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Enantiomerically Pure Substituted Benzo-Fused Heterocycles — A New Class of Anti-Breast Cancer Agents

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Additional information is available at the end of the chapter

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1. Introduction

With more than 10 million new cases each year cancer is at present one of the most devastating diseases worldwide with an immense affliction burden not only for affected individuals, their relatives and friends but also representing heavy challenges to health care systems (Steward & Kleihues, 2003). In the year 2000, cancer was responsible for 12% of nearly 56 million deaths worldwide and in many countries this percentage is even higher with more than a quarter of deaths attributable to cancer. Moreover, it is expected that cancer rates further increase by 50% to 15 million new cases in the year 2020, mainly due to steadily ageing populations in both developed and developing countries (Fresco et al., 2010).

In recent years, many studies have shown an association between cell cycle regulation and cancer inasmuch as the cell cycle inhibitors are being considered as a weapon for the management of cancer (Hajdich et al., 1999). Ultimately a great level of interest has arisen in the G₀/G₁ phase regulatory molecules such as cyclin D1, CdkIs, and p53 as potential therapeutic targets in diseases where control of inappropriate cellular proliferation would be a therapeutic benefit (Sherr, 1996).

Apoptosis is an essential physiological process throughout the life of multi-cellular organisms important in the development and in the maintenance of tissue homeostasis. Apoptosis is involved in controlling the cell number and proliferation during embryogenesis, deletion of activated lymphocytes at the end of the immune response, elimination of self-reactive lymphocytes, in controlled destruction of damaged, aged, infected, transformed, and other harmful cells (Nagata, 1997; Testa, 2004). Zivny et al. have recently reviewed the apoptotic

pathways, molecules involved in the cross-talk between individual apoptosis pathways, apoptosis regulation as well as mechanisms of action of conventional anticancer drugs and new promising agents, which trigger directly or indirectly apoptosis of hematologic cancer cells (Zivny et al., 2010).

We report herein the synthesis and antiproliferative activities of purine derivatives **1-11** (Chart 2) against the cancerous MCF-7 and MDA-MB-231 human breast cancer cell lines and the corresponding normal one (MCF-10A) to define the *in vitro* therapeutic index (TI) as a measure of the selectivity. From a structural point of view, the compounds studied differ from others previously reported (Díaz-Gavilán et al., 2008b) by the addition of an extra halogen or PhS-groups on the purine ring. Finally the most active racemic compound (**1**) was resolved and the antiproliferative activity of its enantiomers was measured (López-Cara et al., 2011).

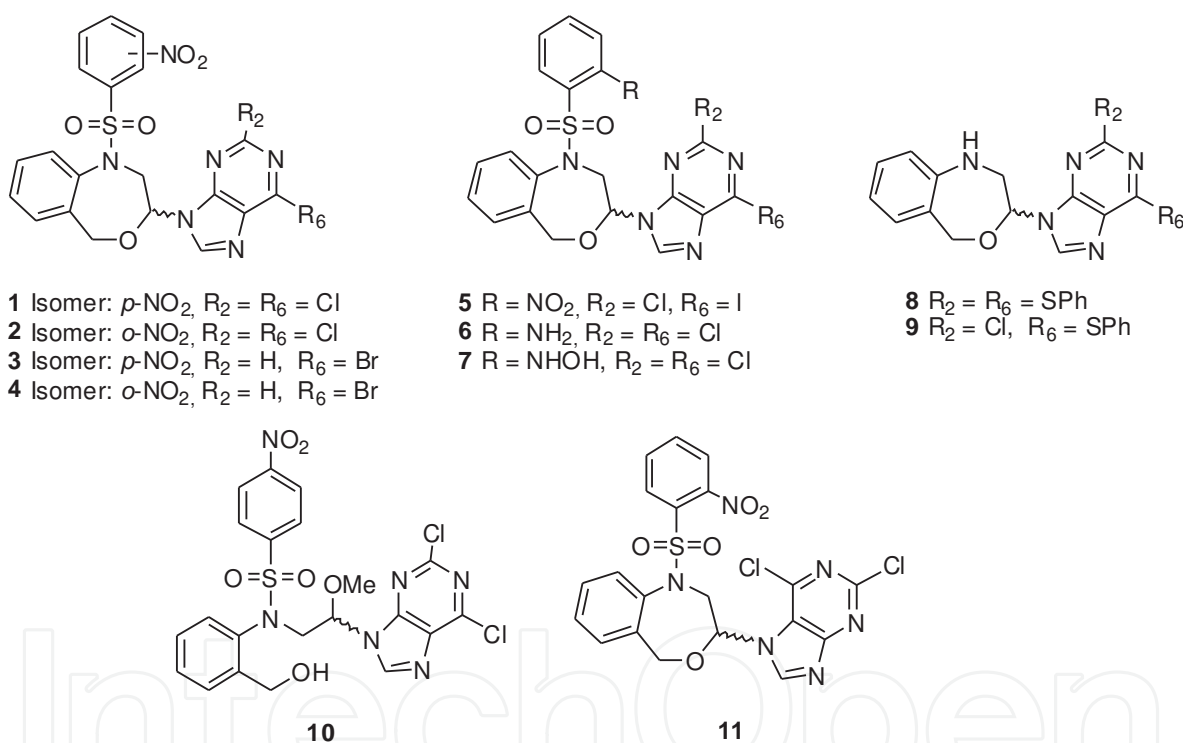
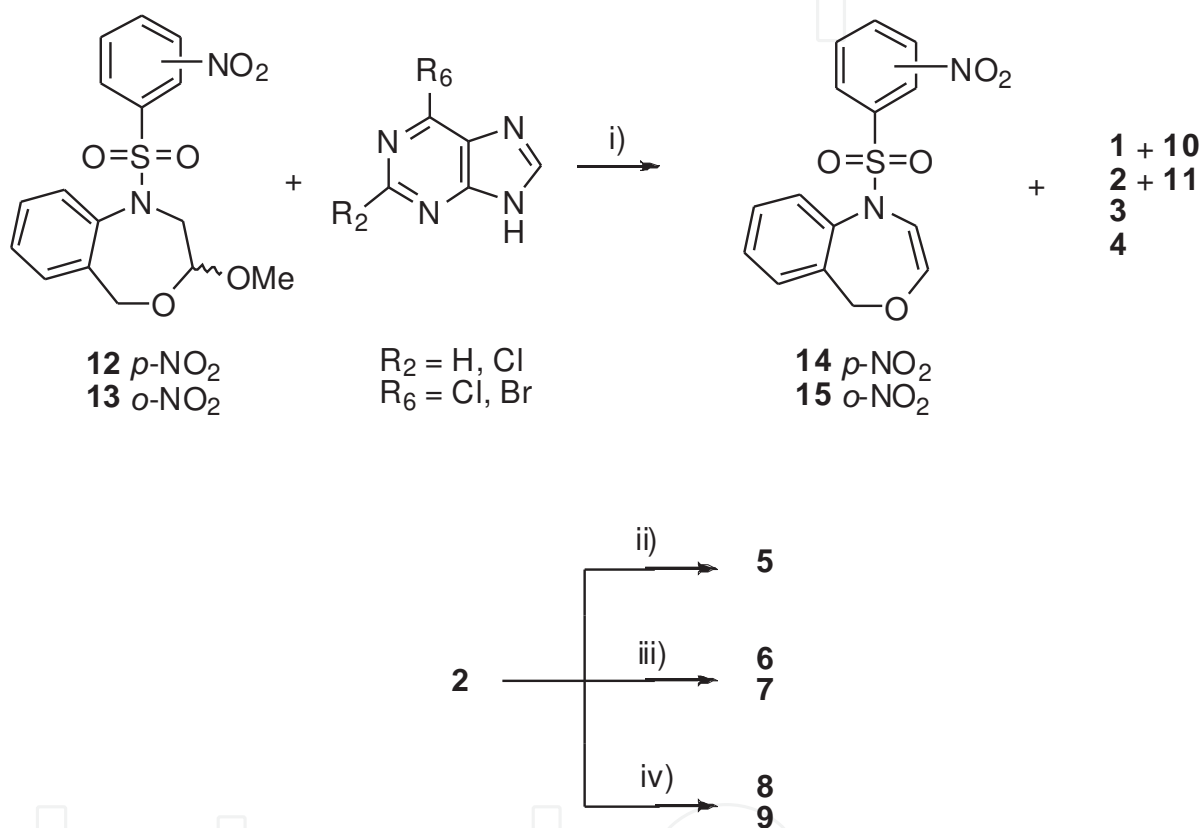


Chart 1. New cyclic (**1-9**) and acyclic (**10, 11**) purinic *O,N*-acetals (López-Cara et al., 2011).

Modern drug discovery relies on high speed organic synthesis. Microwave-assisted organic synthesis is proving to be instrumental for the rapid synthesis of compounds with new and improved biological activities (Al-Obeidi et al., 2003; Kappe & Dallinger, 2006). We previously investigated the Vorbrüggen condensation in microwave-assisted organic synthesis (Conejo-García et al., 2008). Microwave advantage is chiefly the quick access to the target molecules as well as the better yield obtained in the only isomer formed making the purification processes much easier.

