Full Paper

Synthesis and Biological Evaluation of Heteroaryldiamides and Heteroaryldiamines as Cytotoxic Agents, Apoptosis Inducers and Caspase-3 Activators

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The work described here involved the synthesis and biological evaluation of new heteroaryldiamides and heteroaryldiamines. A new general model in which the structures can be adjusted has been applied in this study. Three different structural units can be distinguished: a central nucleus and two symmetric terminal units. The central element is either an aliphatic chain of varying length and flexibility, piperazine, or a polyamine nucleus. However, the terminal units are pyridine, quinoline, indole, benzene or pyrido[2,3-*d*]pyrimidine with different substituents. The antitumoural activities of the compounds were evaluated *in vitro* by examining their cytotoxic effects against human breast, colon, and bladder cancer cell lines. Compounds that showed cytotoxic activity were subjected to both apoptosis and caspase-3 assays. With regard to selectivity, the cytotoxicity was also determined in cell cultures of two nontumoural lines. The most promising compounds are **4c**, **5c** and **7**, which are amino-pyridinium, quinolyl-*N*-oxide, and pyridyl derivatives, respectively, and these reveal a significant *in vitro* cytotoxicity in at least two of the three cell lines tested. These compounds induced apoptosis and also produced a rapid dose-dependent increase in the caspase-3 level in HT-29 cells. Other encouraging profiles were found, such as those presented by **1k** and **8d**, which are cytotoxic and apoptotic but do not provoke an increase in the level of caspase-3, or those presented by **2f**, **3c** and **4a**, which are slightly cytotoxic but do not show any other significant activity. The different types of behaviour of each compound are not necessarily parallel in the three cell lines tested.

Keywords: Heteroaryldiamides / Heteroaryldiamines / Cytotoxicity / Apoptosis / Caspase-3

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Introduction

In recent years, a number of chemotherapeutic agents have been developed for clinical use. However, the benefits of these compounds are limited due to the fact that cancer is still one of the most common causes of mortality. The study of cellular proliferation kinetics in tumours has provided important information on the genetic changes that are involved in several types of cancer [1]. The cellular death programme disorder is also included in this group due to its determinant activity in tumourgenesis processes [2, 3].

There are two types of cellular death: necrosis and apoptosis. It has been demonstrated that apoptosis is

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inhibited in major tumours and this results in an uncontrolled cellular increase in carcinogenic tissue. This proliferation disorder is not only produced by the outbreak of the mitosis rate but can also be produced by the considerable decrease in the rate of cellular death. Therefore, gaining knowledge concerning the biochemistry and molecular apoptotic pathways is extremely important for the discovery and development of new therapeutic strategies in cancer [4–9]. The use of the apoptotic mechanism as a target in cancer research is a complex issue, but most previous studies have revealed that the cystein-protease family (namely caspases) are activated in apoptotic pathways [10, 11]. These systems are probably the most important effect-provoking molecules related to the induction of apoptosis and all of them are synthesised in the form of inactive precursors (proenzymes) that are subsequently activated by self-proteolysis (autocatalytic cleavage) or other proteases [12].

A review of the literature enabled the selection of new molecules with different chemical groups for the present work. Structural analysis was then carried out on compounds with recognized efficacy in cancer treatment and most of their mechanisms of action are intimately related with the induction of apoptosis and/or caspase activation. Among the many structural groups identified, the following were selected for this study:

1) π-deficient monocyclic aromatic systems, such as pyridine [13, 14] or bicyclic aromatic systems, such as quinoline [15, 16], indole [17, 18] and pyrido[2,3-*d*]pyrimidine [19, 20] at the ends of the molecule;

2) Amide groups directly linked to the heterocycle or near to it [21, 22] – these are attached to apolar chains of variable length and flexibility [23, 24];

3) Groups that are capable of participating in oxide-reduction processes, such as *N*-oxides [25], or are capable of altering molecular polarity and the possibility of forming hydrogen bonds, such as *N*-amino and *N*-methyl groups [26]. It was also noted that many of the molecules studied possess a high degree of symmetry [27–30].

In designing new structures, a general pattern derived from the reference literature has been adopted. This pattern, while flexible in geometry and chemical structure, basically responds to molecules which contain three entities: a central nucleus made up of a cyclic or linear aliphatic chain of variable length and flexibility, two identical lateral arms connected to the centre by a variable functional group. In the work described here, an attempt was made to confirm the usefulness, in terms of cytotoxic activity and apoptosis induction, of certain structural parameters relative to the described model. These structural considerations were combined with our experience in the study and preparation of this kind of molecules. As a result, we present the synthesis of new compounds that are based on this general formula (Fig. 1).

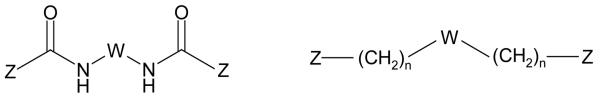


Figure 1. General formula of newly synthesised compounds.

The compounds **1a–n**, **2a–f**, **3a–e**, **4a–c**, **5a–c**, and **6a–b** (Table 1–2) include symmetrical amide derivatives with pyridine, quinoline and indole units connected by central apolar chains of variable length and flexibility. The aromatic end units are in some cases functionalized by *N*-oxide, *N*-methyl, *N*-amino, halogen, thereby modifying the electronic distribution, polarity and ability to form hydrogen bonds. The derivatives **7** and **8c–d** include amine and polyamine derivatives with the aim of evaluating the influence of the replacement of amides for amines in the target activity. By contrast, the derivatives **8a–b**, recently published [31], include the flat and rigid pyrido[2,3-*d*]pyrimidine bicycle functionalized in positions 2 and 4 by amine aliphatic chains with pyridine and indole aromatic end groups in the central nucleus.

Results

Chemistry

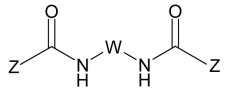
The synthesis of the diamides **1a–n** and **2a–f** was carried out according to Schemes 1a and 1b, by reaction between acyl chlorides, obtained by the corresponding carboxylic acid by heating under reflux with thionyl chloride, and the appropriate amines in the presence of equimolecular amounts of triethylamine. In the case of the acyl chlorides prepared for compounds **2a–f**, chloroform was added as a solvent in order to provide milder conditions and avoid halogenation of the quinoline ring.

Compounds **3a–e** were obtained from the corresponding derivatives **1** by treatment with methyl iodide in refluxing ethanol. Derivatives **4a–c** were obtained from derivatives **1** by treatment with hydroxylamine-*O*-sulfonic acid (potassium salt) in refluxing water, with methanol as co-solvent (Scheme 1a). HI was then added to obtain the desired dihydroiodide salts. Compound **4c** was obtained as a sulfate.

Compounds **5a–c** (Scheme 1b) were obtained from the corresponding compounds **2** by treatment with 3-chloroperoxybenzoic acid. The use of dichloromethane/chloroform as the solvent allowed *N*-oxidation of the quinoline ring without modification of the amide group.

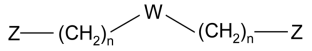
Compounds **6a–b** were obtained by reaction of the corresponding diamine with 2-ethoxycarbonylindole in

Table 1. Experimental data for new compounds **1–6**.



