role in apoptosis inhibition in certain cell types avoiding pro-caspase-9 cleavage when it binds to this protein [128].

Naip has been studied because of the clinical relevance in tumors such as prostate or breast cancer. Naip has two different functions; in the first one it is involved in the inflammatory process by caspase-1, -4 and -5 activation, and the second one is via apoptosis regulation by caspase-3 and -7 inhibition [129]. Davoody *et al* showed the cleavage inhibition of procaspase-3 by apoptosome activated caspase-9 and the inhibition of the autocatalytic processing of procaspase-9 in the apoptosome complex. This fact indicates that unlike other IAPs, Naip is an inhibitor of procaspase-9 [130].

Finally, Ts-IAP, also known as ILP-2, is the product of a human testis-specific mRNA and is related to the C-ter region of another member of the IAPs protein family, XIAP. It is showed that this protein is a weak caspase-9 inhibitor, and also a highly unstable molecule. However, a stabilized form of this protein containing nine additional N-ter residues may form a complex with Smac/DIABLO [131].

4.2. Use of IAPs into clinic: prognostic and therapeutic values

The expression and/or function of IAPs are deregulated in many human cancers because of genetic aberrations, an increase in their mRNA or protein expression or the loss of endogenous inhibitors such as Smac. The expression levels of IAPs and their antagonists have been correlated with clinical parameters and cancer prognosis in several retrospective trials. However, these results should be interpreted with caution due to the low numbers of samples studied in some reports and the limitations of currently available reagents for analyzing the expression of IAP proteins in tissue specimens. Therefore, additional studies are required to evaluate the prognostic value of IAPs in human malignancies [10].

The issue of primary or acquired resistance to current chemotherapeutic-based treatments is a major impediment to effective cancer treatment. Although there are many genetic and biochemical alterations that occur in cancer cells, in vitro experiments demonstrate that the up-regulation of IAPs expression increases resistance to chemotherapy and radiation. The fundamental role of the IAPs in apoptosis regulation and their elevated expression in many tumour types suggest that there is value in exploiting the inhibition of IAP expression and function as a direct therapeutic strategy [125].

Novel drugs have been developed and some of them are in current clinical trials. Several strategies have been chosen for anticancer drug development targeting IAP molecules: (1) small-molecule IAP antagonists, (2) antisense oligonucleotides (ASOs), (3) Smac mimetic molecules and others [132]. Small-molecule IAP antagonists and antisense oligonucleotides have garnered the most attention. Also, IAP antagonists have been extensively studied in combination with other cytotoxic agents including anticancer drugs, small-molecule signal transduction inhibitors, proteasome inhibitors and death receptor ligands as well as with radiation therapy.

4.2.1. Small-molecule IAP antagonists

Owing to the differences in the mechanism of caspase inhibition by the BIR 2 and BIR 3, domain molecules have been developed to specifically target the BIR 2 or BIR 3 region of XIAP. The structural data surrounding the interaction between the BIR 3 domain of XIAP and caspase-9, suggests that small molecules that bind the BIR 3 pocket of XIAP could mimic the action of Smac and inhibit the interaction between XIAP and caspase-9. These structural studies have facilitated a variety of chemical biology approaches including fluorescent polarization, nuclear magnetic resonance, 'in silico' virtual screening and computer modelling to identify BIR 3 inhibitors [133]. The first small-molecule XIAP inhibitors were reported by [134]. These inhibitors were identified using a high throughput enzymatic derepression assay in which recombinant XIAP was combined with active caspase-3 to inhibit caspase-mediated cleavage of the fluorogenic peptide substrate. With this assay, they screened 160.000 compounds in 1536-well format and identified potent XIAP inhibitors including the compounds TWX006 and TWX024, aryl sulphonamides with flexible acyclic diamines in the first and third fragments. These compounds derepressed XIAP-mediated inhibition of caspase-3 more potently than Smac. In addition, these molecules bound the BIR 2-linker region of XIAP, and, in enzymatic assays, relieved the repression of caspase-3 more potently than Smac peptides.

Recently, small-molecule phenylurea-based chemical inhibitors of XIAP were identified by large-scale combinatorial library screening. Subsequent studies have confirmed that the active XIAP inhibitors, but not their inactive structural analogues, could induce apoptosis in a variety of human cancer cell lines and xenograft. Furthermore, it was determined that these XIAP inhibitors act by binding to its BIR-2 domain, resulting in elevated activity of the downstream caspase-3 and caspase-7. Thus, the action of these exogenous XIAP inhibitors was found to be mechanistically distinct from that of the endogenous inhibitor second modulator of apoptotic proteases, which predominantly binds to the BIR-3 domain [135].