2. The chiral switch from the benzo-fused seven-membered *O,N*-acetal (1)

Preparation of the *O,N*-acetals **1-4** was achieved by the microwave-assisted Vorbrüggen one-pot condensation of the cyclic acetals **12** and **13** (Díaz-Gavilán et al., 2004) and the commercially available purine bases 6-chloro-, 6-bromo- and 2,6-dichloro-purines, using chlorotrimethylsilane (TMSCl), 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and tin(IV) chloride as the Lewis acid in anhydrous acetonitrile. The reaction mixture was microwave-irradiated at a temperature of 140 °C or 160 °C for 5 min (Scheme 1).



Scheme 1. Reagents and conditions: i) purine, TMSCl, HMDS, SnCl₄ (1 M solution in CH₂Cl₂), 140 or 160°C, microwave, 5 min; ii) NaI, TFA, butanone, -15°C, 6 hours; iii) SnCl₂·2H₂O, EtOH, reflux, 2 hours; iv) PhSH, K₂CO₃, DMF, rt, 4 hours.

Compounds **14** and **15** were isolated from the reactions and the acyclic *O,N*-acetal **10** was also obtained in the synthesis of **1**. Traces of the *N*-7' regioisomer **11** were detected in the synthesis of **2**. The following modifications were carried out on **2**: a) selective nucleophilic substitution of the chlorine atom at position 6 of the purine ring using NaI and trifluoroacetic acid (TFA) to yield **5**; b) reduction of the nitro group with SnCl₂ to give rise to **6** and **7**; and c) the treatment with the PhSH to produce **8** and **9**.

Compounds **14** and **15** were obtained along with the cyclic and acyclic *O,N*-acetals in the reaction of purines with **12** and **13**, respectively. Their importance lies in the information that they provide of the mechanism of the reaction with purines (López-Cara et al., 2011).

2.1. Resolution of (RS)-1 into its enantiomers: Biological activities

The issue of drug chirality is now a major theme in the design and development of new drugs, underpinned by a new understanding of the role of molecular recognition in many pharmacologically relevant events. In general, three methods are utilized for the production of a chiral drug: the chiral pool, separation of racemates, and asymmetric synthesis. Although the use of chiral drugs predates modern medicine, only since the 1980’s has there been a significant increase in the development of chiral pharmaceutical drugs. An important commercial reason is that as patents on racemic drugs expire, pharmaceutical companies have the opportunity to extend patent coverage through development of the chiral switch enantiomers with desired bioactivity (Núñez et al., 2009).

(RS)-9-[1-(*p*-Nitrobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]-2,6-dichloro-9*H*-purine (**1**) is resolved into its two enantiomers: [(*R*)-**1**: [α]_D²⁵=−43.6 (c=0.22, THF), and (*S*)-**1**: [α]_D²⁵=+41.0 (c=0.23, THF);] using a semipreparative column CHIRALPAK® IA and a mixture of hexane/*t*-BuOMe/*i*PrOH as eluent (Marchal et al., 2010).

Table 1 shows the antiproliferative activity (IC₅₀ values) for **1-11** and 5-fluorouracil (5-FU). All the compounds were first assayed as antiproliferative agents against the human breast adenocarcinoma cell line MCF-7 (p53 wild-type and ras mutated). Compounds (**1**, **2**, **5-7**, and **10**, **11**) were selected to be further assayed on the human breast cancer cell line MDA-MB-231, which has high levels of mutant p53, the most commonly mutated gene in human cancer. Additionally, we used a non-cancerous human mammary epithelial cell line (MCF-10A), in order to study the therapeutic index against breast cancer.

Compound	IC ₅₀ MCF-7 (μM)	IC ₅₀ MDA-MB-231 (μM)	IC ₅₀ MCF-10A(μM)
1	0.355 ± 0.011	0.166 ± 0.063	1.825 ± 0.503
2	0.383 ± 0.027	0.280 ± 0.006	1.530 ± 0.198
3	1.226 ± 0.348	N.D. ^b	N.D. ^b
4	3.618 ± 0.273	N.D. ^b	N.D. ^b
5	0.610 ± 0.043	0.256 ± 0.002	0.351 ± 0.020
6	0.820 ± 0.050	0.467 ± 0.017	1.520 ± 0.498
7	1.530 ± 0.040	0.487 ± 0.006	1.233 ± 0.217
8	9.710 ± 0.380	N.D. ^b	N.D. ^b
9	13.85 ± 1.790	N.D. ^b	N.D. ^b
10	0.355 ± 0.122	0.409 ± 0.074	1.863 ± 0.050
11	0.990 ± 0.090	0.318 ± 0.066	1.265 ± 0.163
5-FU	4.32 ± 0.020	N.D. ^b	N.D. ^b

^aAll experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations. The treatment time was 48 h.

^bN.D.=Not determined.

Table 1. Antiproliferative activities^a for compounds **1-11** and 5-FU against the cancerous cell lines MCF-7 and MDA-MB-231, and the non-cancerous cell line MCF-10A (López-Cara et al., 2011).

It must be pointed out that from the twenty IC_{50} values against the two cancerous cell lines, the majority of the IC_{50} values were below 1 μ M. As shown in Table 1, all the compounds were more active as anti-proliferative agents against MDA-MB-231 than against the MCF-7 human breast cancer cell line, except for the acyclic derivative 10, whose anti-proliferative effect remains the same in both cancer cell lines. The $IC_{50}=0.166$ μ M for compound 1 against the human cancerous cell line MDA-MB-231 stands out over the rest of the values.

A comparison between the cancerous cell lines (MCF-7 and MDA-MB-231) and the corresponding normal one (MCF-10A) was established in an intent to define the *in vitro* therapeutic index as a measure of the selectivity. The *in vitro* TI of a drug is defined as the ratio of the toxic dose to the therapeutic dose (*in vitro* TI= IC_{50} non-tumour cell line/ IC_{50} tumour cell line) (Núñez et al., 2007). TI was better for compounds **1**, **2** and **11** against both cancer cell lines with values up to 11.0, 5.50 and 4.55, respectively against MDA-MB-231 cell line. 2,6-Dichloro derivatives **1** and **10** were the most selective compounds against the human breast adenocarcinoma MCF-7 cancer cell line (TIs=5.1 and 5.2, respectively) in relation to the normal one. The iodine derivative **5** showed the most toxic effect against the non-tumour MCF-10A human mammary epithelial cell line (Table 2).

Compound	Therapeutic index (TI)	
	MCF-7	MDA-MB-231
1	5.14	11.0
2	4.00	5.50
5	0.57	1.37
6	1.85	3.25
7	0.80	2.53
10	5.25	4.55
11	1.27	4.00

Table 2. Therapeutic indexes for the most representative compounds.

When the homochiral forms were analyzed we found differences in the IC_{50} values between (S)-**1** and (R)-**1** enantiomers, although no differences in activity were found between the two enantiomers against the MDA-MB-231 cell line. However both enantiomers present higher anti-proliferative activity than the racemic compound showing the greatest differences against MCF-7 cells. Enantiomer (S)-**1** shows higher anti-tumour activity, up to twice that of (R)-**1** in the MCF-7 cell line (Table 3). Studies with other compounds showed similar results with more potency in cytotoxicity in an enantiomer in comparison with the racemate. This enantioselective cytotoxicity indicates that the enantiomers of some chiral drugs may differ both quantitatively and qualitatively in their biological activity (Liu et al., 2009; Shelley et al., 1999). Moreover, enantiomers demonstrate minimal *in vitro* but a dramatic *in vivo* chiral dependency in their anti-tumour activities (Lai et al., 2007; Brown et al., 2010).

Compound	MCF-7 (μM)	MDA-MB-231 (μM)
(RS)-1	0.355 ± 0.011	0.166 ± 0.063
(R)-1	0.19 ± 0.001	0.11 ± 0.001
(S)-1	0.10 ± 0.001	0.11 ± 0.001

^aAll experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations.

Table 3. Anti-proliferative activities of (RS)-1 and its enantiomers against the cancerous cell lines MCF-7 and MDA-MB-231.

Once the anti-tumour activity of compounds was determined against the different breast cell lines, we carried out a selection between those that showed a great cytotoxic effect against MCF-7, including (R)-1 and (S)-1, in order to determine their influence on the several cell cycle phases. In this study we have included drugs used in clinic against breast cancer, such 5-FU and paclitaxel, with a known mechanism of action at the level of cell cycle.

In order to analyze if the anti-tumour effects of the drugs involve changes in cell cycle distribution, the non-tumour cell line MCF-10A and the breast cancer cell lines MCF-7 and MDA-MB-231 were treated with the compounds during 48 hours and then analyzed by flow cytometry. The non-accumulation in a specific phase was detected during treatment with the drugs in most of the cell lines analyzed in comparison with control-DMSO-treated cells. Only the (R)-1 enantiomer was able to induce in MDA-MB-231 cells an accumulation in both G₀/G₁ and G₂/M phases with the consequently significant decreased in the S phase. Also an accumulation in the phase G₂/M was detected in MCF-7-5 treated cells. Treatment with 5-FU and paclitaxel, as has been described previously (Grem et al., 1999), induced accumulation in the S or G₂/M phases depending on the cell line analyzed. Similar data were obtained when cell lines were treated for 24 hours with 0.5 mM mimosine to synchronize the cells in the G₁/S phase (data not shown). These results indicate that compounds inhibited all phases of the cell cycle, probably through the inhibition of protein synthesis as has been proved with other anti-tumour drugs (Duncan et al., 2009).