Ref	Z	Position	W	Yield [%]	M. p. [°C]	Recrystallisation Solvent	C.H.N.
1a	Pyridyl	3	$-(CH_2)_4-$	52	202-3	EtOH	$C_{16}H_{18}N_4O_2$
1b	Pyridyl	3	1,4-piperazine	38	198-9	EtOH	$C_{16}H_{16}N_4O_2$
1c	Pyridyl	3	$-(CH_2)_3-$	31	157-8	EtOH	$C_{15}H_{16}N_4O_2$
1d	Pyridyl	3	1,4-cyclohexanediamine	36	>300	MeOH	$C_{18}H_{20}N_4O_2$
1e	Pyridyl	3	$-(CH_2)_5-$	7	129-30	AcOEt	$C_{17}H_{20}N_4O_2$
1f	Pyridyl	3	$-(CH_2)_8-$	40	148-9	EtOH	$C_{20}H_{26}N_4O_2$
1g	Pyridyl	2	$-(CH_2)_3-$	23	165-6	$CHCl_3$/EtOH	$C_{15}H_{16}N_4O_2$
1h	(6-Cl)-Pyridyl	3	1,4-cyclohexanediamine	57	300	EtOH	$C_{18}H_{18}Cl_2N_4O_2$
1i	(6-Cl)-Pyridyl	3	1,4-piperazine	77	245-6	EtOH	$C_{16}H_{14}Cl_2N_4O_2$
1j	(6-Cl)-Pyridyl	3	$-(CH_2)_4-$	55	200-1	EtOH	$C_{16}H_{16}Cl_2N_4O_2$
1k	Pyridyl-N-oxide	3	$-(CH_2)_4-$	22	248-9	MeOH/H_2O	$C_{16}H_{18}N_4O_4$
1l	Pyridyl-N-oxide	3	1,4-piperazine	17	281-2	EtOH/H_2O	$C_{16}H_{16}N_4O_4$
1m	Pyridyl-N-oxide	3	$-(CH_2)_3-$	6	206-7	MeOH	$C_{15}H_{16}N_4O_4$
1n	Pyridyl-N-oxide	3	1,4-cyclohexanediamine	8	>300	MeOH/H_2O	$C_{18}H_{20}N_4O_4 \cdot {}^1/_3\,H_2O$
2a	Quinolyl	3	$-(CH_2)_3-$	66	200-1	n-Hexane/EtOH	$C_{23}H_{20}N_4O_2$
2b	Quinolyl	3	1,4-piperazine	73	268-9	MeOH/CH_2Cl_2	$C_{24}H_{20}N_4O_2$
2c	Quinolyl	3	$-(CH_2)_5-$	67	179-80	EtOH	$C_{25}H_{24}N_4O_2$
2d	Quinolyl	2	1,4-piperazine	24	197-8	MeOH	$C_{24}H_{20}N_4O_2$
2e	Quinolyl	2	$-(CH_2)_3-$	23	118-9	n-Hexane/2-Propanol	$C_{23}H_{20}N_4O_2$
2f	Quinolyl	2	$-(CH_2)_2-$	35	214-5	CH_2Cl_2	$C_{22}H_{18}N_4O_2$
3a	1-Methyl-pyridinium	3	$-(CH_2)_4-$	50	226-7	EtOH/H_2O	$C_{18}H_{24}I_2N_4O_2$
3b	1-Methyl-pyridinium	3	1,4-piperazine	51	>300	EtOH/H_2O	$C_{18}H_{22}I_2N_4O_2$
3c	1-Methyl-pyridinium	3	$-(CH_2)_3-$	40	220-1	EtOH/H_2O	$C_{17}H_{22}I_2N_4O_2$
3d	1-Methyl-pyridinium	3	1,4-cyclohexanediamine	21	>300	EtOH/H_2O	$C_{20}H_{26}I_2N_4O_2$
3e	1-Methyl-pyridinium	3	$-(CH_2)_5-$	13	159-60	EtOH	$C_{19}H_{26}I_2N_4O_2$
4a	1-Amino-pyridinium	3	$-(CH_2)_4-$	14	195-6	MeOH	$C_{16}H_{22}I_2N_6O_2$
4b	1-Amino-pyridinium	3	1,4-piperazine	13	224-5	MeOH	$C_{16}H_{20}I_2N_6O_2$
4c	1-Amino-pyridinium	3	$-(CH_2)_3-$	7	264-5	MeOH	$C_{15}H_{20}N_6O_6S \cdot {}^1/_2\,H_2O$
5a	Quinolyl-N-oxide	2	1,4-piperazine	8	290-3	CH_2Cl_2/MeOH	$C_{24}H_{20}N_4O_4$
5b	Quinolyl-N-oxide	2	$-(CH_2)_2-$	89	269-70	EtOH	$C_{22}H_{18}N_4O_4$
5c	Quinolyl-N-oxide	2	$-(CH_2)_3-$	13	157-8	EtOH	$C_{23}H_{20}N_4O_2$
6a	Indolyl	2	$-(CH_2)_3-$	26	>300	Washed with MeOH	$C_{21}H_{20}N_4O_2$
6b	Indolyl	2	1,4-piperazine	13	>300	Washed with MeOH	$C_{22}H_{20}N_4O_2$

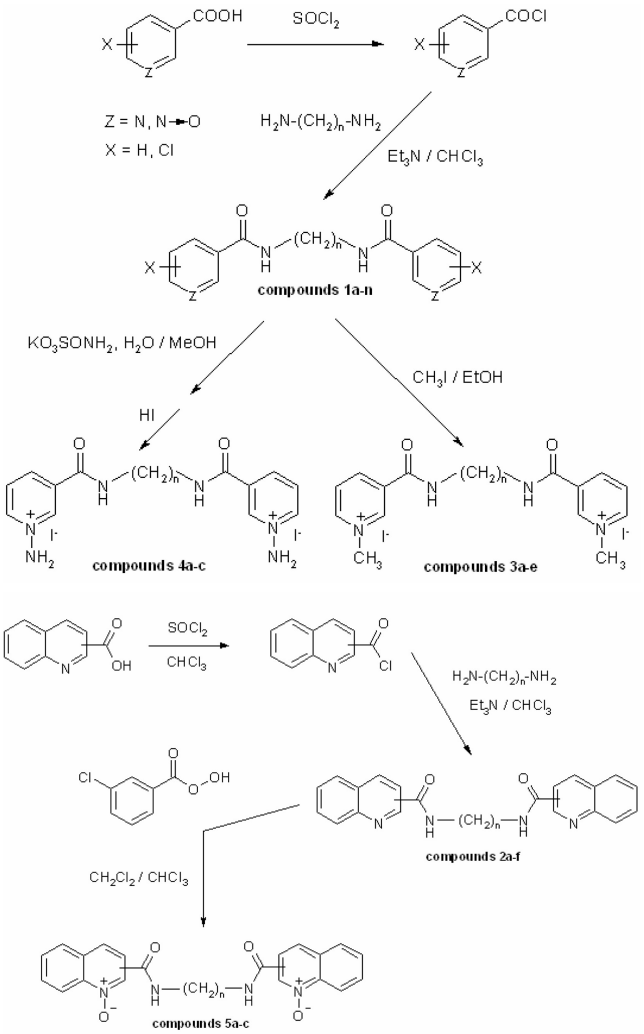
Table 2. Experimental data for new compounds **7–8**.



