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Synthesis and biological evaluation of new symmetrical derivatives as cytotoxic agents and apoptosis inducers

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Abstract—Based on the research of less toxic anticancer therapies, we have looked for novel compounds with anticancer activity based on a proapoptotic mechanism. The described compounds are derivatives of ether, carbamate, urea, amide, or amine. Some of the prepared compounds decreased cell viability of various tumor cell lines in a time- and dose-dependent manner, and also induced DNA fragmentation, which indicated cell apoptosis. The potential antitumoral activity of the compounds was evaluated in vitro by examining their cytotoxic effects against human mama, colon, and bladder cancer cell lines (MD-MBA-231, HT-29, and T-24). Compounds showing cytotoxic activity were subjected to an apoptosis assay. In addition, some of the synthesized compounds provoked a rapid and dose-dependent increase in the level of caspase-3, an enzyme, which is considered to be one of the principal executing caspases in which all of the biochemical routes involved in the apoptosis response converge. The most promising compounds, with respect to cytotoxicity and apoptosis induction capability, were the 4-nitrophenylcarbamate derivative of 2,2′-methylenebis(4-chlorophenyl) **3c**, the naphthylurea derivative **4d**, and the *n*-propylurea derivative **4c**, from 4,4′-methylenebisphenyl, all of which displayed cytotoxic activity and showed very interesting levels of apoptosis. Furthermore, good levels of apoptosis induction were achieved for **3a** and **4b** in the T-24 cell line. Therefore, compounds such as **7b**, a pyrido[2,3-*d*]pyrimidine derivative, show a significant in vitro cytotoxicity, with IC_{50} values between 3 and 8 μm in the three cell lines tested. This compound also produced a rapid and dose-dependent increase of the caspase-3 level and induced apoptosis in HT-29 cells. Other profiles have been found, such as those presented by **5c** and **7c**, which are cytotoxic and apoptotic but do not provoke an increase in the level of caspase-3, or those presented by **1c**, **1d**, and **2a**, which are cytotoxic, without showing any other activity. The different types of behavior of each compound are not necessarily parallel in the three cell lines tested. A great number of these compounds of interest show no cytotoxicity in nontumoral human cells such as CRL-8799, a nontumoral line of mama. Subsequent modulation of these lead structures permits advances in the design of potent cytotoxic and proapoptotic anticancer drugs.

1. Introduction and design considerations

Progressive elucidation of the molecular mechanisms involved in cancer has opened up a new horizon for the development of new antitumoral compounds. While the traditional cancer therapies were based on the theory that rapidly dividing cancer cells are more sensitive to cytotoxic agents, which has led to the use of a group of compounds whose mechanism is related to the alteration, at different levels, of the correct functioning of DNA, there has been a recent shift of emphasis toward novel mechanistic targets that have arisen directly from the in-depth study of the underlying genetic changes related to the cancerous state. Thus, for instance, it is now accepted that tumor growth may principally be due to reduced rates of cell death rather than to enhanced proliferation.[1–3] The process of programmed cell death is now recognized as an important component of multistep carcinogenesis.[4–10] Apoptotic pathways might be significantly altered in cancer cells with respect to

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untransformed cells, and these differences might present a therapeutic window that can be exploited for the development of cancer drugs.[11–13]

It is a well-known fact that the regulating mechanisms of apoptosis are extremely complex, but it is also known that the way in which the apoptotic processes function differs, depending on whether the cells involved are tumoral or healthy. These differences are due to the fact that the tumoral lines are more sensitive when these mechanisms are activated, while the healthy cells possess the ability to make repairs, which often counteracts this apoptotic process, maintaining survival of the cell.[14]

Among the mechanisms involved, the activation of a group of enzymes pertaining to the cistein-protease family, known as caspases, stands out. They are probably the most important effect-provoking molecules related to the induction of apoptosis, and all of them are synthesized in the form of inactive precursors (proenzymes), which are subsequently activated by self-proteolysis (autocatalytic cleavage) or other proteases.[15] It is assumed that a series of adaptive molecules exist, which interact with a specific point of the amino-terminal prodomains of the procaspases. If two proenzymes, which carry these adaptive structures, meet each other within an adequate distance, they are able to aggregate to each other. This fact is sufficient enough so that, by means of a self-activating internal self-proteolysis process, the two large units and the two small ones are released. These four units associate with each other, forming a heterotetramer, the active form of the caspase. At the same time, this active form of caspase can act on other procaspases, thereby initiating what is known as a cascade of the caspases, an amplifying system of the apoptotic signals, fruit of the original signal.