Finally, to determine if the observed growth inhibition was due to apoptosis, both flow cytometry and confocal microscopy studies were carried out. Cells were treated with the IC₅₀ values of compounds and stained using Annexin V and propidium iodide (PI) at 24 and 48 hours post-drug treatment. Apoptosis assays were accomplished in the MCF-7 human breast cancer cell line, where the demonstration of programmed cell death by known apoptosis-inducing agents has proved difficult and only few cytotoxic agents act preferentially through an apoptotic mechanism in human breast cancer cells (Saunders et al., 1997; Chaderton et al., 2000). Paclitaxel (Taxol) induced programmed cell death of up to 43% of the cell population. Simultaneous staining with annexin V-FITC and the PI non-vital dye made it possible to distinguish between early apoptosis (stained positive for annexin V-FITC and

negative for PI), and late apoptosis or cell death (stained positive for both annexin V-FITC and PI). In MCF-7 control-DMSO cultures neither early nor late apoptosis were detected after 24 h or 48 h. Similarly, compounds did not induce apoptosis after 24 h of treatment. In contrast, MCF-7 cells treated during 48 h with the novel compounds showed a significant increase of early apoptotic cells in relation to the control culture with percentages varying from 13.93% in cells treated with **11** to 43.30% and 41.99% after treatment with **10** and (*R*)-**1**, respectively. It should be noted that levels of early apoptosis induced by (*R*)-**1** were almost double in comparison with the corresponding racemic **1**, which may explain the enantioselective anti-proliferative activity shown by this enantiomer. These high apoptotic percentages shown by (*R*)-**1** are consistent with the G₁ and G₂/M arrest since cells exposed to specific agents typically enter apoptosis from a given phase of the cell cycle (Saunders et al., 1997; Marchal et al., 2004; Lundberg & Weinberg, 1999). Differences in cytotoxicity, cell cycle analysis or apoptotic levels between (*R*)-**1** and (*S*)-**1** suggest distinct signalling pathways as has been shown with other anti-tumour enantiomers (De Fátima et al., 2008). Moreover, it is possible that the amount of cells undergoing apoptosis in response to the compounds have been higher than these values, because only adherent cells were stained and counted.

The effects of compounds on the pattern of cell death were also confirmed by confocal microscopy after staining with FITC-conjugated annexin V and the nuclear non-vital stain PI. MCF-7 cells treated with compounds showed several staining patterns. Some cells displayed an intense FITC staining located at the plasma membrane and a nucleus with intensely PI-labelled marginated chromatin, suggesting that they were in the course of apoptosis. Other cells showed a peculiar staining pattern, because they exhibited nuclei with the same features observed in true apoptotic cells and, at the same time, cytoplasm homogeneously stained for annexin V. In fact, the FITC staining was located not only at the cell surface, but also within the cytoplasm. Therefore, these cells were considered as aponecrotic cells as has been previously established (Formigli et al., 2002). In addition, patches of localised partially condensed chromatin were found in other cells abutted along the inner part of the nuclear membrane. In the control cultures, most of the cells turned out to be negative for both staining except for some dying cells with the staining features of apoptosis (data not shown). The present data support the effect of the compounds in some of the series of steps of the apoptotic process where a wide range of intermediate morphological and biochemical types of cell death occurs (Marchal et al., 2004; Gooch & Yee, 1999).

Toxicity was determined selecting (*RS*)-**1**, which was the most in vitro cytotoxic compound against MCF-7 cells. We examined the acute-toxicity profile of (*RS*)-**1** in BALB/c mice when it was administered in a single i.p. bolus injection (n=25) at dose levels of 50, 75, 100, 150 and 200 mg/kg or *via* gavage (n=25) in a single p.o. bolus at dose levels of 0.05, 0.5, 5 and 50 mg/kg. Compound (*RS*)-**1** was nontoxic to BALB/c mice even at the highest i.p. bolus dose of 200 mg/kg and p.o. bolus dose of 50 mg/kg after 2 weeks. Control mice (n=10; 5 mice for the i.p. group and 5 mice for the p.o. group) were treated with the vehicle alone. All 50 (*RS*)-**1**-treated mice remained healthy and gained weight throughout the 15-day observation period, with no evidence of morbidity.

3. Purines linked to racemic benzo-fused six-membered heterocycles

Very recently, a series of 2-and 6-substituted (*RS*)-9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purine derivatives (**16-26**, Chart 2) was obtained by applying a standard Mitsunobu protocol that led to a six-membered ring contraction from (*RS*)-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol *via* an episulfonium intermediate (Díaz-Gavilán et al., 2008a). The most active compounds were **17** and **18** with $IC_{50}=6.18 \pm 1.70$ and 8.97 ± 0.83 μ M, against MCF-7 cells respectively. These results suggest that the presence of bulky substituents on position 6 of the purine ring reduces the anti-proliferative activity. An approach that has guided the origin of novel drugs is bioisosterism, which we have carried out as suitable structural modifications of the seven-membered building block, such as the modification O-1/S (Núñez et al., 2005; Núñez et al., 2007).

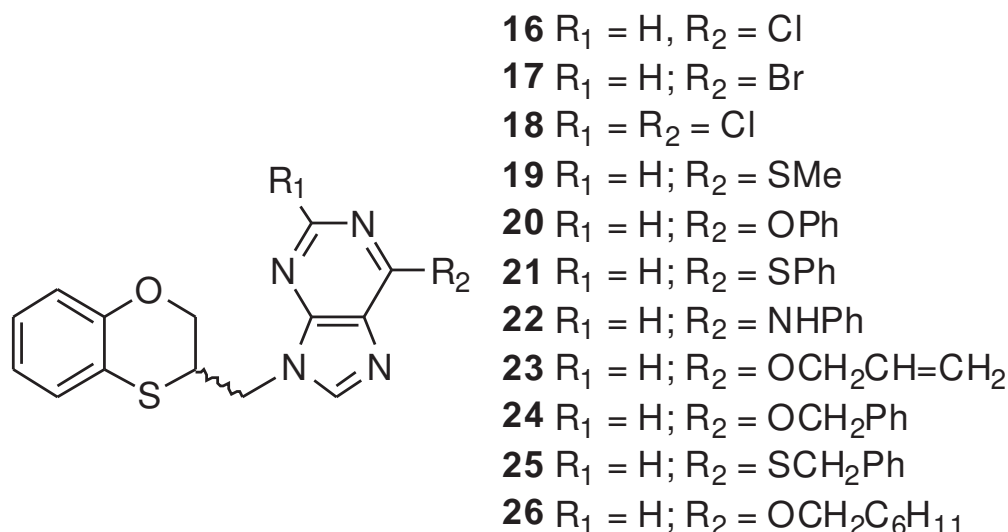


Chart 2. Series of substituted (*RS*)-9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purine derivatives **16-26** (Díaz-Gavilán et al., 2008a).

The design, synthesis and biological evaluation of two series of substituted (*RS*)-9-(2,3-dihydro-1,4-benzoxathiin-2-ylmethyl)-9*H*-purines **27-30** (Series A, Chart 3), and (*RS*)-9-(2,3-dihydro-1,4-benzodioxin-2-ylmethyl)-9*H*-purines **31-33** (Series B, Chart 3) have been described (Conejo-García et al., 2011). In series A, the methylene linker that connects the six-membered ring and the purine moiety has been changed from position 3 to 2 in relation to derivatives **16-26** (Chart 2). Series B is the isosteric group in which sulfur is replaced by oxygen. We will show the activity of these compounds in the inhibition of MCF-7 breast cancer cell growth to ascertain potential directions for synthetic lead-optimization studies.