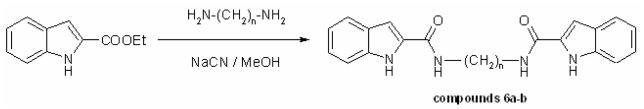
Ref	Z	n	W	Yield [%]	M. p. [0°C]	Recrystallisation Solvent	C.H.N.
7	2-Pyridyl	1	1,4-piperazine	37	94-5	n-Hexane/CH_2Cl_2	$C_{16}H_{20}N_4$
8a	2-Pyridyl	2	2,4-diaminopyrido-pyrimidine	15	156-7	AcOEt/2-propanol	$C_{21}H_{21}N_7$
8b	3-Indolyl	2	2,4-diaminopyrido-pyrimidine	10	162-3	n-Hexane/MeOH	$C_{27}H_{25}N_7 \cdot HCl$
8c	Pyrido[2,3-d]pyrimidyl		1,4-bis(3-aminopropyl)-piperazine	30	268-9	EtOH/MeOH	$C_{24}H_{30}N_{10} \cdot 2\,HCl$
8d	Pyrido[2,3-d]pyrimidyl	3	$-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH-$	9	284-5	Washed with boiling acetic acid	$C_{24}H_{32}N_{10} \cdot 3\,HI$

refluxing methanol with sodium cyanide as the catalyst (Scheme 2).

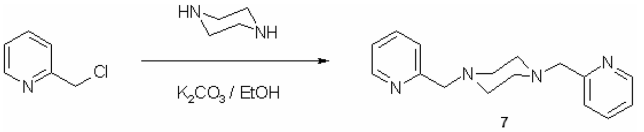
Compound **7** was obtained by direct reaction of piperazine with the alkyl halide in refluxing ethanol in the presence of K_2CO_3 (Scheme 3).



Scheme 1. a) Synthesis route of compounds **1a**–**n**, **3a**–**e** and **4a**–**c**. **b)** Synthesis route of compounds **2a**–**f** and **5a**–**c**.

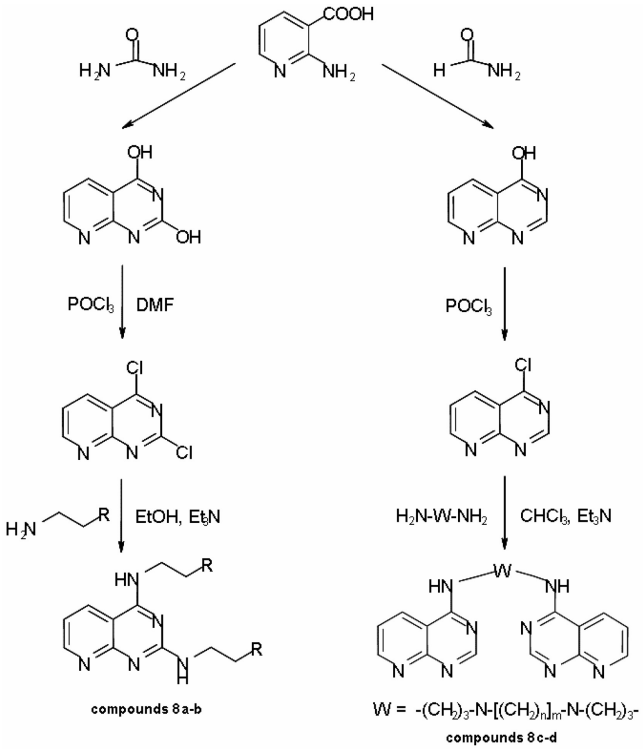


Scheme 2. Synthesis route of compounds **6a**, **b**.



Scheme 3. Synthesis route of compound **7**.

The synthesis of compounds **8a**–**b** and **8c**–**d** was carried out in three steps (Scheme 4). 2-Aminonicotinic acid was condensed with an excess of urea or formamide to afford pyrido[2,3-*d*]pyrimidine-2,4-diol and pyrido[2,3-*d*]pyrimidine-4-ol, respectively. The hydroxyl groups were



Scheme 4. Synthesis route of compounds **8a**–**b** and **8c**–**d**.

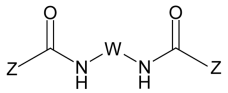
replaced with chloro-substituents by treatment with refluxing phosphorus oxychloride. *N*,*N*-dimethylformamide was added as a catalyst to the reaction of the diol because substitution in position 2 is less favored [32]. In the third step, the chloro-substituents were replaced by the corresponding amines in the presence of equimolecular amounts of triethylamine. In the case of compound **8d**, which was obtained by reaction of 4-chloropyrido[2,3-*d*]pyrimidine and 1,4-butanediamine, the addition of KI was necessary in order to facilitate the substitution of primary amines rather than secondary amines.

Biological evaluation

Cytotoxicity

The cytotoxic activities of the synthesised compounds were determined on three human cancer cell lines [breast (MD-MBA-231), bladder (T-24) and colon (HT-29)] using the neutral red assay [33]. The survival percentage was determined after a period of 72 h at screening concentrations of 100 and 20 μM, using the survival percentage obtained from the cells treated only with the solvent (DMSO at 0.5%) as a reference. The results are expressed as the average of assays carried out in triplicate. IC_{50} values were calculated for those compounds that showed

Table 3. Biological profile for the most active compounds.



Ref	Z	Position	W	IC_{50} [μM]			Apoptosis in cell line			Caspase-3	% survival[a]	% survival[b]
				(a)	(b)	(c)	(a)	(b)	(c)			
1k	Pyridyl-N-oxide	3	$-(CH_2)_4-$	18.0	na[c]	35.5	1.8	–[d]	1.8	na	100	65
1m	Pyridyl-N-oxide	3	$-(CH_2)_3-$	25.0	47.9	na	1.6	1.5	–	+(MD-MBA-231)	100	90
2a	Quinolyl	3	$-(CH_2)_3-$	31.0	na	na	1.3	–	–	+(MD-MBA-231)	100	48
2f	Quinolyl	2	$-(CH_2)_3-$	49.0	na	na	1.5	–	–	na	100	82
3a	1-Methyl-pyridinium	3	$-(CH_2)_4-$	36.4	na	na	1.6	–	–	+(MD-MBA-231)	95	73
3c	1-Methyl-pyridinium	3	$-(CH_2)_3-$	42.3	na	62.8	1.5	–	na	na	82	64
4a	1-amino-pyridinium	3	$-(CH_2)_4-$	55.1	na	na	na	–	–	na	100	89
4c	1-amino-pyridinium	3	$-(CH_2)_3-$	25.8	49.2	na	2.0	1.7	–	++(HT-29)	95	90
5c	Quinolyl-N-oxide	2	$-(CH_2)_3-$	7.9	37.8	50.5	2.6	2.9	na	++(HT-29)	95	86
6a	Indolyl	2	$-(CH_2)_3-$	na	na	78.0	–	–	1.9	na	75	64
camptothecin				0.291	0.014	0.009	2.6	2.6	3.3	nd[e]	nd	nd

(a) Cell line: MD-MBA-231, (b) Cell line: HT-29m (c) Cell line: T-24.

a) Survival percentage in CRL-8799 cell line (concentration: the highest IC_{50} value obtained from the three tumoural lines).