4.2.2. Antisense oligonucleotides

The single-stranded antisense oligodeoxynucleotides (AS ODNs) are short stretches of synthetic DNA, approximately 12–30 nucleotides long, and are complementary to a specific mRNA strand. Hybridization of the AS ODNs to the mRNA by Watson–Crick base pairing prevents the target gene from being translated into protein, thereby blocking the action of

the gene, and resulting in the degradation of the target mRNA. The specificity in the AS ODN approach is based on the fact that any sequence of approximately 13 bases in RNA and 17 bases in DNA is estimated to be represented only once in the human genome. AS ODNs targeting survivin expression in human lung adenocarcinoma cell lines decreased survivin protein levels in a dose-dependent manner, induced apoptosis, stimulated higher levels of caspase-3 activation, and increased the sensitivity of cells to chemotherapeutics. XIAP AS ODNs effectively down-regulated both specific mRNA and protein levels in human non-small cell lung cancer growth both in vitro and in vivo. XIAP AS ODNs effectively induced apoptosis on their own and sensitized the tumor cells to the cytotoxic effects of several chemotherapeutics, including Taxol, etoposide and doxorubicin [26]. Furthermore, the administration of XIAP AS ODNs in a xenograft model of human non-small cell lung cancer results in a significant down-regulation of XIAP protein [124].

AEG35156 is a 19-mer oligonucleotide targeting XIAP. Its sequence was designed to achieve maximal stability and potency and to minimize immunostimulation through avoiding CpG motifs. Phase I studies in patients with refractory malignancies established safety [136]. A phase I/II study of AEG35156 in combination with idarubicin and high-dose cytarabine in patients with relapsed or refractory AML demonstrated a dose-dependent knock-down of XIAP mRNA and protein and a promising response rate [137]. The molecule may rapidly enter randomized studies in AML while being also tested in lymphomas. The dose-limiting toxicity of an antisense oligonucleotide designed to inhibit surviving mRNA expression (LY2181308) was headache and the compound demonstrated some biological efficacy in decreasing survivin expression [138]. A phase II study has opened in solid tumours. The locked nucleic acid strategy was used to design other survivin-targeting antisense oligonucleotides, including SPC3042 [139] and EZN-3042 [140], which are currently being evaluated in Phase I clinical trials as single agents and in combination with cytotoxic drugs.

4.2.3. Smac-mimicking IAP antagonist

Smac mimetics may be a useful therapeutic target as over-expression of Smac may potentiate apoptosis by neutralizing the caspase-inhibitory function of IAPs. Following the discovery that an IAP-binding motif consisting of four NH2-terminal amino acid residues was sufficient to bind to the BIR3 domain of XIAP, Smac-peptide mimetics were constructed which were capable of competing with caspase-9, displacing it from the BIR3 domain of XIAP. The three members of the IAP family, XIAP, cIAP1, and cIAP2, are structurally homologous (XIAP amino acid sequence identity to cIAP1 and cIAP2 of 36% and 39%, respectively, the amino acid sequence identity between cIAP1 and cIAP2 is 70%). In particular, the BIR3 IBM region is well conserved among the three IAPs. The XIAP BIR3 residues involved in van der Waals contacts (Val298, Lys299, and Trp310) and hydrogen bonds (Gly306, Leu307, and Trp323) with the inhibitory compounds are conserved. Minor exceptions are Leu292, replaced by Val in the cIAPs, Glu314 substituted by Asp in both cIAPs, and Gln319, which is Glu325 in cIAP1, and Gln311 in cIAP2. Finally, residues Thr308 and Asp309 that were found relevant for Smac-mimetics interaction with XIAP BIR3, are replaced by Arg314/Arg300 and Cys315/ Cys307 in cIAP1/cIAP2, respectively [127].

Smac interacts with IAPs via its N-terminal tetrapeptide, Ala1- Val2-Pro3-Ile4 (AVPI). Using this structural information, several groups designed small molecules mimicking AVPI to derive proteolytically stable compounds. Monovalent Smac mimetics were designed to mimic the Smac AVPI-binding motif and so target the XIAP-BIR3 domain. They exhibit high affinities, not only to XIAP-BIR3 but also to cIAP1, cIAP2 and MLIAP proteins. Bivalent Smac mimetics, containing two AVPI-binding motifs, bind to XIAP-BIR2–BIR3 with an extremely high affinity, exceeding that of Smac protein. Preclinical profiling studies have shown that Smac mimetics effectively sensitize cancer cells to other therapeutic agents, but they are also capable as single agents of inducing apoptosis in some but not all human cancer cell lines. To date, two Smac mimetics have reached phase I clinical development, and approximately ten are in an advanced preclinical development stage and are expected to enter human clinical testing soon [141].