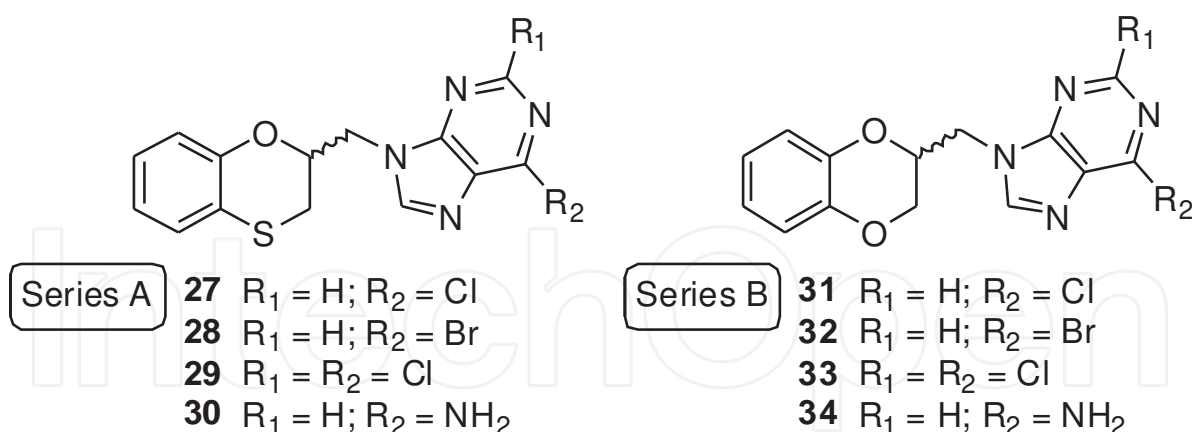
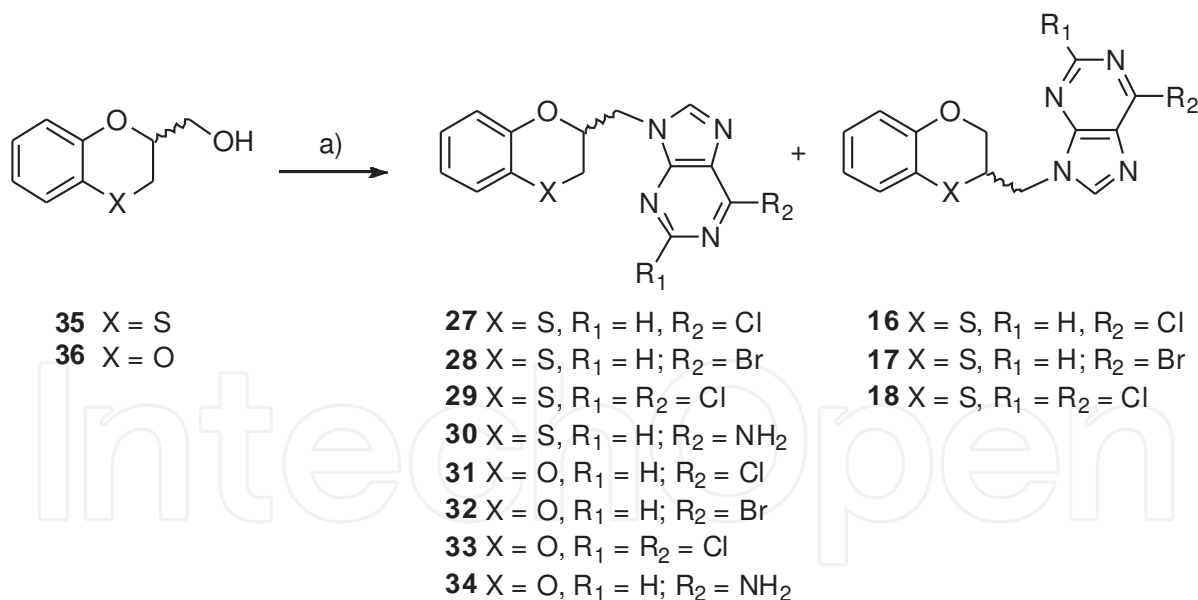


Chart 3. Substituted (*RS*)-9-(2,3-dihydro-1,4-benzoxathiin-2-ylmethyl)-9*H*-purines **27-30** (series A) and (*RS*)-9-(2,3-dihydro-1,4-benzodioxin-2-ylmethyl)-9*H*-purines **31-34** (series B).

The starting material (*RS*)-2,3-dihydro-2*H*-1,4-benzoxathiin-2-methanol (**35**) was prepared as previously reported (Díaz-Gavilán et al., 2008a) whilst (*RS*)-(2,3-dihydro-1,4-benzodioxin-2-yl)methanol (**36**) was synthesized by the reaction of cathecol with epichlorohydrin in NaOH and water (Díaz-Gavilán et al., 2007).



Scheme 2. Reagents and conditions: a) Substituted purines, Ph_3P , DIAD, anhydrous THF, microwave irradiation, 140 °C, 5 min, or in the case of **32**, 160 °C, 15 min (Conejo-García et al., 2011).

Final compounds **27-34** were synthesized by the Mitsunobu reaction in dry THF between **35** or **36** and the corresponding purines (6-chloropurine, 6-bromopurine, 2,6-dichloropurine and adenine) under microwave-assisted conditions (Scheme 2).

It must be pointed out that when starting from **35** and using 6-chloro-, 6-bromo-, and 2,6-dichloro-purines, apart from the target compounds **27**, **28** and **29**, their corresponding isomers **16**, **17** and **18** (Díaz-Gavilán et al., 2008) previously reported were also obtained as side-products. Therefore we have justified the formation of such "abnormal" products through a neighbouring-group mechanism (Conejo-García et al., 2011).

The anti-carcinogenic potential of the target molecules is reported against the MCF-7 human breast cancer cell line (Table 4). In general, (RS)-9-(2,3-dihydro-1,4-benzoxathiin-2-ylmethyl)-9H-purines **27-29** (series A) show a better activity than their isosteres (RS)-9-(2,3-dihydro-1,4-benzodioxin-2-ylmethyl)-9H-purines **31-33** (series B). The anti-cancer activity depends on the substituent of the purine ring. The most active compound **29**, bearing two chlorine atoms at positions 2 and 6 of the purine ring, shows an $IC_{50}=2.75 \pm 0.02 \mu M$. In general, compounds bearing halogen atoms on the purine ring (**27-29** and **31-33**) present better activity than compounds substituted bearing an amino group (**30** and **34**).

Comp.	$IC_{50} (\mu M)$	Comp.	$IC_{50} (\mu M)$	Comp.	$IC_{50} (\mu M)$
16	10.6 ± 0.66	28	4.87 ± 0.02	32	7.64 ± 0.03
17	6.18 ± 1.70	29	2.75 ± 0.03	33	19.58 ± 0.02
18	8.97 ± 0.83	30	">30	34	">30
27	9.24 ± 0.01	31	18.75 ± 0.02		

Table 4. Anti-proliferative activities against the MCF-7 cell line for the (RS)-9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9H-purines (**16**, **17** and **18**), (RS)-9-(2,3-dihydro-1,4-benzoxathiin-2-ylmethyl)-9H-purines (**27-30**), and (RS)-9-(2,3-dihydro-1,4-benzodioxin-2-ylmethyl)-9H-purines (**31-34**).

In recent years, many studies have shown an association between cell cycle regulation and cancer inasmuch as the cell cycle inhibitors are being considered as a weapon for the management of cancer (Hajdуч et al., 1999). To study the mechanisms of the anti-tumour activity of the compounds (**27-29** and **32**), the effects on the cell cycle distribution were analysed by flow cytometry (Table 5). DMSO-treated cell cultures contain a $62.79 \pm 1.30 \%$ of the cells in the G_0/G_1 -phase, and a $19.29 \pm 2.98 \%$ of the cells in the S-phase, a $13.26 \pm 2.98 \%$ of the cells in the G_2/M -phase. In contrast, MCF-7 cells treated during 48 h with **27-29** and **32** show important differences in the cell cycle progression compared with DMSO-treated control cells. The following can be deduced from the analysis of the cell cycle distribution: compounds **27**, **28**, **29** and **32** accumulate the cancerous cells in the G_2/M -phase (23.35 ± 1.97 , 31.37 ± 1.45 , 43.89 ± 1.96 and 36.71 ± 7.40 , respectively) at the expense of the S-phase cells (13.77 ± 1.13 , 17.06 ± 0.75 , 10.83 ± 4.70 and 10.27 ± 6.24 , respectively) and of the G_0/G_1 -phase cells in the case of compounds **28**, **29** and **32** (51.56 ± 1.06 , 45.28 ± 2.73 and 53.02 ± 1.16 , respectively), except in the case of **27**, which induces a cell cycle arrest in the G_2/M -phase cells (23.35 ± 1.97) at the expense of the S-phase cells (13.77 ± 1.13).

Compound	Cell cycle ^a			Apoptosis ^{b,c}
	G ₀ /G ₁	S	G ₂ /M	
Control	62.79 ± 1.30	19.29 ± 1.68	13.26 ± 2.98	0.92 ± 1.29
27	62.87 ± 0.60	13.77 ± 1.13	23.35 ± 1.97	37.99 ± 8.56
28	51.56 ± 1.06	17.06 ± 0.75	31.37 ± 1.45	14.33 ± 1.23
29	45.28 ± 2.73	10.83 ± 4.70	43.89 ± 1.96	70.08 ± 0.33
32	53.02 ± 1.16	10.27 ± 6.24	36.71 ± 7.40	21.66 ± 0.30

^aDetermined by flow cytometry (Marchal et al., 2004).

^bApoptosis was determined using an Annexin V-based assay (Marchal et al., 2004). The data indicate the percentage of cells undergoing apoptosis in each sample.

^cAll experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations.

Table 5. Cell cycle distribution and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h with the three most active compounds as anti-proliferative agents.

The protein expression analysis by western blot showed that **27-29** have an important role in the activation and phosphorylation of the initiation factor eIF2 α . The initiation factor eIF2 α was phosphorylated in MCF-7 human breast cancer cell line after treatment with **27-29**. It is well established that eIF2 α phosphorylation correlates with a translational block and consequently produces inhibition of protein synthesis (Holcik & Sonenberg, 2005). These results are in concordance with the delay in the G₂/M cell cycle phase produced by compounds. Furthermore, a prolonged induction of eIF2 α finally triggers the cell cycle arrest and/or the apoptosis phenomena (Gil et al., 1999; Dagon et al., 2001).