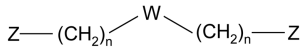
b) Survival percentage in CRL-11233 cell line (concentration: the highest IC_{50} value obtained from the three tumoural lines).

c) na = no activity observed after 48 h incubation at the highest IC_{50} found.

d) – = apoptosis assay not pertinent because compound is not cytotoxic.

e) nd = no data.

Table 4. Biological profile for the most active compounds.



Ref	Z	n	W	IC_{50} [μM]			Apoptosis in cell line			Caspase-3	% survival[a]	% survival[b]
				(a)	(b)	(c)	(a)	(b)	(c)			
7	2-Pyridyl	1	1,4-piperazine	15.3	28.9	na[c])	2.4	5.4	–[d]	++(HT-29)	80	58
8a	2-Pyridyl	2	2,4-diaminopyridopyrimidine	na	68.0	na	–	1.5	–	+(HT-29)	100	100
8b	3-Indolyl	2	2,4-diaminopyridopyrimidine	5.1	3.0	7.7	na	3.0	na	++(HT-29)	56	nd[e]
8d	Pyrido[2,3-d]pyrimidyl	3	$-NH-(CH_2)_4-NH-$	9.2	15.4	62.7	na	3.0	2.7	na	62	68
camptothecin				0.29	0.014	0.009	2.6	2.6	3.3	nd	nd	nd

(a) Cell line: MD-MBA-231, (b) Cell line: HT-29, (c) Cell line: T-24.

a) Survival percentage in CRL-8799 cell line (concentration: the highest IC_{50} value obtained from the three tumoural lines).

b) Survival percentage in CRL-11233 cell line (concentration: the highest IC_{50} value obtained from the three tumoural lines).

c) na = no activity observed after 48 h incubation at the highest IC_{50} found.

d) – = apoptosis assay not pertinent because compound is not cytotoxic.

e) nd = no data.

survival levels of < 45% (100 μM). The results obtained for the most active compounds are shown in Tables 3 – 4.

With regard to selectivity, the cytotoxicity was determined in cell cultures of two nontumoural lines, one of breast (CRL-8799) and another of liver (CRL-11233). The same experimental procedure was used in these cytotoxicity assays with nontumoural lines. For each compound, the highest IC_{50} value obtained from the three tumoural lines was selected as the test concentration. The results obtained are expressed in survival percentage at this concentration because we only expect a rough measure about selectivity of these compounds.

Apoptosis and Caspase-3

Once the active compounds in the cytotoxicity assay had been identified, the compounds were subjected to a test aimed at determining whether or not they also act as inducers of apoptosis and/or activators of caspase-3.

Apoptosis

The ability of the selected compounds to induce apoptosis in cell cultures (lines MD-MBA-231, HT-29 and T-24), was assessed [31] by using the Cell Death Detection ELISA Plus Kit from Roche Biochemical (Roche Diagnostics, Barcelona, Spain); this cellular test detects nucleosomes in

cytoplasm prior to disintegration of the plasma membrane, a well-known hallmark of apoptosis. The test concentrations correspond to the IC_{50} values determined in the cytotoxicity assay; the incubation time was of 24 h. The results express the number of times in which the culture containing the test compound surpasses the control culture in its ability to induce DNA fragmentation, with a relative value of 1 assigned to the level of apoptosis detected in the control culture. A result was considered positive when the level of DNA fragmentation obtained was at least double the values obtained for the corresponding control cultures, which were treated only with the solvent. The results obtained for the selected compounds and the reference substance (camptothecin) are shown in Tables 3 – 4.

Caspase-3

The compounds that showed cytotoxicity were subjected to the caspase-3 assay because caspase-3 is considered to be one of the principal executing caspases in which all of the biochemical routes involved in the apoptosis response converge. The Active-Caspase-3 FITC Mab apoptosis kit from Pharmingen (San Diego, CA, USA) was used [31]. This test detects the quantity of caspase-3 dimerized in the apoptotic cells, by means of the number of cells that contain the dimerized and activated form of caspase-3 after treatment with a cytotoxic compound. The test allows confirmation of the involvement of this enzyme in the cell death process. The range of effective measurements for this enzyme was found to be between 14 and 48 h. We have selected 48 h because at this time all the enzyme is activated. Therefore, for the most active compounds, measurements are taken at 14, 24 and 48 h, in order to detect other forms of cell death, and the obtained values were compared with those of control cells incubated without the test compounds. The test concentrations correspond to the IC_{50} values determined in the cytotoxicity assay. The results of this semi-quantitative assay are expressed using the following symbols: (na) when an increase was not detected in caspase-3 level with respect to the control, (+) when an increase of approximately 50% was detected, and (++) when an increase of 100% or more was observed (Tables 3 – 4). This is considered to be preliminary data for the determination of the mechanism of action.

Discussion

The study described here included the synthesis of 36 new compounds – however, reference is only made to those that showed a certain degree of cytotoxicity in at least one of the tested cell lines. The results for the most active compounds are summarised in Tables 3 – 4.

Cytotoxicity

The structure-activity relationships that are considered useful for improving the activity were established on the basis of the results from the biological assays carried out on the compounds prepared from the aforementioned design. These relationships have already been applied in this study but, more importantly, they are useful in the planning of future studies. These conclusions can be summarised as follows:

In general, the amide derivatives that contain aliphatic chains as the central linking unit with pyridine, *N*-alkyl and *N*-amines as the terminal groups have moderate cytotoxic activity. The *N*-oxidation of the pyridinic nitrogen causes an increase in activity in comparison to the analogous reduced compounds in cases where the central chains have 3 or 4 carbon atoms. Compounds **1k** and **1m** were cytotoxic, especially on cellular line MD-MBA-231 (18.0 and 25.0 μM, respectively).

The compounds with central chains of 3 carbon atoms that separate the heterocyclic end units and are located in the 2-position with respect to the heteroatom gave rise to the best results. In particular, **5c** was active on MD-MBA-231, HT-29 and T-24 lines (7.9, 37.8 and 50.5 μM, respectively). It is worth noting that in this derivative the quinolinic nitrogen is also in the *N*-oxide form.

Replacement of the amide group by a polyamine, a structure highlighted by numerous literature references [34] on cancer therapy, made reach the desired markedly higher activity levels and, therefore, this system appears to be a very interesting central block in terms of increasing the activity. These results, considering the small number of structures tested (**7**, **8c** and **8d**), justify future research in this area.

In order to study the degree of selectivity of the cytotoxic activity of the compounds under investigation, assays using healthy cells were carried out on some representative examples. The compounds selected were those that showed activity in tumoural cells. The healthy cells corresponded to CLR-8799 and CLR-11233, and the survival values were between 95 and 100% for **1k**, **1m**, **2a**, **2f**, **3a, 4a, 4c, 5c**, and **8a** in at least one of the tested lines.