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. These molecules have a central nucleus made up of an aromatic system or a cyclic or linear aliphatic chain of variable length and flexibility, connected by a variable functional group, upon which two identical lateral arms, consisting of heterocycles (pyridine, quinoline, indole, pyrido[2,3-*d*]pyrimidine) with different substituents, are introduced. These arms can bond to the central element by means of a variable functional group (ether, amine, amide, urea, and carbamate) (Figure 1).

The general scheme of distribution used for these structural elements initially results in the formation a triangle, with one of its vertices being the central nucleus and the other two vertices being made up of the ends of the arms.

Selection of the chemical groups of the new molecules is based on the diverse reference literature that was consulted. Structural analysis is then carried out on compounds with recognized efficacy in cancer treatment via induction of apoptosis. Among the many structural groups identified, the following are included: (1) carboaromatics and π-deficient monocyclic aromatic systems, such as pyridine[16,17] or bicyclic aromatic systems, such as quinoline,[18,19] indole,[20,21] and pyrido[2,3-*d*]pyrimidine,[22] as well as variable aliphatic chains at the ends (R) of the molecule. (2) Functional groups, Y, have been selected for the ends, maintaining a gradient in their ability to form hydrogen bonds and establish electrostatic interactions. These groups include: ether group,[23–25] a low polarity structure of great flexibility, with the ability to establish hydrogen bonds; amide groups directly linked to the heterocycle or near it;[26,27] carbamate group,[28–30] a structure of medium polarity, capable of forming hydrogen bonds as donor and receptor; a urea group,[31,32] in which the substitution of oxygen for nitrogen increases the ability of interaction and the formation of hydrogen bonds and finally, an amine

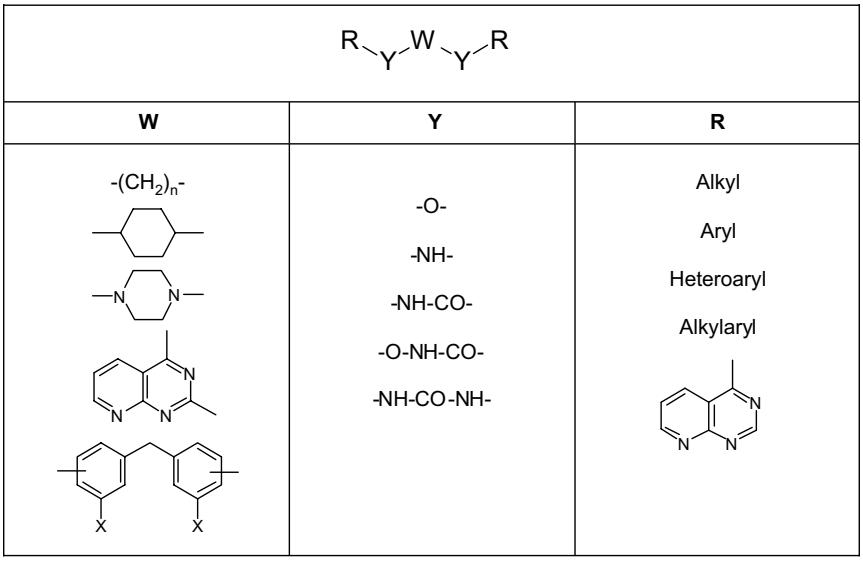
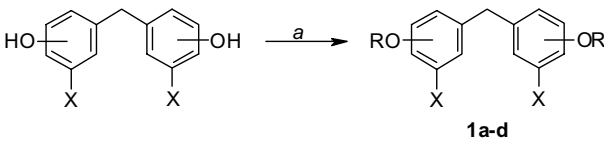


Figure 1.

group,[33,34] which greatly increases polarity and the possibility of the formation of hydrogen bonds. (3) Diverse antitumoral compounds described in the reference literature,[35,36] which present two central aromatic systems separated by aliphatic chains or other structural groups. Variable, linear, and cyclic aliphatic chains as well as the heterocycle, pyrido[2,3-*d*]pyrimidine in position W of the general formula, have also been assayed. Finally, it has been observed that many of the molecules studied possess high symmetry.[32,37–42]



Ref	Chain position	X	R
1a	4,4′	H	-CH_2-(4-C_6H_4Cl)
1b	4,4′	H	-$(CH_2)_3$-CH_3
1c	4,4′	H	2-CH_2-C_5H_4N
1d	2,2′	Cl	-CH_2-C_6H_5

Scheme 1. Reagents and conditions: (a) R–Cl, Br/KOH/dimethylsulfoxide/rt.