Smac mimetic binds to XIAP and induces cIAP degradation. There are two cellular events that result from cIAP degradation: (1) activation of the non-canonical NF-κB pathway and subsequently increased production of autocrine TNF-α and (2) release of RIP1 from the TNF-α receptor complex, leading to caspase-8 activation, which requires TNF-α receptor to be activated in the first place. Accumulating evidence suggests that whether or not Smac mimetic induces autocrine TNF-α production is the key factor in deciding the cell's fate upon Smac mimetic treatment. Depending on their response to Smac mimetics cells can be grouped into three general classes: in class I cells, Smac mimetic induces autocrine TNF-α production and massive cell loss; in class II cells, Smac mimetic still induces autocrine TNF-α production but this does not have a major effect on the cell population; and in class III cells, Smac mimetic has no effect on autocrine TNF-α production or on cell death. The ability of cells to produce TNF-α is necessary but not sufficient for Smac mimetic to induce cell death as a single agent [142].

5. miRNA-based therapy

The miRNAs, is a class of endogenous, small, non-coding RNAs of 18–25 nucleotides in length, that negatively regulates gene expression by degradation of mRNA or suppression of mRNA translation. Mature miRNA products are formed in from a longer primary miRNA (pri-miRNA) transcript through sequential processing by the ribonucleases Drosha and Dicer1 [143,144]. miRNAs are known to repress thousands of target genes because only partial complementarity to the target mRNA is required. Thus, one miRNA may be simultaneously targeting a complexity of mRNAs as well as the expression of a single mRNA may be regulated by many miRNAs [143].

The miRNAs are involved in normal processes, including cellular development, differentiation, proliferation, apoptosis, and stem cell self-renewal [28].The aberrant expression or alteration of miRNAs contributes to a range of human pathologies, including cancer. Furthermore, the deregulation of miRNA causes evasion of apoptosis which involved tumourigenesis and drug resistance [122]. During tumour initiation and progression, the functionality of aberrant miRNAs may act as oncogenes (OncomiRs) or tumour suppressors (TSmiRs), a numbers of

them are strongly related to the apoptosis phenomenon. Therefore, manipulation of miRNA expression levels which target genes and pathways that are involved in apoptosis could be a potential therapeutic strategy for developing efficient therapies against cancer. In addition, given that cancer cells often exhibit a distinctive pattern of miRNA expression, unique profiles of altered miRNAs expression could be useful as molecular biomarkers for tumour diagnosis, prognosis of disease-specific outcomes, and evaluation of tumour aggressiveness. Based on this, several anticancer therapies focusing on restoring miRNA activities and repairing gene regulatory networks or drug sensitivity are being developed.

There are two strategies of molecular therapy targeted at miRNA, one by the inhibition of oncogenic miRNAs and the other the over-expression of tumour suppressor miRNAs.

5.1. Targeting oncogenic miRNAs

The oncomiRs could be blocked by different approaches such as (i) antisense oligonucleotides, (ii) antagomirs, (iii) locked nucleic acid (LNA) constructs or (iv) sponges [145]. Antisense oligonucleotides work as competitive inhibitors of miRNA, leading to the up-regulation of tumour suppressor proteins, inducing apoptosis and blocking tumour formation in vitro and in vivo. Some of the potential ways to enhance miRNA stability include chemical modifications. In order to improve their effectiveness and stability, antisense oligonucleotides, have been modified on their 50 end, by adding 20-O-methyl and 20-O-methoxyethyl groups [146]. Oligonucleotides with 2′-O-methyl groups have proved to be effective inhibitors of miRNA expression in several cancer cell lines [30,147,148,149]. The utility of anti-miRNA oligonucleotide in vivo through intravenous injection of modified anti-miRNA oligonucleotide O-methyl-modified cholesterol-conjugated single stranded RNA analogues has been studied, with phosphorothioate linkages, an 'antagomirs', to target the liver-specific miR-122. Specific miR-122 silencing for up to 23 days was conferred with only a single injection of 240 mg•kg-1 body weight [150].