Apoptosis and Caspase-3

Eight compounds showed notable activity (>1.50) in inducing apoptosis against the tested cell tumoural lines. The best apoptosis inducer was compound **7**, which has a value of 5.4 in HT-29 and 2.4 in MD-MBA-231. Other interesting compounds are **8d** (3.0 in HT-29 and 2.7 in T-24) and **5c** (2.9 in HT-29 and 2.6 in MD-MBA-231). Camptothe-

cin, which was used as a reference, gives values between 2.6 and 3.3 for these same cell lines.

Certain compounds, particularly **1m**, **2a** and **3a**, caused notable increases in the caspase-3 levels in the MD-MBA-231 cell line and compounds **4c**, **5c** and **7** caused higher increases in HT-29. This fact is of great interest given the characteristics of this enzyme; the enzyme is considered to be one of the principal executing caspases, in which all of the biochemical routes involved in the apoptosis response converges.

Analysis of the results obtained from the biological evaluation shows different preliminary profiles in the behaviour of the products, thereby suggesting the existence of diverse mechanisms of action. For example, compounds **1k**, **2f**, **3c**, **6a,** and **8d** are cytotoxic and apoptotic in at least one of the tested lines and yet do not modify the levels of caspase-3. This indicates cell death by apoptosis, in which caspase plays no part whatsoever. Such behaviour is similar to that shown by irofulven [35], an agent used in the treatment of prostate cancer. This compound binds to DNA and protein targets, forming a macromolecular adduct. This binding interferes with DNA replication (S-phase arrest) and cell division of tumour cells, leading to apoptotic cell death. Finally, derivatives **1m**, **2a**, **3a**, **4c**, **5c,** and **7** are cytotoxic, apoptotic and activators of caspase-3 in at least one of the tested cell lines. In addition **1m**, **3a**, **4c**, and **5c** have survival values in the range 95-100% in the nontumoural CRL-8799 cell line, thereby perfectly fitting our target compound profile.

The general trend on which the design of these structures is based has proven to be valid in obtaining the desired activity. However, it must be stressed that the model still requires some adjustment and refinement in order to obtain the most potent structures. It is clear that the synthesis of more analogues is required to obtain a good structure-activity relationship, but the initial results presented here have strengthened our interest in these structures in the search for the target activity.

Conclusion

We have completed the synthesis and biological evaluation of 36 novel heteroaryldiamides and heteroaryldiamines as potential antineoplastic agents, apoptosis inducers and caspase-3 activators. These compounds were studied in an *in vitro* cytotoxicity assay against three cancer cell lines: breast (MD-MBA-231), colon (HT-29) and bladder (T-24). Most of the synthesised compounds showed low cytotoxicity against three human cancer cell lines although these data have been useful to make progress about the structure-activity relationships. Compounds that showed cytotoxicity were evaluated in apoptosis and caspase-3 assays. Progressive design of the structures enabled us to obtain some lead derivatives, such as **1m**, **3a**, **4c**, **5c**, and **7** which have the desired cytotoxic activity, proapoptotic behaviour and caspase-3 activator characteristics. These compounds show a similar biological profile even though they have a markedly different structure. The best apoptosis inducer found in this study is compound **7**, which shows an apoptosis value of 5.4 against HT-29 cell line and is also cytotoxic and a caspase-3 activator in the same cell line. It is envisaged that taking these lead compounds as a starting point will enable more potent analogues to be generated.

The precise mechanism of action of some of these derivatives is currently under investigation in our laboratory. The ability of some of these compounds to cause accumulation of caspase-3 will be taken into account even though comparable structures in literature have the ability to bind tightly but reversibly to DNA by intercalation between the base pairs of the double helix [25, 36, 37]. Optimisation of the design of these compounds from a structure-activity relationship point of view, as well as the elucidation of their mechanisms of action, could very well lead to the development of novel types of antineoplastic drug.

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Experimental

Chemistry

Melting points were determined with Mettler FP82 and FP80 apparatus (Mettler-Toledo, Greifense, Switzerland) and are not corrected. ^{1}H-NMR spectra were recorded on either a Bruker AC-200E or a Bruker 400 Ultrashield™ spectrometer (Bruker, Rheinstetten, Germany) using TMS as the internal standard. The IR spectra were obtained using a Thermo Nicolet FT-IR Nexus (Thermo Electron Corporation, Waltham, MA, USA) on KBr pellets. Elemental microanalyses were obtained using an Elemental Analyzer (LECO model CHN-900; LECO Corporation, St. Joseph, MI, USA,) on vacuum-dried samples and were an an accepteable range of ±0.4% for all compounds. Silica gel 60 (0.040 – 0.063 mm) 1.09385.2500 (Merck, Darmstadt, Germany) was used for Column Chromatography and Alugram® SIL G/UV$_{254}$ (Layer: 0.2 mm) (Macherey-Nagel, Düren, Germany) was used for Thin Layer Chromatography. Chemicals were purchased from E. Merck, Scharlau (F.E.R.O.S.A., Barcelona, Spain), Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain), Sigma-Aldrich Química, S.A., (Alcobendas, Madrid, Spain), Acros Organics (Janssen Pharmaceuticalaan 3a, Geel, Belgium), and Lancaster (Bischheim-

Table 5. IR and ^{1}H-NMR data for selected compounds.