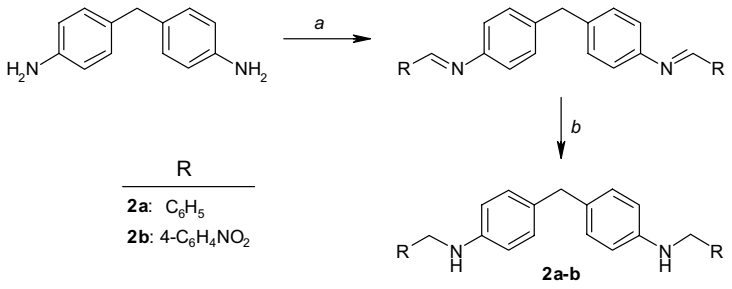
2. Chemistry

Compounds **1a–d** were prepared from 4,4′-methylenebisphenol or 2,2′-methylenebis(4-chlorophenol) and the corresponding alkyl halide by Williamson synthesis, as shown in Scheme 1. The use of dimethylsulfoxide and potassium hydroxide enhances the yield and allows mild reaction conditions for the ether synthesis.[43]

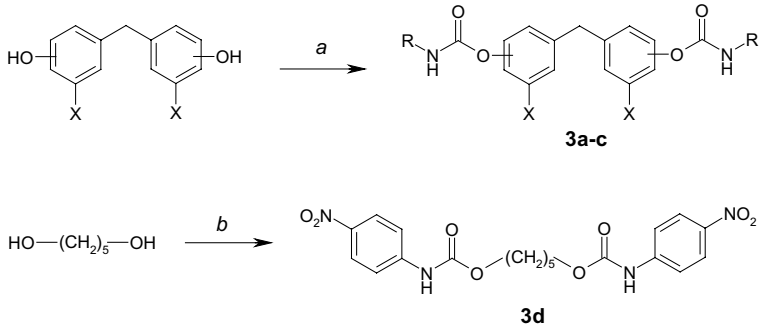
For the synthesis of secondary amines **2a–b**, 4,4′-methylenebisaniline was first condensed with the aldehyde in the presence of molecular sieves (4 Å) to give an imine, which was isolated and then reduced by treatment with sodium cyanoborohydride,[44] as shown in Scheme 2. Reduction requires acidic conditions, which were achieved by using a mixture of methanol and concentrated hydrochloric acid (8:2, v:v). Pure compounds were afforded after purification by flash chromatography, as reported in the Experimental section.

Carbamates **3a–d** were afforded by reaction of the corresponding diphenol or dialcohol, with an isocyanate and catalytic copper(I) chloride in dry dioxane at room temperature[45] (Scheme 3).

Compounds **4a–d** were prepared by nucleophilic substitution from a primary amine[46] and the corresponding isocyanate. Reaction was carried out at room temperature in diethyl ether, and the derivatives were obtained in good yields. The starting reagent for compounds



Scheme 2. Reagents and conditions: (a) R–CHO/diethyl ether/molecular sieves (4 Å)/rt; (b) methanol:HCl(c) (8:2, v:v)/$NaCBH_3$/rt.



Ref	Chain position	X	R
3a	4,4′	H	4-$C_6H_4NO_2$
3b	4,4′	H	-CH_2-CH_2-Cl
3c	2,2′	Cl	4-$C_6H_4NO_2$

Scheme 3. Reagents and conditions: (a) R–NCO/dioxane/CuCl/rt; (b) (4-$NO_2C_6H_4$)–NCO/dioxane/CuCl/rt.

4a–**c** was 4,4′-methylenebisaniline, whereas compound **4d** was synthesized from 4,4′-methylenebis-(phenylisocyanate), as reported in Scheme 4.