MCF-7 cells treated for 48 h with compounds **27-29** induced apoptosis, **29** being the compound that showed a significant increase of apoptotic cells in relation to the control culture with a percentage of 70.08 ± 0.33 (Table 5). Apoptosis is a major form of cell death characterized by changes in signalling pathways that lead to the recruitment and activation of caspases, a family of cysteine-containing, aspartate-specific proteases. Caspases exist as inactive proenzymes in cells, and are activated through their processing into two subunits in response to apoptotic stimulation. Activated caspases cleave a variety of important cellular proteins, other caspases, and Bcl-2 family members, leading to a commitment to cell death. Caspase-9 is involved in one of the relatively well-characterized caspase cascades. It is triggered by cytochrome C release from the mitochondria, which promotes the activation of caspase-9 by forming a complex with Apaf-1 in the presence of dATP. Once activated, caspase-9 initiates a caspase cascade that finally induces cell death (Altieri, 2003). Western blot assays showed that compounds **27-29** induced activation of caspase 9 at late times (16 h and 36 h of treatment) similarly to paclitaxel used as control compound. These data confirm that levels of apoptosis showed by annexin V assays that are dependent of intrinsic pathway of cell death. p53 was not activated by the compounds which indicate that apoptosis was induced in a p53 independent manner (Conejo-García et al., 2011).

4. Different apoptosis modulation in breast cancer cells of enantiomers of benzo-fused six-membered heterocycles linked to purines

The intrinsically chiral and non-racemic nature of the living world often results in its different interactions with the enantiomers of a given substance. If this substance is a drug, it might well be that only one of the two isomers is capable of exerting the desired therapeutic effect. The other may be inert, harmful or responsible for possibly undesirable side effects.

García-Rubiño et al. have described the preparation of homochiral **27-29** and **31-33** (García-Rubiño et al., 2013). Compounds (*R*)-**27-29**, (*R*)-**16-18**, (*S*)-**27-29** and (*S*)-**16-18** have been subjected to anti-proliferative, apoptosis (Tables 6 and 7) and cell cycle studies in the MCF-7 and SKBR-3 human breast cancer cell lines.

Comp.	IC ₅₀ (μM) ^a	Total apoptosis	Comp.	IC ₅₀ (μM) ^a	Total apoptosis
(<i>RS</i>)- 27	9.24 ± 0.01	67.4 ± 0.90 ^b 10.3 ± 0.14 ^c	(<i>RS</i>)- 16	10.6 ± 0.66	73.8 ± 0.42 ^b 22.6 ± 0.07 ^c
(<i>R</i>)- 27	4.73 ± 0.02	43.0 ± 0.63 ^b 9.70 ± 0.42 ^c	(<i>R</i>)- 16	15.2 ± 0.03	72.0 ± 0.21 ^b 20.2 ± 0.21 ^c
(<i>S</i>)- 27	11.4 ± 0.06	89.5 ± 0.70 ^b 19.0 ± 0.63 ^c	(<i>S</i>)- 16	3.30 ± 0.02	31.6 ± 1.40 ^b 14.0 ± 0.60 ^c
(<i>RS</i>)- 28	4.87 ± 0.02	99.4 ± 0.07 ^b 38.4 ± 4.73 ^c	(<i>RS</i>)- 17	6.18 ± 1.70	63.4 ± 1.50 ^b 30.6 ± 6.78 ^c
(<i>R</i>)- 28	4.45 ± 0.07	63.8 ± 6.00 ^b 16.0 ± 2.33 ^c	(<i>R</i>)- 17	6.17 ± 0.07	55.8 ± 12.0 ^b 26.6 ± 0.20 ^c
(<i>S</i>)- 28	3.33 ± 0.13	50.2 ± 1.13 ^b 25.2 ± 0.49 ^c	(<i>S</i>)- 17	6.32 ± 0.04	60.5 ± 9.00 ^b 41.8 ± 0.56 ^c
(<i>RS</i>)- 29	2.75 ± 0.03	97.7 ± 0.56 ^b 29.4 ± 0.30 ^c	(<i>RS</i>)- 18	8.97 ± 0.83	51.4 ± 0.21 ^b 15.8 ± 0.49 ^c
(<i>R</i>)- 29	3.33 ± 0.04	99.1 ± 0.65 ^b 77.0 ± 2.80 ^c	(<i>R</i>)- 18	10.3 ± 0.01	27.4 ± 0.07 ^b 6.25 ± 3.30 ^c
(<i>S</i>)- 29	1.85 ± 0.05	89.4 ± 1.50 ^b 33.2 ± 0.20 ^c	(<i>S</i>)- 18	6.93 ± 0.09	58.8 ± 2.75 ^b 60.4 ± 2.40 ^c

^aAll experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations. IC₅₀ was determined after 6 days of treatment. ^bCells were treated with the 3 × IC₅₀ values of compounds. ^cCells were treated with the IC₅₀ values of compounds. Apoptosis was measured after 48 h of treatment.

Table 6. Anti-proliferative effect and apoptosis induction for the target compounds **27-29** and **16-18** in the MCF-7 cell line

Comp.	IC ₅₀ (μM) ^a	Total apoptosis	Comp.	IC ₅₀ (μM) ^a	Total apoptosis
(<i>RS</i>)- 27	8.04 ± 0.00	55.2 ± 0.70 ^b 23.6 ± 0.10 ^c	(<i>RS</i>)- 16	8.17+/-0.00	40.8 ± 0.12b 13.4 ± 0.14c
(<i>R</i>)- 27	6.56 ± 0.11	60.0 ± 1.13 ^b 11.7 ± 0.23 ^c	(<i>R</i>)- 16	12.1 ± 0.04	29.2 ± 0.11b 9.35 ± 0.12c
(<i>S</i>)- 27	9.46+/-0.00	37.2 ± 0.11 ^b 12.4 ± 0.87 ^c	(<i>S</i>)- 16	4.50 ± 0.12	42.0 ± 2.31b 18.4 ± 0.44c
(<i>RS</i>)- 28	7.25+/-0.00	95.8 ± 0.21 ^b 36.2 ± 1.03 ^c	(<i>RS</i>)- 17	8.98+/-0.00	28.6 ± 0.50 ^b 7.62 ± 0.70 ^c
(<i>R</i>)- 28	5.18+/-0.00	47.5 ± 2.11 ^b 8.42 ± 0.41 ^c	(<i>R</i>)- 17	9.24+/-0.00	42.7 ± 0.15 ^b 7.95 ± 0.02 ^c
(<i>S</i>)- 28	7.78+/-0.00	25.7 ± 0.55 ^b 10.6 ± 0.09 ^c	(<i>S</i>)- 17	9.05 ± 0.14	26.6 ± 1.30 ^b 27.2 ± 0.05 ^c
(<i>RS</i>)- 29	5+/-0.00	78.2 ± 1.26 ^b 27.5 ± 0.33 ^c	(<i>RS</i>)- 18	5.73± 0.22	59.8 ± 0.11 ^b 20.2 ± 0.04 ^c
(<i>R</i>)- 29	4.34+/-0.00	87.4 ± 0.35 ^b 37.2 ± 0.30 ^c	(<i>R</i>)- 18	7.52+/-0.01	37.5 ± 0.05 ^b 10.6 ± 0.32 ^c
(<i>S</i>)- 29	7.03+/-0.00	56.1 ± 0.09 ^b 4.85 ± 0.19 ^c	(<i>S</i>)- 18	4.35+/-0.00	69.0 ± 0.57 ^b 27.5 ± 0.60 ^c

^aAll experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations. IC₅₀ was determined after 6 days of treatment. ^bCells were treated with the 3 × IC₅₀ values of compounds. ^cCells were treated with the IC₅₀ values of compounds. Apoptosis was measured after 48 h of treatment.

Table 7. Anti-proliferative effect and apoptosis induction for the target compounds **27-29** and **16-18** in the SKBR3 cell line.

Compounds **27-29**, **16** and **18** show one major bioactive enantiomer against both MCF-7 and SKBR-3 human breast cancer cells whereas compound **17** has presented equally bioactive enantiomers. In general, the IC₅₀ values of racemates (*RS*)-**27-29**, **16** and **18** are similar to the average IC₅₀ of the corresponding enantiomers (*R*)-**27-29**, **16**, **18** and (*S*)-**27-29**, **16**, **18**. Structure-activity relationship between the configuration of the enantiomers and the anti-proliferative effect indicates that in general, (*S*)-enantiomers are more active in the MCF-7 cell line. Thus, (*S*)-**28**, (*S*)-**29**, (*S*)-**16** and (*S*)-**18** are more potent than their corresponding enantiomers while (*R*)-**27** is more active than (*RS*)-**27** in the MCF-7 cell line. However, (*R*)-**27-29** and (*S*)-**16** and (*S*)-**18** show more cytotoxicity in the SKBR-3 cell line.

In the MCF-7 cell line racemic and homochiral compounds **27**, **28**, and **29**, with the purine moiety at position 2, are more active than their corresponding regioisomers **16**, **17** and **18**, with the purine moiety at position 3, except for (*S*)-**27**. The most active compound (*S*)-**29**, with 2,6-dichloropurine moiety at position 2, shows an IC₅₀=1.85 ± 0.05 μM being 2.5-fold more potent than the clinically used drug 5-FU (IC₅₀=4.32 ± 0.02 μM) (García-Rubiño et al., 2013). In contrast,

in the SKBR-3 cell line both racemic and homochiral compounds **27**, **28** and **29** are more active than their corresponding regioisomers **16**, **17** and **18**, except for (*S*)-**16** and (*S*)-**18**. The most active compound in this case is (*R*)-**29** with 2,6-dichloropurine moiety at position 2, shows an $IC_{50}=4.34 \pm 0.00 \mu M$.