Ref.	IR [cm^{-1}]	^{1}H-NMR [J in Hz]
1k	3305, 3072, 3000 – 2850, 1636	[a] 1.55 (brs, 4H, CH_2); 3.30 (brs, 4H, CH_2); 7.50 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 6, 2H, H_5, $H_{5'}$); 7.79 (d, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$); 8.34 (d, $J_{6\text{-}5}$ = 6, 2H, H_6, $H_{6'}$); 8.55 (s, 2H, H_5, $H_{5'}$); 8.74 (brs, 2H, NH, NH′).
1m	3308, 3074, 2928, 2883, 1642	[a] 1.79 (q, J = 7, 2H, CH_2); 3.32 (m, 4H, CH_2); 7.55 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 6, 2H, H_5, $H_{5'}$); 7.76 (d, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$); 8.34 (d, $J_{6\text{-}5}$ = 6, 2H, H_6, $H_{6'}$); 8.57 (s, 2H, H_2, $H_{2'}$); 8.75 (t, J = 5, 2H, NH, NH′).
2a	3295, 3068, 3000 – 2900, 1634	[a] 1.91 (q, J = 7, 2H, CH_2); 3.45 (m, 4H, CH_2); 7.68 (dd, $J_{6\text{-}5}$ = 8, $J_{6\text{-}7}$ = 8, 2H, H_6, $H_{6'}$); 7.86 (dd, $J_{7\text{-}6}$ = 8, $J_{7\text{-}8}$ = 8, 2H, H_7, $H_{7'}$); 8.07 (d, $J_{5\text{-}6}$ = 8, 2H, H_5, $H_{5'}$); 8.07 (d, $J_{8\text{-}7}$ = 8, 2H, H_8, $H_{8'}$); 8.81 (s, 2H, H_4, $H_{4'}$); 8.89 (t, J = 5, 2H, NH, NH′); 9.28 (s, 2H, H_2, $H_{2'}$).
2f	3396, 3068, 2940, 1665	[b] 3.84 (d, J = 6, 4H, CH_2); 7.58 (dd, $J_{6\text{-}5}$ = 8, $J_{6\text{-}7}$ = 8, 2H, H_6, $H_{6'}$); 7.72 (dd, $J_{7\text{-}6}$ = 8, $J_{7\text{-}8}$ = 8, 2H, H_7, $H_{7'}$); 7.84 (d, $J_{5\text{-}6}$ = 8, 2H, H_5, $H_{5'}$); 8.07 (d, $J_{8\text{-}7}$ = 8, 2H, H_8, $H_{8'}$); 8.28 (d, 2H, H_4, $H_{4'}$); 8.28 (d, 2H, H_3, $H_{3'}$); 8.65 (brs, 2H, NH, NH′).
3a	3212, 3064, 1660	[a] 1.64 (brs, 4H, CH_2); 3.38 (brs, 4H, CH_2); 4.41 (s, 6H, CH_3); 8.25 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 5, 2H, H_5, $H_{5'}$); 8.89 (d, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$); 9.09 (t, J = 5, 2H, NH, NH′); 9.09 (d, $J_{6\text{-}5}$ = 5, 2H, H_6, $H_{6'}$); 9.39 (s, 2H, H_2, $H_{2'}$).
3c	3262, 3040, 2952, 1660	[a] 1.89 (q, J = 7, 2H, CH_2); 3.43 (m, 4H, CH_2); 4.42 (s, 6H, CH_3); 8.26 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 5, 2H, H_5, $H_{5'}$); 8.89 (d, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$); 9.12 (t, J = 8, 2H, NH, NH′); 9.12 (d, $J_{6\text{-}5}$ = 5, 2H, H_6, $H_{6'}$); 9.40 (s, 2H, H_2, $H_{2'}$).
4a	3258, 3062, 1656	[a] 1.61 (brs, 4H, CH_2); 3.35 (brs, 4H, CH_2); 8.12 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 6, 2H, H_5, $H_{5'}$); 8.61 (d, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$); 8.61 (s, 4H, NH_2, $NH_{2'}$); 8.86 (d, $J_{6\text{-}5}$ = 6, 2H, H_6, $H_{6'}$); 9.14 (s, 2H, H_2, $H_{2'}$); 9.14 (brs, 2H, NH, NH′).
4c	3214, 3072, 1655	[a] 1.94 (q, J = 6, 2H, CH_2); 3.48 (t, J = 6, 4H, CH_2); 8.02 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 6, 2H, H_5, $H_{5'}$); 8.58 (d, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$); 8.80 (d, $J_{6\text{-}5}$ = 6, 2H, H_6, $H_{6'}$); 9.06 (s, 2H, H_2, $H_{2'}$).
5c	3414, 3053, 3000 – 2900, 1656	[b] 2.19 (q, J = 6, 2H, CH_2); 3.82 (m, 4H, CH_2); 7.77 – 7.99 (dd, 2H, H_6, $H_{6'}$); 7.77 – 7.99 (dd, 2H, H_7, $H_{7'}$); 7.77 – 7.99 (d, 2H, H_5, $H_{5'}$); 7.77 – 7.99 (d, 2H, H_8, $H_{8'}$); 8.53 (d, $J_{4\text{-}3}$ = 8, 2H, H_4, $H_{4'}$); 8.75 (d, $J_{3\text{-}4}$ = 8, 2H, H_3, $H_{3'}$); 11.83 (brs, 2H, NH, NH′).
6a	3400, 3290, 3100 – 3000, 3000 – 2900, 1623	[c] 1.62 (brs, 4H, CH_2); 3.14 (brs, 2H, CH_2); 7.02 (dd, $J_{6\text{-}5}$ = 8, $J_{6\text{-}7}$ = 8, 2H, H_6, $H_{6'}$); 7.10 (s, 2H, H_3, $H_{3'}$); 7.16 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 8, 2H, H_5, $H_{5'}$); 7.41 (d, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$); 7.59 (d, $J_{7\text{-}6}$ = 8, 2H, H_7, $H_{7'}$); 8.50 (t, J = 5, 2H, NH, NH′ amide); 11.53 (s, 2H, NH, NH′ indole).
7	3150 – 3000, 3000 – 2750	[d] 2.57 (brs, 8H, CH_2); 3.68 (s, 4H, CH_2); 7.12 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 5, 2H, H_5, $H_{5'}$); 7.39 (d, $J_{3\text{-}4}$ = 8 2H, H_3, $H_{3'}$); 7.62 (dd, $J_{4\text{-}3}$ = 8, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$); 8.54 (d, $J_{6\text{-}5}$ = 5, 2H, H_6, $H_{6'}$).
8a	3254, 3100 – 3000, 3000 – 2900	[b] 3.15 (brs, 4H, CH_2-pyridine); 3.96 (brs, 4H, CH_2-N); 7.11 (brs, 1H, H_6 pyridopyrimidine); 7.19 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 5, 2H, H_5, $H_{5'}$ pyridine); 7.23 (d, $J_{3\text{-}4}$ = 8, 2H, H_3, $H_{3'}$ pyridine); 7.56 (brs, 1H, H_5 pyridopyrimidine); 7.64 (dd, $J_{4\text{-}3}$ = 8, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$ pyridine); 8.54 (brs, 1H, H_7 pyridopyrimidine); 8.57 (d, $J_{6\text{-}5}$ = 5, 2H, H_6, $H_{6'}$ pyridine); 7.00 – 7.92-8.29 – 8.71 (dd- brs- brs- brs, 2H, NH, NH′)
8b	3415, 3300, 3100 – 3000, 3000 – 2900	[b] 3.01 (t, 2H, CH_2-indole); 3.10 (t, 2H, CH_2-indole); 3.74 (m, 2H, CH_2-N); 3.85 (m, 2H, CH_2-N); 6.86 (dd, $J_{5\text{-}4}$ = 8, $J_{5\text{-}6}$ = 8, 1H, H_5 indole); 6.97 (dd, $J_{5'\text{-}4'}$ = 8, $J_{5'\text{-}6'}$ = 8, 1H, $H_{5'}$ indole); 7.03 (brs, 1H, H_6 pyridopyrimidine); 7.03 (brs, 2H, H_6, $H_{6'}$ indole); 7.14 (brs, 1H, $H_{2'}$ indole); 7.19 (brs, 1H, H_2 indole); 7.33 (d, $J_{5\text{-}6}$ = 8, 1H, H_5 pyridopyrimidine); 7.35 (d, $J_{7\text{-}6}$ = 8, 2H, H_7, $H_{7'}$ indole); 7.57 (d, $J_{4\text{-}5}$ = 8, 2H, H_4, $H_{4'}$ indole); 8.63 (d, $J_{7\text{-}6}$ = 5, 1H, H_7 pyridopyrimidine); 10.80 (brs, 2H, NH, NH′ indole); 7.96 – 8.40 – 8.52 – 9.55 (brs, 3H, NH, NH‘,HCl).
8d	3270, 3124, 3100 – 3000, 2986, 2950, 2838	[b] 1.59 (brs, 4H, CH_2); 1.97 (m, 4H, CH_2); 2.94 (brs, 4H, CH_2); 3.00 (brs, 4H, CH_2-NH-CH_2); 3.63 (brs, 4H, CH_2-NH-pyridopyrimidine); 7.60 (dd, $J_{6\text{-}5}$ = 8, $J_{6\text{-}7}$ = 4, 2H, H_6, $H_{6'}$); 8.35 (brs, 4H, N*H*-*H*I); 8.65 (s, 2H, H_2, $H_{2'}$); 8.68 (d, $J_{5\text{-}6}$ = 8, 2H, H_5, $H_{5'}$); 8.79 (brs, 2H, NH-pyridopyrimidine, NH′-pyridopyrimidine); 9.01 (d, $J_{7\text{-}6}$ = 4, 2H, H_7, $H_{7'}$).