Synthesis of the diamides, **5a**–**b**, was carried out by reaction of acyl chlorides, obtained from the corresponding carboxylic acid by means of reflux with thionyl chloride, with the appropriate amines and in the presence of equimolecular amounts of triethylamine. In the case of the acyl chloride prepared for compound **5b**, chloroform was added as the solvent in order to provide milder conditions and avoid halogenation of the quinoline ring (Scheme 5). Compound **5c**, 4-methoxy-*N*-(3-{[(4-methoxy-7-nitroquinolin-2-yl)carbonyl]amine}propyl)-5-nitroquinoline-2-carboxamide, was obtained by reacting 1,3-propanediamine with the reaction mixture obtained by means of the following steps: (1) nitration of 4-methoxy-2-quinolinic acid with nitric acid, and later, (2) reaction with thionyl chloride for acyl chloride formation from carboxylic acid. Compound **5c** and *N*,*N*′-propane-1,3-diylbis(4-methoxy-5-nitroquinoline-2-carboxamide) were principally formed, and the latter, a symmetrical isomer, which did not present any cytotoxic activity, was then separated.

Compound **6a** was obtained by direct reaction of piperazine with 1-bromo-3-phenylpropane, in refluxing chloroform and in the presence of triethylamine.

The synthesis of compounds **7a**–**b** and **7c** was carried out in three steps (Scheme 6). 2-aminonicotinic acid was condensed with an excess of urea or formamide, affording pyrido[2,3-*d*]pyrimidine-2,4-diol and pyrido[2,3-*d*]pyrimidine-4-ol, respectively.[47] The hydroxyl groups were replaced by chlorine after treatment with refluxing phosphorus oxychloride and *N*,*N*-dimethylformamide was added as the catalyst.[47] In the third step, the chlorines were substituted with the corresponding amines in the presence of equimolecular amounts of triethylamine.

3. Biological evaluation

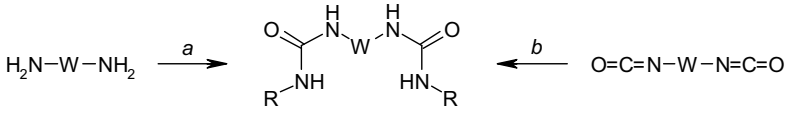
3.1. Cytotoxicity

The cytotoxic activity of the synthesized compounds is determined on three human cancer cell lines: mama (MD-MBA-231), bladder (T-24), and colon (HT-29), using the neutral red assay. Survival percentage is determined after a 72-h period, at screening concentrations of 100 and 20 μm and using the survival percentage obtained with the cell treated only with the solvent (DMSO at 0.5%) as reference. The results are expressed as the average of triplicate assays. IC_{50} values were calculated for those compounds showing survival <45% (100 μm). Table 1 shows the biological results obtained for the compounds.

With regard to selectivity and as an orientative measure, the cytotoxicity has been determined in cell cultures of two nontumoral lines, one of mama (CRL-8799) and another of liver (CRL-11233). The same experimental procedure has been used and the highest IC_{50} calculated in the three tumoral lines was selected as the test concentration. The data obtained using these healthy cell lines can be found in the section titled 'Results and discussion'.

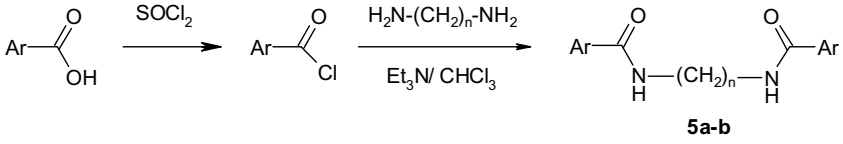
3.2. Apoptosis

Once it has been determined, which compounds are active in the cytotoxicity assay, they are subjected to a test, which determines whether or not they also act as inducers of apoptosis. Their ability to induce DNA fragmentation in cell cultures of the lines MD-MBA-231, T-24