The cell cycle does not show significant differences among the compounds (data not shown). Since it is well established that the eukaryotic initiation factor 2 alpha (eIF2 α) phosphorylation correlates with a translational block and consequently leads to the inhibition of protein synthesis and induction of apoptosis (García-Rubiño et al., 2013), we have analyzed the protein activation of this factor by western blot. eIF2 α is significantly phosphorylated in MCF-7 cancer cells after treatment with (*S*)-**29**, (*S*)-**17** and (*R*)-**16** at 16 h and 36 h.

Interestingly, (*S*)-**29** induces high eIF2 α phosphorylation in the MCF-7 cell line in comparison with its racemate and its enantiomer, where no activation is shown. These results support the highest anti-proliferative activity displayed by (*S*)-**29** and suggest that this activity is in part due to the suppression of protein synthesis provoked by eIF2 α phosphorylation (Baltzis et al., 2007). Furthermore, a prolonged induction of eIF2 α finally triggers the apoptosis phenomena (Gil et al., 1999; 20, Dagon et al., 2001).

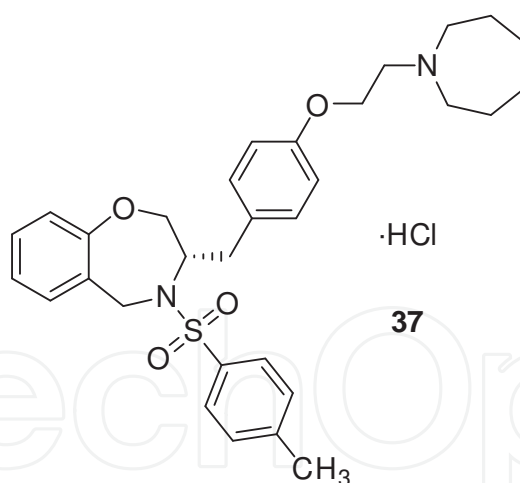
The following can be stated from Tables 6 and 7:

- a. In the MCF-7 cell line, compounds are more potent as programmed cell-death inducers than in SKBR-3, and more specifically, (*R*)-**29** and (*S*)-**18** are the more effective apoptotic inducers (77% and 60% at their IC_{50} , respectively) in the MCF-7 cell line.
- b. In the SKBR-3 cell line the best apoptotic values are observed at their $3 \times IC_{50}$ concentrations.
- c. Compounds (*RS*)-**28**, (*RS*)-**29** and (*R*)-**29** present the best apoptotic percentages in both cancerous cell lines at their $3 \times IC_{50}$ concentrations (99%, 98%, and 99%, respectively in MCF-7, and 96%, 78%, and 87%, respectively, in SKBR-3).

Previous works scarcely reports a different pattern in apoptosis levels between enantiomers. An exception is D-(-)-lentiginosine, the non-natural enantiomer of the iminosugar indolizidine alkaloid that acts as an apoptosis inducer on different tumour cells in contrast to its natural enantiomer (Macchi et al., 2010). All homochiral compounds included in this study show a different apoptosis effect between the two enantiomers. Apoptotic defects in cancer cells are the primary obstacle that limits the therapeutic efficacy of anticancer agents, and hence the development of novel agents targeting novel canonical and non-canonical programmed cell death pathways has become an imperative mission for clinical research (Cummings et al., 2004). Compounds **27-29**, and **16-18** induce strong levels of cell death measured by cytotoxicity analysis and by phosphatidylserine externalization (Annexin V binding) (Tables 6 and 7) even in the MCF-7 breast cancer cells that have shown deficiency in the caspase-activation mechanisms (Kagawa et al., 2001).

Whereas compound (*S*)-**27** activates the canonical intrinsic caspase-8/caspase-3 apoptotic pathway on the MCF-7 cell line, compound (*RS*)-**29** induces caspase-2 activation. However, a strong apoptosis induction is also detected in the rest of the compounds analysed. The caspase-

independent apoptosis in cells exposed to different drugs with diverse cellular effects has been previously described (Macchi et al., 2010). While caspase-2 activation could induce cell death through cytochrome c/mitochondria damage (Robertson et al., 2002), non-caspase-mediated increase in phosphatidylserine externalization can occur in response to high intracellular Ca^{2+} levels that alters scramblase and translocase (Vanags et al., 1996; 26, Kagan et al., 2000). Additionally, non-caspase proteases may activate and cleave the cytoskeleton proteins attached to phospholipids, including focal adhesion kinase and the actin-capping protein α -adducin (van de Water, 1999). To further confirm the involvement of caspases, including caspase-3, in the apoptosis induced by the most apoptotic compounds in the caspase-3 wild type SKBR-3 cell line, cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk for 2 h, followed by the (RS)-**28** and (RS)-**29** treatment, and cell viability metabolic-analysis was carried out. Our results show that (RS)-**28** and (RS)-**29** were sensible to the effect of z-VAD-fmk caspase inhibitor, which could rescue SKBR-3 cells from the cytotoxicity of compounds. These results demonstrate the involvement of caspase activation during cell death induced by the compounds in the SKBR-3 cells as previously described for numerous anti-tumour apoptotic drugs (Yang et al., 2012; Kumar et al., 2013; Lamberto et al., 2013). These and other anti-tumour effects such as autophagy or senescence events could be involved in the caspase-dependent and caspase-independent cell death induced by the compounds included in this study. This fact opens an important line of research that is yet to be explored.



Indian researchers have very recently investigated the effect of α tyrosine-based benzoxazine derivative in MCF-7 and MDA-MB-231 cells (Dwivedi et al., 2013). The anti-proliferative effect of **37** on MCF-7 cells was associated with G_1 cell-cycle arrest. This G_1 growth arrest was followed by apoptosis as **37**-dose dependently increased phosphatidylserine exposure. PARP cleavage and DNA fragmentation that are hallmarks of apoptotic cell death. Compound **37** activated components of both intrinsic and extrinsic pathways of apoptosis characterized by activation of caspase-8 and -9, mitochondrial membrane depolarization and increase in Bax/Bcl2 ratio. However, use of selective caspase inhibitors revealed that the caspase-8-dependent

pathway is the major contributor to **37**-induced apoptosis. Compound **37** also significantly reduced the growth of MCF-7 xenograft tumours in athymic nude mice (Dwivedi et al., 2013).

5. Conclusion

Cancer continues to be a major health problem in developing as well as undeveloped countries. Although major advances have been made in the chemotherapeutic management of some patients, the continued commitment to the laborious task of discovering new anticancer agents remains critically important, in the course of identifying various chemical substances, which may serve as leads for designing novel anti-tumour agents.

The ever-increasing use of asymmetric syntheses over many years has been manifested by the biological importance of enantiomerically pure single compound entity factors and further has been strongly guided by drug regulatory bodies because of strict rules and regulations about single isomers. A contributing factor to this effect has been, and continues to be the development of new, novel and efficient methods for accessing single isomers. In general, the binomial enantiomers → different biological activities and in particular, enantiomers → different anti-proliferative activities are rarely known, in spite of their great importance. It seems that in the future this topic will receive increasing attention and will help better understanding of the molecular recognition between drugs and biological targets.

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References

- [1] Al-Obeidi, F.; Austin, R.E.; Okonya, J.F. & Bond, D.R.S. (2003). Microwave-assisted solid-phase synthesis (MASS): parallel and combinatorial chemical library synthesis. *Mini-Reviews in Medicinal Chemistry*, Vol.3, No.5, (August 2003), pp. 449-460, ISSN 1389-5575.