[a] 200 MHz, DMSO-d_6.
[b] 400 MHz, DMSO-d_6.
[c] 200 MHz, $CDCl_3$.
[d] 400 MHz, $CDCl_3$.

Strasbourg, France). The spectroscopic properties of the most active compounds are summarised in Table 5.

General procedures for N,N′-alkyl-diyldinicotinamides and related compounds

*Method A, for compounds **1a–g***

A solution of nicotinic acid (**1a**–**f**) or isonicotinic acid (**1g**) (4.00 g, 32.5 mmol) in thionyl chloride (15-20 mL) was stirred and heated under reflux for 2 h. The solvent was removed and the residue was suspended in dry chloroform (30 mL). Triethylamine was then added (4.52 mL, 32.5 mmol). The resulting solution was added dropwise at room temperature over 1 h to a mixture of the appropriate diamine (16.2 mmol) and triethylamine (4.52 mL, 32.5 mmol) in dry chloroform. After completion of the addition, the mixture was heated under reflux for 5 h. The solvent was removed and the resulting residue was subjected to the purification method described below for each compound.

*Method B, for compounds **1h–j***

A solution of 6-chloronicotinic (**1h**) and (**1i**) or 2-chloronicotinic acid (**1j**) (4.00 g, 25.2 mmol) in thionyl chloride (15 – 20 mL) was stirred and heated under reflux for 2 h. The solvent was removed and the residue was suspended in dry chloroform (30 mL). Triethylamine was then added (3.50 mL, 25.2 mmol). This solution was added dropwise to a mixture of the appropriate diamine (12.6 mmol) and triethylamine (3.50 mL, 25.2 mmol) in dry chloroform and the mixture was stirred at room temperature for 1 h. The mixture was then heated under reflux for 5 h. The solvent was removed and the residue was suspended in water (100 mL). The resulting solid was filtered off, washed with water and recrystallized.

*Method C, for compounds **1k–n***

A solution of nicotinic acid *N*-oxide (4.00 g, 28.7 mmol) in thionyl chloride (15 – 20 mL) was stirred and heated under reflux for 3 h. The solvent was removed under reduced pressure. The residue was suspended in dry chloroform (30 mL) and triethylamine was added (4.00 mL, 28.7 mmol). The resulting solution was added dropwise to a mixture of the appropriate diamine (14.4 mmol) and triethylamine (4.00 mL, 28.7 mmol) in dry chloroform and the mixture was stirred at room temperature for 1 h. The mixture was then heated under reflux for 5 h. The solvent was removed and the resulting residue was subjected to the purification method described for each compound.

*General procedure for N,N′-alkyl-diyldiquinoline-carboxamide and related **2a–f***

A solution of 3-quinolinic acid (**2a**–**c**) or 2-quinolinic acid (**2d**–**f**) (2.00 g, 11.5 mmol) and thionyl chloride (15 – 20 mL) in dry chloroform (50 mL) was stirred and heated under reflux for 2 h.

The solvent was removed and the residue was suspended in dry chloroform (30 mL). Triethylamine (1.61 mL, 11.6 mmol) was added to the residue. This solution was added dropwise to a mixture of the appropriate diamine (5.8 mmol) and triethylamine (1.61 mL, 11.6 mmol) in dry chloroform and the mixture stirred at room temperature for 1 h. The mixture was then heated under reflux for 4 h and the solvent was removed. For compounds **2a – d** and **2f**, the residue was suspended in water (100 mL) and the solid filtered off. The resulting solid was washed with boiling water (4 × 25 mL) and recrystallized from the solvent described for each compound.

*General procedure for bis(1-methylpyridinium)diiodide derivatives **3a – e***

A solution of **1a**, **1b**, **1c**, **1d** or **1e** (1.7 mmol) and iodomethane (0.60 mL, 9.0 mmol) in ethanol (40 mL) was stirred and heated under reflux for 96 h. The mixture was cooled to 0°C and the solid filtered off. The solid was washed with ethanol (2 × 10 mL) and recrystallized.

*General procedure for bis(1-aminopyridinium) derivatives **4a – c***

A solution of hydroxylamine-*O*-sulfonic acid (0.26 g, 2.2 mmol) and 85% KOH (0.15 g, 2.2 mmol) in water (10 mL) was added to a solution of the appropriate compound (**1a**, **1b** or **1c**; 1.0 mmol) in water (7 mL) and methanol (3 mL). The mixture was stirred and heated to 60°C. The mixture was then heated under reflux for 18 h, cooled and washed with dichloromethane (2 × 15 mL). Concentrated HI was added until pH 1 – 2 was attained. The solvent was removed and the residue was dissolved in boiling 2-propanol and then filtered. The resultant solid was suspended in boiling isopropanol, filtered off, dissolved in boiling methanol, cooled and then refiltered. The solvent was removed and the resulting solid was isolated and recrystallized.

*General procedure for diquinoline-dioxide derivatives **5a – c***

A solution of 3-chloroperoxybenzoic acid (77%, 0.18 g, 0.81 mmol) in dichloromethane (15 mL) was added dropwise to a stirred solution of the appropriate compound (**2d**, **2e** or **2f**; 0.40 mmol) in dry chloroform (15 mL). The mixture was stirred for 10 h at room temperature. A second quantity of 3-chloroperoxybenzoic acid (0.09 g, 0.4 mmol) was then added and the solution was stirred for 8 h. The mixture was washed with a solution of K_2CO_3 (10%; 2 × 20 mL) and water (2 × 20 mL). The organic layer was dried over anhydrous sodium sulfate, the solvent was removed and the residue was recrystallized.

*General procedure for indole derivatives **6a** and **6b***

A stirred mixture of ethyl 1*H*-indole-2-carboxylate (1.00 g, 5.28 mmol), the appropriate diamine (2.6 mmol) and sodium cyanide (25 mg, 0.53 mmol) in methanol (10 mL) was heated to 60°C for 96 h. The solution was filtered and the solid was washed with boiling methanol (6 × 20 mL). The solvent was removed and the resulting residue was suspended in methanol (30 mL) and the solid filtered off.