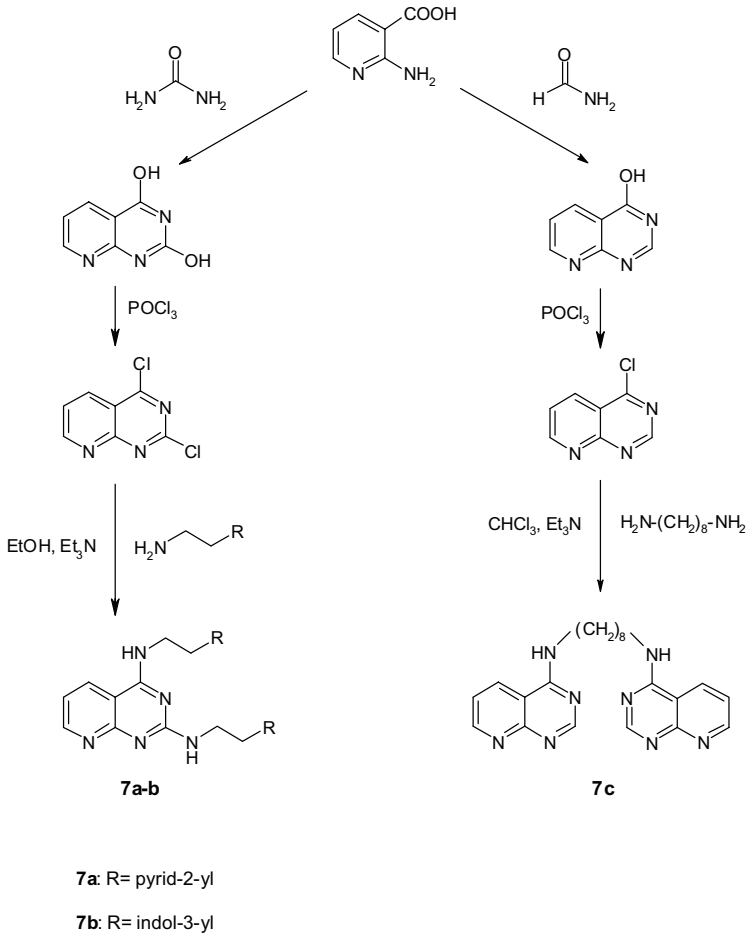


Ref	W	R	Method
4a	4,4'-methylenebisphenyle	-CH_2-CH_2Cl	a
4b	4,4'-methylenebisphenyle	4-$C_6H_4NO_2$	a
4c	4,4'-methylenebisphenyle	-$(CH_2)_2$-CH_3	a
4d	4,4'-methylenebisphenyle	Naphth-1-yl	b

Scheme 4. Reagents and conditions: (a) R–NCO/diethyl ether/rt; (b) Ar–NH_2/diethyl ether/rt.



Scheme 5. **5a**: Ar: pyrid-3-yl; $n = 12$; **5b**: Ar: quinol-3-yl; $n = 3$.



Scheme 6. **7a**: R = pyrid-2-yl; **7b**: R = indol-3-yl.

and HT-29, which were incubated with the compound under study for 24 h, is also tested, using the *Cell Death Detection ELISA Plus* Kit (Roche). With regard to apoptosis induction, a result is considered positive when the obtained level of DNA fragmentation at least doubles the values obtained for the control cultures that were treated only with the solvent. The results express the number of times in which the culture containing the test product surpasses the control culture in the ability to induce DNA fragmentation, having assigned a relative value of 1 at the level of apoptosis detected in said control culture. Table 1 shows the results obtained for the selected compounds as well as for the reference substance, camptothecin.

3.3. Caspase-3

The compounds which show cytotoxicity and sufficient ability to induce apoptosis are 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 test used, *Active-Caspase-3 FITC Mab apoptosis kit* from Pharmingen, detects the quantity of caspase-3 dimerized in the apoptotic cells and permits confirmation of the involvement of this enzyme in the cell death process; this is considered to be preliminary data for the determination of the mechanism of action. The results of this semi-quantitative assay are expressed using the following symbols: (−) when no increase is detected in caspase-3 level with respect to the control, (+) when there is an increase of approximately 50%, and (++) when there is an increase of 100% or more (Table 1).

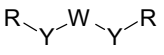
4. Results and discussion

4.1. Cytotoxicity

Of all of the compounds synthesized by our group for this research, we only make reference to those, which have presented an acceptable level of cytotoxicity in at least one of the tested cell lines. In general, the compounds which contain the ether group did not present notable cytotoxicity values.