On the other hand, the locked-nucleic-acid antisense oligonucleotides exhibit relatively low toxicity and have been optimized by reducing their molecular size which has increased their therapeutic potential [151]. LNA anti-miR' constructs have been used successfully in several in vitro studies to knock down specific miRNA expression [151,152]. Also it has showed that miR-221 and miR-222 knockdown through antisense LNA oligonucleotides increases p27Kip1 in human prostate cancer (PC3) cells and strongly reduces their clonogenicity in vitro [153]. In vivo, the use of LNAs has achieved unexpected success for the treatment of hepatitis C in non-human primates [154]. These finding demonstrate the impressive potential of this strategy to overcome a major hurdle for clinical miRNA therapy.

Moreover, other techniques have emerged as an effective way to repress expression levels of miRNA families. A new form of miRNA inhibitors that can be transiently expressed in cultured mammalian cells, "miRNA sponges", was developed. Sponges are ectopically expressed mRNAs that contain multiple miRNA target sites of miRNA that share the same seed sequence [155]. In contrast to miRNA sponges, Xiao et al. designed alternative strategy called "miRNA masking" which covers up the miRNA-binding site to depress its target mRNA [156].

Although several in vitro and in vivo technologies have been developed to inhibit the oncomiRs, it is still a long and arduous way to go for substantial applications of miRNAs in cancer treatments. To date, many OncomiRs seem to have a role in apoptosis (Table 5), therefore some have been regarded as hallmarks in tumour progressions and hot targets in cancer therapy. Thus, miR-21 is functionally considered oncogenic because it is overexpressed in various tumours [157,158]. In fact, antisense inhibition of miR-21 leads to the induction of programmed cell death in neuroepithelial cells, through activation of caspases [159]. This apoptosis induction was also confirmed in breast cancer, colon cancer, pancreas cancer, lung cancer, liver cancer, prostate cancer, stomach cancer and oral squamous cell carcinoma (OSCC) [31,160,161,162,163]. So far, multiple targets of miR-21 have been identified and mapped to anti-apoptotic signalling pathways which suggest a promising miRNA treatment for cancer [31,164]. Other examples have been reported; in breast cancer cells, the overexpresion of the anti-apoptotic Bcl-2 is restored by the silencing of miR-15a and miR-16 through the use of specific inhibitors [165]. As the anti-apoptotic Bcl-2 is frequently overexpressed in a number of human cancers, such as Hodgkin's lymphoma, cell lymphoma and breast, miR-15 and miR-16, it could be used for therapy of cancer-associated phenotypes.

miRNA	Function	Gene target	Cancer Type	Reference(s)
miR-221, miR-222	OncomiRs	PTEN p27kip1 Bim PUMA	Hepatocarcinoma, melanoma, glioblastoma, lung, prostate cancer, leukemia, gastric carcinoma	[153,166,167,168, 169,170]
miR-21	OncomiRs	PTEN PDCD4 Bcl-2 Fas L	Non-small cell lung cancer, breast, prostate, gastric, hepatocellular cancer, colorectal cancer, glioblastoma and leukemia	[31,157,164,165,171,172,173,174,175]
miR-17-92 cluster	OncomiRs	PTEN, BIM, p21	Lymphoma, lung, breast, stomach, colon and pancreatic cancer	[176,177]
miR-29a, miR-29b and miR-29c	Tumour suppressor	CDC42 and p85a (up-regulating p53), Bcl-2	Lymphocytic leukaemia, cholangiocarcinomahepatocarcinoma, colon, breast, and lung cancer	[148,149,178,179]
miR-34 family	Tumour suppressor	Bcl-2, SIRT1	Prostate cancer non-small-cell lung cancer and neuroblastoma	[180,181,182,183]
miR-15a, miR-16-1,	Tumour suppressor	Bcl-2, Mcl1, PDCD6IP	Leukemia, gastric cancer cells and prostate cancer	[30,184,185]
Let 7	Tumour suppressor	Bcl-Xl	Lung, colon, stomach, ovarian and breast cancer.	[186,187,188,189, 190]

Table 5. Key microRNAs involved in apoptosis

5.2. Restoration of tumour suppressor miRNAs

The restoration of tumour suppressor miRNAs, as a therapeutic strategy, includes viral delivery or synthetic miRNA mimics. Elevation of the expression levels of miRNAs can restore tumour inhibitory functions in cancer cells. Adeno-associated virus delivery of miRNAs or miRNA antagonists has the advantage of being efficient and because the virus does not integrate into the genome, non-mutagenic. In Myc-induced liver tumours, intravenous injection of adeno-associated virus 8 (AAV8)-expressing miR-26 resulted in the suppression of tumourigenicity by inducing tumour-apoptosis, without signs of toxicity [191]. These findings indicate a possibility strategy for the treatment of liver cancer, however, before this approach achieve widespread clinical use, the delivery and safety of different treatments needs to be improved.