*1,4-Bis(pyridin-2-ylmethyl)piperazine **7***

A solution of 2-chloromethylpyridine hydrochloride (1.25 g, 7.62 mmol), K_2CO_3 (1.57 g, 11.4 mmol) and piperazine (0.33 g, 3.8 mmol) in ethanol (50 mL) was stirred and heated under reflux for 72 h. The solvent was removed and the resulting residue was dissolved in water (40 mL). 5N KOH (10 mL) was added until a basic pH was achieved. The solution was extracted with dichloromethane (3 × 30 mL). The combined extracts were washed with water (2 × 25 mL) and dried over anhydrous sodium sulfate. The solvent was removed and the residue was recrystallized.

Preparation of pyrido[2,3-d]pyrimidin-4-ol

A mixture of 2-aminonicotinic acid (8.00 g, 57.9 mmol) and formamide (16.0 g, 355 mmol) was pulverized and heated at 170°C (2 h at this temperature). The mixture was allowed to cool to room temperature and water (50 mL) was added. The solid was filtered off and washed with water (2 × 10 mL). The solid was recrystallized from water to give pyrido[2,3-*d*]pyrimidin-4-ol. Yield 55%. IR: 3422, 3100 – 3000, 3000 – 2900. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 7.56 (dd, $J_{6\text{-}5}$ = 8 Hz, $J_{6\text{-}7}$ = 5 Hz, 1H, H_6); 8.32 (s, 1H, H_2); 8.51 (d, $J_{5\text{-}6}$ = 8 Hz, 1H, H_5); 8.95 (d, $J_{7\text{-}6}$ = 5 Hz, 2H, H_7); 12.55 (brs, 1H, OH). Anal. Calcd. for $C_7H_5N_3O$ (%): C, 57.16; H, 3.40; N, 28.56; found (%): C, 56.88; H, 3.42; N, 28.89.

Preparation of 4-chloropyrido[2,3-d]pyrimidine

A solution of pyrido[2,3-*d*]pyrimidin-4-ol (0.75 g, 5.1 mmol) in phosphorus oxychloride (40 mL) was stirred and heated under reflux for 1 h. The solvent was removed under reduced pressure and ice (50 g) was added to the resulting residue. The solution was extracted with chloroform (10 × 40 mL). The combined extracts were washed with water (2 × 80 mL) and dried over anhydrous sodium sulfate. The solvent was removed and 4-chloropyrido[2,3-*d*]pyrimidine (0.84 g) was obtained and used immediately without further purification.

*N,N′-[Piperazine-1,4-diylbis(propane-3,1-diyl)]dipyrido-[2,3-d]pyrimidin-4-aminedihydro-chloride **8c***

A solution of 4-chloropyrido[2,3-*d*]pyrimidine (0.84 g, 5.1 mmol), triethylamine (0.70 mL, 5.1 mmol) and 1,4-bis(3-aminopropyl)piperazine (0.51 g, 2.5 mmol) in chloroform (50 mL) was stirred and heated under reflux for 48 h [38]. The solvent was removed. The resulting residue was suspended in ethanol (50 mL), the solid filtered off, washed with ethanol (3 × 20 mL) and recrystallized.

*N,N′-Bis[3-(pyrido[2,3-d]pyrimidin-4-ylamino)propyl]butane-1,4-diamine **8d***

A solution of 4-chloropyrido[2,3-*d*]pyrimidine (0.84 g, 5.1 mmol), triethylamine (0.70 mL, 5.1 mmol), KI (0.86 g, 5.1 mmol) and spermine (0.51 g, 2.5 mmol) in chloroform (50 mL) was stirred and heated under reflux for 48 h [38]. The mixture was filtered. The solid was washed with water (2 × 10 mL), suspended in boiling methanol and filtered off. The solid was suspended in boiling acetic acid, filtered and washed with boiling acetic acid (10 × 20 mL).

Biological evaluation

Evaluation of cytotoxic potential against human cancer cell lines

An assay utilizing neutral red [33] staining was employed. Cells were cultured in McCoy medium for HT-29 and T-24 cell lines

and Leibovitz medium for MD-MBA-231 cell line supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. For this assay, cells were obtained from 80 – 90% confluent T-75 flasks by detaching the cells with PBS/EDTA. 100 μM of cells were seeded at a density of 20×10^3/well in 96-well plates (Microtest™ 96 FALCON®; Becton Dickinson S.A., Madrid, Spain), but only the 60 inner wells were used in order to avoid any border effects. Cells were allowed to attach to the bottom of the wells for 12 h prior to the addition of the compounds. Compounds were diluted in complete medium. The plating density permitted several rounds of cell proliferation before confluent monolayers were formed. After 3 days of incubation, 0.05 mL of neutral red solution (0.05 mg /mL diluted in saline) was added to the cells in the existing growth medium (0.2 mL) for 1 h 30 min at 37°C. The plates were flicked and 0.1 mL of 0.05 M sodium phosphate (monobasic) in 50% ethanol was added. The plates were vortexed in a plate shaker, incubated for 10 minutes at room temperature and read using a plate reader at 540 nm absorbance. Data were calculated as a percentage of total absorbance found for cells in non-drug-treated wells.

With regard to selectivity, and as an orientative measure, the cytotoxicity was determined in cell cultures of two nontumoural lines, one of breast (CRL-8799, cultured in MEGM, Mammary Epithelial Grow Medium, Clonetics Corporation, San Diego, CA, USA) and another of liver (CRL-11233, cultured in BEGM medium, Bullet kit, Clonetics Corporation). The same experimental procedure was used and the highest IC_{50} calculated in the three tumoural lines was selected as the test concentration. Once it had been determined which compounds were active in the cytotoxicity assay, they were subjected to a test to determine whether or not they also acted as inducers of apoptosis and/or were caspase-3 activators.

Evaluation of apoptosis induction in human cancer cell lines

Apoptosis was quantified using a detection kit called Cell Death Detection ELISA^{Plus} (Roche Biochemicals). This cellular test detects nucleosomes in cytoplasm prior to disintegration of the plasma membrane, a well-known hallmark of apoptosis [39]. The assay is based on a quantitative sandwich-enzyme-immunoassay principle: monoclonal mouse antibodies directed against DNA and histones (H1, H2A, H2B, H3 and H4) specifically detect mono- and oligonucleosomes. Apoptosis was measured with the aid of this kit, following the instructions provided by the manufacturer and using as the test concentrations the IC_{50} values determined in the previous cytotoxicity assay for each of the cell lines. The apoptosis measurements were taken after 48 h of incubation. As previously indicated, a relative value of 1 was attributed to the apoptosis detected in the control cultures in which the test compound was not present.

Evaluation of caspase-3 activation

Detection was carried out by flow cytometry (FACScan, Becton Dickinson), using the Active-Caspase-3 FITC Mab apoptosis kit from Pharmingen. This test evaluates the number of cells that are contained in the dimerized and caspase-3-activated form. It has been determined that the range of measurements considered to be effective for this enzyme is between 14 and 48 h. Therefore, measurements were taken at 14, 24 and 48 h, and the values obtained were compared with the control cells that express this enzyme when they are incubated without the test compound. The test concentrations correspond to the IC_{50} values determined in the cytotoxicity assay.

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