- [2] Altieri, D.C. (2003). Validating survivin as a cancer therapeutic target. *Nature Reviews Cancer*, Vol.3, No.1, (January 2003), pp. 46-54, ISSN 1474-175X.
- [3] Baltzis, D.; Pluquet, O.; Papadakis, A.I.; Kazemi, S.; Qu, L.-K & Koromilas, A.E. (2007). The eIF2 α Kinases PERK and PKR Activate Glycogen Synthase Kinase 3 to Promote the Proteasomal Degradation of p53. *Journal of Biological Chemistry*, Vol.282, No.43, (October 2007), pp. 31675-31687, ISSN 0021-9258.
- [4] Brown, N.M.; Belles, C.A.; Lindley, S.L.; Zimmer-Nechemias, L.D.; Zhao, X.; Witte, D.P.; Kim, M.O. & Setchell, K.D.R. (2010). The chemopreventive action of equol enantiomers in a chemically induced animal model of breast cancer. *Carcinogenesis*, Vol.31, No.5, (May 2010), pp. 886–893, ISSN 0143-3334.
- [5] Chadderton, A.; Villeneuve, D.J.; Gluck, S.; Kirwan-Rhude, A.F.; Gannon, B.R.; Blais, D.E. & Parissenti, A.M. (2000). Role of specific apoptotic pathways in the restoration of paclitaxel-induced apoptosis by valspodar in doxorubicin-resistant MCF-7 breast cancer cells. *Breast Cancer Research and Treatment*, Vol.59, No.3, (February 2000), pp. 231-244, ISSN 1573-7217.
- [6] Conejo-García, A.; Núñez, M.C.; Marchal, J.A.; Rodríguez-Serrano, F.; Aránega, A.; Gallo, M.A.; Espinosa, A. & Campos, J.M. (2008). Regioespecific Microwave-Assisted Synthesis and Cytotoxic Activity against Human Breast Cancer Cells of (RS)-6-Substituted-7-or 9-(2,3-Dihydro-5H-1,4-Benzodioxepin-3-yl)-7H-or-9H-Purines. *European Journal of Medicinal Chemistry*, Vol.43, No.8, (August 2008), pp. 1742-1748, ISSN 0223-5234.
- [7] Conejo-García, A.; García-Rubiño, M.E.; Marchal, J.A.; Núñez, M.C.; Ramírez, A.; Ciminio, S.; García, M.A.; Aránega, A.; Gallo, M.A. & Campos, J.M. (2011). Synthesis and anticancer activity of (RS)-9-(2,3-dihydro-1,4-benzoheteroin-2-ylmethyl)-9H-purines. *European Journal of Medicinal Chemistry*, Vol.46, No.9, (September 2011), pp. 3795-3801, ISSN 0223-5234.
- [8] Cummings, B.S.; Kinsey, G.R.; Bolchoz, L.J. & Schnellmann, R.G. (2004). Identification of caspase-independent apoptosis in epithelial and cancer cells. *Journal of Pharmacology and Experimental Therapeutics*, Vol.310, No.1, (July 2004), pp. 126-134, ISSN 1521-0103.
- [9] Dagon, Y.; Dovrat, S.; Vilchik, S.; Hachohen, D.; Shlomo, G.; Sredni, B.; Salzberg S. & Nir, U. (2001). Double-stranded RNA-dependent protein kinase, PKR, down-regulates CDC2/cyclin B1 and induces apoptosis in non-transformed but not in v-mos transformed cells. *Oncogene*, Vol.20, No.56, (December 2001), pp. 8045-8056, ISSN 0950-9232.
- [10] De Fátima, A.; Zambuzzi, W.F.; Modolo, L.V.; Tarsitano, C.A.B.; Gadelha, F.R.; Hyslop, S.; de Carvalho, J.E.; Salgado, I.; Ferreira, C.V. & Pilli, R.A. (2008). Cytotoxicity of goniothalamine enantiomers in renal cancer cells: involvement of nitric oxide, apopto-

- sis and autophagy. *Chemico-Biological Interactions*, Vol.176, No.2-3, (November 2008), pp. 143-150, ISSN 0009-2797.
- [11] Díaz-Gavilán, M.; Choquesillo-Lazarte, D.; González-Pérez, J.M.; Gallo, M.A.; Espinosa, A. & Campos, J.M. (2007). Synthesis and Reactivity of (RS)-6-Chloro-7-or 9-(1,2,3,5-Tetrahydro-4,1-Benzoxazepin-3-yl)-7H-or 9H-Purines Bearing a Nitrobenzenesulfonyl Group on the Nitrogen Atom. *Tetrahedron*, Vol.63, No.24, (June 2007), pp. 5274-5286, ISSN 0040-4020.
- [12] Díaz-Gavilán, M.; Conejo-García, A.; Cruz-López, O.; Núñez, M.C.; Choquesillo-Lazarte, D.; González-Pérez, J.M.; Rodríguez-Serrano, F.; Marchal, J.A.; Aránega, A.; Gallo, M.A.; Espinosa, A. & Campos, J.M. (2008). Synthesis and Anticancer Activity of (R,S)-9-(2,3-Dihydro-1,4-Benzoxathiin-3-ylmethyl)-9H-Purines. *ChemMedChem*, Vol.3, No.1, (January 2008a), pp. 127-135, ISSN 1860-7187.
- [13] Díaz-Gavilán, M.; Gómez-Vidal, J.A.; Rodríguez-Serrano, F.; Marchal, J.A.; Caba, O.; Aránega, A.; Gallo, M.A.; Espinosa, A. & Campos, J.M. (2008). Anticancer Activity of (1.2.3.5-Tetrahydro-4.1-Benzoxazepine-3-yl)-Pyrimidines and-Purines against the MCF-7 Cell Line: Preliminary cDNA Microarray Studies. *Bioorganic & Medicinal Chemistry Letters*, Vol.18, No.4, (February 2008b), pp. 1457-1460, ISSN 0960-894X.
- [14] Díaz-Gavilán, M.; Rodríguez-Serrano, F.; Gómez-Vidal, J.A.; Marchal, J.A.; Aránega, A.; Gallo, M.A.; Espinosa, A. & Campos, J.M. (2004). Synthesis of Tetrahydrobenzoxazepine Acetals with Electron-Withdrawing Groups on the Nitrogen Atom. Novel Scaffolds Endowed with Anticancer Activity against Breast Cancer Cells. *Tetrahedron*, Vol.60, No.50, (December 2004), pp. 11547-11557, ISSN 0040-4020.
- [15] Duncan, K.J.; Eckert, K.A. & Clawson, G.A. (2009). Mechanisms of growth inhibition in human papillomavirus positive and negative cervical cancer cells by the chloromethyl ketone protease inhibitor, succinyl-alanine-alanine-proline-phenylalanine chloromethyl ketone. *Journal of Pharmacology and Experimental Therapeutics*, Vol.330, No.1, (July 2009), pp. 359-366, ISSN 1521-0103.
- [16] Dwivedi, S.K.D.; Samanta, K.; Yadav, M.; Jana, A.K.; Singh, A.K.; Chakravarti, B.; Mondal, S.; Konwar, R.; Trivedi, A.K.; Charttopadhyay, N.; Sanyal, S. & Panda, G. (2013). Amino acids derived benzoxazepines; Design, synthesis and antitumor activity. *Bioorganic & Medicinal Chemistry Letters*, Vol.23, No.24, (December 2013), pp. 6816-6821, ISSN 0960-894X.
- [17] Formigli, L.; Zecchi Orlandini, S.; Capaccioli, S.; Poupon, M.F. & Bani, D. (2002). Energy-dependent types of cell death in MCF-7 breast cancer cell tumors implanted into nude mice. *Cells Tissues Organs*, Vol.170, No.2-3, (January 2002), pp. 99-110, ISSN 1422-6405.
- [18] Fresco, P.; Borges, F.; Marques, M.P.M. & Diniz, C. (2010). The Anticancer Properties of Dietary Polyphenols and its Relation with Apoptosis. *Current Pharmaceutical Design*, Vol.16, No.1, (January 2010), pp. 114-134, ISSN 1381-6128.

- [19] García-Rubiño, M.E.; Conejo-García, A.; Núñez, M.C.; Carrasco, E.; García, M.A.; Choquesillo-Lazarte, D.; García-Ruiz, J.M.; Gallo, M.A.; Marchal, J.A. & Campos, J.M. (2013). Enantiospecific Synthesis of Heterocycles Linked to Purines: Different Apoptosis Modulation of Enantiomers in Breast Cancer Cells. *Current Medicinal Chemistry*, Vol.20, No.38, (December 2013), pp. 4924-4934, ISSN 0929-8673.
- [20] Gil, J.; Alcamí, J. & Esteban, M. (1999). Induction of apoptosis by double-stranded-RNA-dependent protein kinase (PKR) involves the alpha subunit of eukaryotic translation initiation factor 2 and NF-kappaB. *Molecular and Cellular Biology*, Vol.19, No.7, (July 1999), pp. 4653-4663, ISSN 0270-7306.
- [21] Gooch, J.L. & Yee, D. (1999). Strain-specific differences in formation of apoptotic DNA ladders in MCF-7 breast cancer cells. *Cancer Letters*, Vol.144, No.1, (September 1999), pp. 31-37, ISSN 0304-3835.
- [22] Grem, J.L.; Nguyen, D.; Monahan, B.P.; Kao, V. & Geoffroy, F.J. (1999). Sequence-dependent antagonism between fluorouracil and paclitaxel in human breast cancer cells. *Biochemical Pharmacology*, Vol.58, No.3, (August 1999), pp. 477-486, ISSN 0006-2952.
- [23] Hajdúch, M.; Havlíček, L.; Vesely, J.; Novotný, R.; Mihal, V. & Strnad, M. (1999). Synthetic cyclin-dependent kinase inhibitors: new generation of potent anti-cancer drugs. *Advances in Experimental Medicine and Biology*, Vol.457, No.II, (1999), pp. 341-353, ISSN 0065-2598.
- [24] Holcik, M. & Sonenberg, N. (2005). Translational control in stress and apoptosis. *Nature Reviews Molecular Cell Biology*, Vol.6, No.4, (April 2005), pp. 318-327, ISSN 1471-0072.
- [25] Kagan, V.E.; Fabisiak, J.P.; Shvedova, A.A.; Tyurina, Y.Y.; Tyurin, V.A.; Schor, N.F. & Kawai, K. (2000). Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. *FEBS Letters*, Vol.477, No.1-2, (July 2000), pp. 1-7, ISSN 0014-5793.
- [26] Kagawa, S.; Gu, J.; Honda, T.; McDonnell, T.J.; Swisher, S.G.; Roth, J. A. & Fang, B. (2001). Deficiency of caspase-3 in MCF7 cells blocks Bax-mediated nuclear fragmentation but not cell death. *Clinical Cancer Research*, Vol.7, No.5, (May 2001), pp. 1474-1480, ISSN 1078-0432.
- [27] Kappe, C. & Dallinger, O.D. (2006). The impact of microwave synthesis on drug discovery. *Nature Reviews Drug Discovery*, Vol.5, No.1, (January 2006), pp. 51-63, ISSN 1474-1776.
- [28] Kumar, S.; Kumar, A.; Pathania, A.S.; Guru, S.K.; Jada, S.; Sharma, P.R. Bhushan, S.; Saxena, A.K.; Kumar, H.M. & Malik, F. (2013). Tiron and trolox potentiate the autophagic cell death induced by magnolol analog Ery5 by activation of Bax in HL-60 cells. *Apoptosis*, Vol.18, No.5, (May 2013), pp. 605-617, ISSN 1360-8185.