Another strategy to increase the expression of a tumour-suppressor miRNA in cancer could be overcome by miRNA mimics, which are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNAs [192]. Introduction of synthetic miRNA mimics with tumour-suppressor function in cancer cells have been implicated to induce cell death and block proliferation in several studies [30,147,178,184,186]. In prostate and AMl cell lines mimics of miR-15a and miR-29 respectively, induced apoptosis by repression of anti-apoptotic genes Mcl-1 and Bcl-2 [184].

Multiple miRNAs have been found to inhibit the apoptotic pathway following their over expression during cancer development. Reduced expression of miR-15, miR-16, and let-7 has been observed in different types of cancers and as one consequence anti-apoptotic genes and apoptotic signalling pathways have been activated in these cancer cells [30,187,193]. As well, transfection of anti-miR-24 oligonucleotides has been proved to induce apoptosis in several cell lines [194]. It has been reported that miR-195, miR-24-2 and miR-365-2 act as negative regulators of the anti-apoptotic proto-oncogene Bcl2. The overexpression of these miRNAs caused an increase in apoptosis and also augmented the apoptotic effect of etoposide in breast cancer MCF7 cells [195]

5.3. miRNAs and drug resistance

Several miRNA, some of them related to apoptosis, have been associated with drug resistance. Deregulation of miR-214 is a recurrent event in human ovarian cancer and it has been shown that miR-214 induces cell survival and cisplatin resistance primarily through targeting the PTEN/Akt pathway [196]. Also, is known that the let-7 family of miRs plays a role in a host of cellular functions such as modulation of drug sensitivity. The miRNA let-7a which directly targeting caspase-3 is over-expressed in some human cancers and has been shown to induce resistance to a variety of drugs caspase-3-dependent apoptosis, including doxorubicin, paclitaxel and interferon-gamma. Let-7e was up-regulated in some ovarian cancer cell lines with increased resistance to doxorubicin. On other hand, it has been reported that let-7i is down-regulated in chemotherapy-resistant ovarian cancer, and reintroduction of let-7i can sensitize resistant ovarian cancer cell lines to platinum-based

chemotherapy [188]. In a non small cell lung cancer cell line, the down-regulation of miR-186* which increased the expression of its direct target, Caspase-10, has been indicated the cause of the apoptosis induced by the chemopreventive agent curcumin [189]. Thus, the effect of anti-cancer drugs that modulate cell proliferation apoptosis on miRNA expression profiles was explored and could help for predicting apoptosis resistance. As a result, the knowledge of potential miRNAs implicated in apoptosis resistance could avoid unnecessary morbidity and may represent a novel class of biomarkers for facilitating personalized treatment.

6. Cancer stem cells and apoptosis

Some cancers are originated in cells with intrinsic self-renewal activity or in differentiated cells in which self-renewal is activated by oncogenic mechanisms; hence, the study of normal self-renewal is important to improve our understanding of these mechanisms. Cancers express a spectrum of aberrantly differentiated cells, ranging from those that appear well differentiated to those that appear undifferentiated, and these phenotypes are commonly evident in the same tumour. This suggests that the transformation process can induce defects throughout the multistep differentiation process. Recent data suggest that cancers arise from rare self-renewing stem cells that are biologically distinct from their more numerous differentiated progeny. A small number of cells identified as cancer stem cells (CSCs) from solid tumours usually express organ-specific markers, contribute to chemotherapy resistance and are able to generate a new tumour in immunodeficient mice. Moreover, there is growing evidence that pathways regulating normal stem cell self-renewal and differentiation are also present in cancer cells and CSCs [197].

Currently, there are two theories on the origin of cancer: the classic clonal evolution theory or stochastic model, by which malignant transformation results from random mutations and subsequent clonal selection of cancer cells with similar potential to regenerate the tumour growth [198,199]; and the CSC hypothesis, which considers the tumour to be formed by a small population of cells with stem cell-like properties. The features in common with stem cells are: indefinite self-replication, asymmetric cell division, and resistance to toxic agents, owing, in part, to elevated expression of ABC transporters [199,200,201]. In addition, they are also characterized by genetic instability (chromosomal and microsatellite), changes of chromatin, transcription and epigenetics, mobilization of cellular resources, and modified microenvironment interactions (tumour cells, stromal cells, extracellular, endothelium) [202]. Both paradigms of tumour propagation are likely to exist in human cancer but only the CSC model is hierarchical. It is important to note that the two models are not mutually exclusive, as CSCs undergo clonal evolution, as shown for leukaemia stem cells [203].

The theory of cancer stem cells is not new, having started wonderings in the 19th century when comparing cancerous and embryonic tissue in the microscope and certain similarities were observed, annotating the idea that tumours arising from embryonic-like cells. This theory continued to evolve, and the isolation of four different tumour subpopulations from a single breast cancer in a mouse was reported in the decade of the 80s[204]. Tumour

heterogeneity is reflected in different phenotypic aspects such as cell morphology, gene expression, metabolism, motility and proliferation, immunogenic, angiogenic and metastatic potential [202].

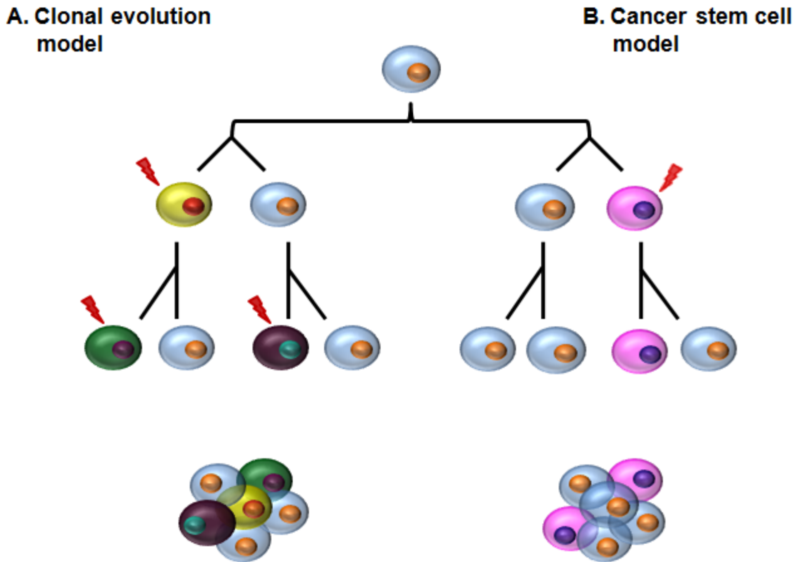


Figure 7. **A.** The clonal evolution model of cancer is based on the fact that accumulation of a series of mutations (inhibition of apoptosis, angiogenesis,…) in any somatic cell can cause a tumour. **B.** The cancer stem cell model is based on the principle that a progenitor cell capable of self-renewal and proliferation (stem cell characteristics) is the cause of formation of a tumour.

TUMOUR TYPE	MARKERS	Reference
Hematological malignancies	$CD34^+$ / $CD38^-$	[207]
CNS	$CD133^+$	[208]
Colon	$CD133^+$/ $EpCAM^{hi}$ / $CD44^+$ / $CD166^+$	[209,210]
Breast	$CD24^{-/low}$ /$CD44^+$	[211]
Lung	$CD133^+$	[212]
Pancreas	$CD44^+$ / $CD24^+$ / $EpCAM^+$/ $CD133^+$	[213]
Liver	$CD90^+$	[214]
Prostate	$CD44^+$ / CD133 / $\alpha2\beta1^{hi}$	[215]
Bladder	$CD44^+$ / $CK5^+$/ $CD20^-$	[216]
Ovaries	$CD44^+$ / $CD117^+$	[217]
Head and neck	$CD44^+$	[218]
Melanoma	$ABCB5^+$	[219]

Table 6. Phenotypic markers of CSCs in various tumours.

Molecular characterization of CSCs is necessary to develop a targeted therapy (Table 6). They have been isolated from several tumour types including haematological malignancies (the first evidence) [205], breast, brain, colon, lung, head and neck, prostate, pancreas and liver cancers and melanoma. The subpopulation of CSCs self renews, differentiates, and regenerates a phenocopy of the original tumour when injected into immunodeficient mice [206].