- [29] Lai, J.C.; Brown, B.D.; Voskresenskiy, A.M.; Vonhoff, S.; Klussman, S.; Tan, W.; Colombini, M.; Weeratna, R.; Miller, P.; Benimetskaya, L. & Stein, C.A. (2007). Comparison of D-G3139 and its Enantiomer L-G3139 in melanoma cells demonstrates minimal in vitro but dramatic in vivo chiral dependency. *Molecular Therapy*, Vol.15, No.2 (February 2007), pp. 270-278, ISSN 1525-0016.
- [30] Lamberto, I.; Plano, D.; Moreno, E.; Font, M.; Palop, J.; Sanmartín, C. & Encío, I. (2013). Bisacylimidoselenocarbamates cause G2/M arrest associated with the modulation of CDK1 and Chk2 in human breast cancer MCF-7 cells. *Current Medicinal Chemistry*, Vol.20, No.12, (2013), pp. 1609-1619, ISSN 0929-8673.
- [31] Liu, H.; Xu, L.; Zhao, M.; Liu, W.; Zhang, C. & Zhou, S. (2009). Enantiomer-specific, bifenthrin-induced apoptosis mediated by MAPK signalling pathway in Hep G2 Cells. *Toxicology*, Vol.261, No.3, (July 2009), pp. 119-125, ISSN 0200-483X.
- [32] López-Cara, L.C.; Conejo-García, A.; Marchal, J.A.; Macchione, G.; Cruz-López, O.; Boulaiz, H.; García, M.A.; Rodríguez-Serrano, F.; Ramírez, A.; Cativiela, C.; Jiménez, A.I.; García-Ruiz, J.M.; Choquesillo-Lazarte, D.; Aránega, A. & Campos, J.M. (2011). New (RS)-Benzoxazepin-Purines with Antitumour Activity: The Chiral Switch from (RS)-2,6-Dichloro-9-[1-(p-Nitrobenzenesulfonyl)-1,2,3,5-Tetrahydro-4,1-Benzoxazepin-3-yl]-9H-Purine. *European Journal of Medicinal Chemistry*, Vol.46, No.1 (January 2011), pp. 249-258, ISSN 0223-5234.
- [33] Lundberg, A.S. & Weinberg, R.A. (1999). Control of the cell cycle and apoptosis. *European Journal of Cancer*, Vol.35, No.4 (April 1999), pp. 531-539, ISSN 0959-8049.
- [34] Macchi, B.; Minutolo, A.; Grelli, S.; Cardona, F.; Cordero, F.M.; Mastino, A. & Brandi, A. (2010). The novel proapoptotic activity of nonnatural enantiomer of Lentiginosine. *Glycobiology*, Vol.20, No.5, (May 2010), pp. 500-506, ISSN 0959-6658.
- [35] Marchal, J.A.; Aránega, A.; Conejo García, A.; García Chaves, M.A.; Cruz-López, O.; Boulaiz, H.; Rodríguez-Serrano, F.; Cativiela, C.; Perán, M.; Jiménez, A.I.; García-Ruiz, J.M.; Choquesillo-Lazarte, D. & Campos, J.M. Enantiómeros de derivados benzoheteroepínicos y su uso como agentes anticancerígenos. P201030415, 2010, Universidad de Granada.
- [36] Marchal, J.A.; Boulaiz, H.; Suárez, I.; Saniger, E.; Campos, J.; Carrillo, E.; Prados, J.; Gallo, M.A.; Espinosa, A. & Aránega, A. (2004). Growth inhibition, G₁-arrest, and apoptosis in MCF-7 human breast cancer cells by novel highly lipophilic 5-fluorouracil derivatives. *Investigational New Drugs*, Vol.22, No.4, (November 2004), pp. 379-389, ISSN 0167-6997.
- [37] Nagata, S. (1997). Apoptosis by death factor. *Cell*, Vol.88, No.3, (February 1997), pp. 355-365, ISSN 0092-8674.
- [38] Núñez, M.C.; Entrena, A.; Rodríguez-Serrano, F.; Marchal, J.A.; Aránega, A.; Gallo, M.A.; Espinosa, A. & Campos, J.M. (2005). Synthesis of novel 1-(2,3-dihydro-5H-4,1-

- benzoxathiepin-3-yl)-uracil and -thymine, and their corresponding S-oxidized derivatives. *Tetrahedron*, Vol.61, No.43, (October 2005), pp. 10363-10369, ISSN 0040-4020.
- [39] Núñez, M.C.; García-Rubiño, M.E.; Conejo-García, A.; Cruz-López, O.; Kimatrai, M.; Gallo, M.A.; Espinosa, A. & Campos, J.M. (2009). Homochiral Drugs: a Demanding Tendency of the Pharmaceutical Industry. *Current Medicinal Chemistry*, Vol.16, No.16, June 2009), pp. 2064-2074, ISSN 0929-8673.
- [40] Núñez, M.C.; Rodríguez-Serrano, F.; Marchal, J.A.; Caba, O.; Aránega, A.; Gallo, M.A.; Espinosa, A. & Campos, J.M. (2007). 6'-Chloro-7-or 9-(2,3-dihydro-5H-4,1-benzoxathiepin-3-yl)-7H-or 9H-purines and their corresponding sulfones as a new family of cytotoxic drugs. *Tetrahedron*, Vol.63, No.1, (January 2007), pp. 183-190, ISSN 0040-4020.
- [41] Robertson, J.D.; Enoksson, M.; Suomela, M.; Zhivotovsky, B. & Orrenius, S. (2002). Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. *Journal of Biological Chemistry*, Vol.277, No.33, (August 2002), pp. 29803-29809, ISSN 0021-9258.
- [42] Saunders, D.E.; Lawrence, W.D.; Christensen, C.; Wappler, N.L.; Ruan, H. & Deppe, G. (1997). Paclitaxel-induced apoptosis in MCF-7 breast cancer cells. *International Journal of Cancer*, Vol.70, No.2, (January 1997), pp. 214-220, ISSN 1097-0215.
- [43] Shelley, M.D.; Hartley, L.; Fish, R.G.; Groundwater, P.; Morgan, J.J.G.; Mort, D.; Mason, M. & Evans, A. (1999). Stereo-specific cytotoxic effects of gossypol enantiomers and gossypolone in tumour cell lines. *Cancer Letters*, Vol.135, No.2, (January 1999), pp. 171-180, ISSN 0304-3835.
- [44] Sherr, C.J. Cancer cell cycles. *Science*, Vol.274, No.5293, (December 1996), pp. 1672-1677, ISSN 0036-8075.
- [45] Stewart, B.W. & Kleihues P. (2003) (Eds.), World Cancer Report, International Agency for Research on Cancer, World Health Organization, 2003.
- [46] Testa, U. (2004). Apoptotic mechanisms in the control of erythropoiesis. *Leukemia*, Vol.18, No.7, (July 2004), pp. 1176-1199, ISSN 0887-6924.
- [47] van de Water, B.; Nagelkerke, J.F. & Stevens, J.L. (1999). Dephosphorylation of focal adhesion kinase (FAK) and loss of focal contacts precede caspase-mediated cleavage of FAK during apoptosis in renal epithelial cells. *Journal of Biological Chemistry*, Vol. 274, No.19, (May 1999), pp. 13328-13337, ISSN 0021-9258.
- [48] Vanags, D.M.; Poern-Ares, M.I.; Coppola, S.; Burgess, D.H. & Orrenius, S. (1996). Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *Journal of Biological Chemistry*, Vol.271, No.49, (December 1996), pp. 31075-31085, ISSN 0021-9258.
- [49] Yang, F.; Jove, V.; Chang, S.; Hedvat, M.; Liu, L.; Buettner, R.; Tian, Y.; Scuto, A.; Wen, W.; Yip, M.L.; Van Meter, T.; Yen, Y. & Jove, R. (2012). Bortezomib induces

apoptosis and growth suppression in human medulloblastoma cells, associated with inhibition of AKT and NF- κ B signaling, and synergizes with an ERK inhibitor. *Cancer Biology Therapy*, Vol.13, No.6, (April 2012), pp. 349-357, ISSN 1538-4047.

- [50] Zivny, J.; Klener Jr, P.; Pytlik, R. & Andera, L. (2010). The Role of Apoptosis in Cancer Development and Treatment: Focusing on the Development and Treatment of Hematologic Malignancies. *Current Pharmaceutical Design*, Vol.16, No.1, (January 2010), pp. 11-33, ISSN 1381-6128.