Tumour stem cells may display multidrug resistance that is conferred by ABC transporters. These ABC transporters have been reported as CSCs markers in melanoma and osteosarcoma, among others. These transporters are up-regulated in CSCs, and the low-staining fraction of cells with the ability to efflux Hoechst 33342 dye is commonly known as the side population (SP) [33]. Targeted inactivation of ABC transporters could reinstate drug sensitivity in CSCs, resulting in their death. Moreover, pathways that regulate self-renewal of normal stem cells, such as Wnt, Notch and Hedgehog, tumor suppressor genes, such as PTEN and p53, have been implicated in the control of CSCs self-renewal. These pathways are believed to be deregulated in CSCs, leading to uncontrolled self-renewal and generation of tumours that are resistant to conventional therapies [220]. The above mentioned characteristics and the ability of CSCs to evade cell death signals contribute to the failure of existing therapies to eradicate malignant tumours, causing resistance to treatment and an increased morbidity and mortality [201,221]. Apoptosis is one of the most critical and well-studied mechanisms, governing tissue development and homeostasis through a complex network of molecules that mediate death and survival signals. Escape from death program is a prerequisite for any tumour-initiating cell and may support the survival of CSCs in response to chemo- or radio-therapy. Thus, manipulating the apoptotic machinery to eradicate tumour-initiating cells holds enormous therapeutic potential [206].

Several studies have focused on apoptosis induction in CSCs by intervening in the extrinsic or intrinsic pathways to treat cancer.

6.1. TRAIL

TRAIL has been demonstrated to induce apoptosis in a wide range of cancer types both in vitro and also in various mouse models of human cancers [106]. In certain types of tumours a correlation has been established between the expression of DR and CSCs. Chemotherapy-resistant colon cancer SP cells express high levels of DR4 [222]**;** in some bladder cancer cell lines there is an increased expression of DR5 [223], and, in glioblastoma and lung CSCs express DRs [206]. More importantly, several clinical trials have explored the response to the use of DRs as a treatment. Studies concluded that SP cells of colon cancer displayed higher sensitivity to TRAIL compared to the non-SP cells [222]. In bladder cancer treatment sensitivity was greater in those showing increased expression of DR5 [223]. Moreover, isolated neurospheres from glioblastomas with characteristics of stem cells showed differences in apoptosis after treatment with TRAIL and it was effective in those who keep the route intact for caspase 8. Genomic heterogeneity in glioblastomas suggests the presence of multiple mechanisms in TRAIL resistance in both CSCs and non-stem cells. Future

clinical trials of TRAIL apoptotic pathway targeted therapies may consider genomic analysis of tumour tissue to identify the genomic status of TRAIL apoptotic genes such as *caspase 8* and use it as a genomic marker to predict tumour resistance to TRAIL apoptotic pathway-targeted therapies [224]. In addition, recent studies are focused on combining mesenchymal stem cells (MSCs) expressing TRAIL and chemotherapy. These MSCs migrated to tumours and reduced the growth of primary cancers and metastases by induction of apoptosis, death and reduced colony formation of the SP and were synergistic when combined with traditional chemotherapy in apoptosis induction [225].

6.2. cFLIP

cFLIP is overexpressed in many types of cancers like melanoma, colon lymphoma and thyroid cancer [226]. The CD133+ populations within the T-cell acute leukemia cell line Jurkat and the breast cancer cell line MCF7 were reported to express higher levels of cFLIP, which was associated with TRAIL resistance. The down-regulation of cFLIP using siRNA restored TRAIL signalling in both cell lines resulting in a dramatic reduction in experimental metastases and the loss of CSC self-renewal [227]. This suggests that a combined TRAIL/FLIPi therapy could prevent metastatic disease progression in cancers.

6.3. IAP

In CD133 + cells isolated from glioblastoma an increased mRNA expression of livin, survivin and the multidrug resistance-associated protein 1 (MRP1) was detected. Therefore, the effects of etoposide, a pro-apoptosis agent, on these associated protein genes in glioblastoma stem-like cells have been studied. Results showed that after etoposide treatment, glioblastoma CSCs displayed a stronger resistance to apoptosis and death. The anti-apoptotic gene livinβ was more related with the high survival rate and MRP1 was more related with transporting chemotherapeutic agent out of glioblastoma stem-like cells [228]. In pancreatic cancer it has been demonstrated that targeting XIAP by RNAi inside the cancer cells, the combination of TRAIL with MSCs suppressed metastatic growth in these tumours [229]. Recently, it has been demonstrated that survivin is regulated by the interleukin-4 (IL-4) pathway in colon CSCs. Blockage of IL-4-mediated signaling pathway with leflunomide, Stat6 inhibitor, increased the nuclear survivin pool suggesting that the IL-4/STAT-6 pathway could escape cell death. IL-4 neutralization, mediated by STAT-6, could down-regulate survivin expression and localization, increasing the nuclear pool and in this way inducing chemo-sensitivity of CSCs [230].

6.4. Bcl-2

The proteins Bcl-2 family members are anti-apoptotic molecules known to be over-expressed in most cancers, and are associated directly with the CSCs [231]. Therefore, recent studies are aimed to target these proteins. A representative example is found in pancreatic cancer, against which the most potent and clinically acceptable Bcl-2 inhibitor AT-101 is currently in 20 different clinical trials around the world [232]. In glioblastoma, high

expression levels of the anti-apoptoticc Bcl-2 protein, Mcl-1, were associated with resistance to treatment with Bcl-2 inhibitor ABT-737 in glioma stem cells [233]. In hematopoietic malignances, it has been showed that despite the over-expression of Bcl-2 this is not the critical point for the generation, selection and maintenance of leukemia stem cells [234].

6.5. NF-kB

The transcription factor NF-κB has been connected to multiple aspects of oncogenesis, including inhibition of apoptosis by increasing the expression of survival factor. In fact, aberrant regulation of NF-κB has been observed in many cancers, including both solid and hematopoietic tumours [235]. A fairly representative example is found in pancreatic cancer, where there is a clear correlation between the basal activity of NF-kB and the ability to generate angiogenesis and metastasis of pancreatic tumour cells [236] and, more recently it has been found that not only in this way, but also in the non-canonical NF-kB is also activated and functional [237]. A very interesting study by [238] showed that in CD44+ breast CSCs the expression of CD24 potentiated DNA-induced apoptosis by suppressing anti-apoptotic NF-κB signaling. Several therapies are being developed to inhibit this factor because there are many tumours which relate the decrease in the activity of NF-kB with a decrease in the size and tumor growth [239,240,241,242,243].

6.6. DNA repair capacity

A classic mechanism involved in the induction of apoptosis is in response to DNA damage by p53 action. This gene is mutated in most human cancers and inactivated in about 50% of cancers [244]. p53 was found to repress the CSC marker gene CD44 in an experimental breast tumour model and the over-expression of CD44 blocked p53-dependent apoptosis, leading to expansion of tumour-initiating cells [245]. Moreover, glioma CSCs resist radiation through preferential activation of the retainer DNA damage response and an increase in DNA repair capacity. In addition, the radioresistance of CD133+ glioma stem cells could be reversed with a specific inhibitor of Chk1 and Chk2 checkpoint kinases [208]. Another report reinforces the tumour-promoting effect of DNA damage response activation in leukemia stem cells by demonstrating that cell-cycle inhibitor p21 was indispensable for maintaining self-renewal of these CSCs [246].

6.7. miRNAs, apoptosis and CSCs

Recently, miRNAs have emerged as key regulators of "stemness", collaborating in the maintenance of pluripotency, control of self-renewal, and differentiation of stem cells. Moreover, certain miRNAs involved in apoptosis appear to influence the CSC fate by controlling self-renewal. It has been shown that restoration of miR-34 modulates self-renewal in pancreatic CSCs by directly regulating down-stream target gene Notch and Bcl-2 [247]. Also the restoration of miR-34, inhibit p53-mutant gastric cancer tumourspheres growth in vitro and tumour formation in vivo, which is reported to be correlated to the self-renewal of CSCs [248]. As miR-34 is a significant tumour suppressor of CSCs by regulation

of both apoptosis and self-renewal properties, restoration of miR-34 may hold significant promise for a novel molecular therapy. Data also suggest that let-7 regulates apoptosis and CSC differentiation, which is considered as a key "keeper" of the differentiated state. In this context decreased expression of these TSmiRs implicated in self-renewal could lead to further cancer progression.

7. Conclusion

In this chapter we have summarized the research in the discovery of molecules, biomarkers for predicting therapeutic response and regulatory pathways implicated in apoptosis induction. These strategies are contributing to design cancer-targeted therapies that diminish or circumvent toxicity and improve life quality and overall survival of patients. The selective eradication of cancer cells can be achieve with small molecules and monoclonal antibodies, used as single agents or in combination with conventional chemotherapy, that interfere with the deregulated cellular signals that promote proliferation and survival to block tumor growth or sensitize cancer cells to apoptosis while leaving normal cells unaffected. Moreover, targeted therapies reactivating death program in CSCs may synergize with established therapies and increase efficacy in the clinic.

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