Compound **1b** displayed selective cytotoxic activity against the MD-MBA-231 cell line, while compounds **1a** and **1d** exhibited cytotoxicity only against T-24 cells. Nevertheless, compound **1c** has demonstrated cytotoxicity against all cancer cell lines tested, showing more selectivity for MD-MBA-231 (12.3 μm) and HT-29 (15.4 μm). Good cytotoxicity levels were obtained for

Table 1. Biological profile of compounds



Ref.	W	Y	R	IC_{50} (μm)			Apoptosis in cell line			Caspase-3	(d) % Surviv	(e) % Surviv
				(a)	(b)	(c)	(a)	(b)	(c)			
1a	4,4′-Methylenebisphenyl	–O–	4-Chlorobenzyl	n.a. (f)	n.a.	29.9	—	—	2. 3	++ (T-24)	90	100
1b	4,4′-Methylenebisphenyl	–O–	Butyl	27.3	n.a.	n.a.	1.5	—	—	n.a.	79	82
1c	4,4′-Methylenebisphenyl	–O–	Pyrid-2-ylmethyl	12.3	15.4	72.5	n.a.	n.a.	n.a.	—	89	96
1d	2,2′-Methylenebis-4-chlorophenyl	–O–	Benzyl	n.a.	n.a.	50.5	—	—	n.a.	—	—	—
2a	4,4′-Methylenebisphenyl	–NH–	Benzyl	7.9	37.3	28.9	n.a.	n. a	n.a.	—	53	64
2b	4,4′-Methylenebisphenyl	–NH–	4-Nitrophenyl	0.63	n.a.	n.a.	1.3	—	—	n.a.	71	93
3a	4,4′-Methylenebisphenyl	–O–CO–NH–	4-Nitrophenyl	14.0	n.a.	44.1	n.a.	—	8.6	+ (T-24)	73	100
3b	4,4′-Methylenebisphenyl	–O–CO–NH–	2-Chloroethyl	n.a.	n.a.	81.5	—	—	2.5	++(T-24)	80	0
3c	2,2′-Methylenebis-4-chlorophenyl	–O–CO–NH–	4-Nitrophenyl	4.2	29.5	23.5	6.9	1.5	14.3	+ (T-24)	95	0
3d	$-(CH_2)_5-$	–O–CO–NH–	4-Nitrophenyl	n.a.	n.a.	n.a.	—	—	—	—	—	—
4a	4,4′-Methylenebisphenyl	–NH–CO–NH–	2-Chloroethyl	66.7	n.a.	34.0	1.3	—	1.2	++ (T-24)	92	93
4b	4,4′-Methylenebisphenyl	–NH–CO–NH–	4-Nitrophenyl	7.0	31.4	33.1	n.a.	2.0	3.3	++ (T-24); ++ (HT-29)	100	9
4c	4,4′-Methylenebisphenyl	–NH–CO–NH–	Propyl	68.2	n.a.	18. 6	5.8	—	2.7	++ (T-24)	86	0
4d	4,4′-Methylenebisphenyl	–NH–CO–NH–	Naphth-2-yl	3.1	42.9	7. 4	6.6	9.0	25.4	++ (HT-29)	90	0
5a	$-(CH_2)_{12}-$	–NH–CO–	Pyrid-3-yl	13.0	n.a.	n.a.	n.a.	—	—	+ (MD-MBA-321)	100	45
5b	$-(CH_2)_3-$	–NH–CO–	Quinol-3-yl	31.0	n.a.	n.a.	1.3	—	—	+ (MD-MBA-321)	100	48
5c	$-(CH_2)_3-$	–NH–CO–	(g)	n.a.	n.a.	39.0	—	—	2.8	n.a.	100	n.d. (h)
6a	$-[(CH_2)_2]_2-$	=N–	3-Phenylpropyl	n.a.	32.1	n.a.	—	13.2	—	++ (HT-29)	42	n.d.
7a	Pyrido[2,3-*d*]pyrimidine (2,4)	–NH–	Pyrid-2-ylethyl	n.a.	68.0	n.a.	—	1.5	—	+ (HT-29)	100	100
7b	Pyrido[2,3-*d*]pyrimidine (2,4)	–NH–	3-Indolyl	5.1	3.0	7.7	n.a.	3.0	n.a.	++ (HT-29)	56	n.d.
7c	$-(CH_2)_8-$	–NH–	Pyrido[2,3-*d*]-pyrimid-4-yl	7.7	n.a.	86.0	n.a.	—	3.8	n.a.	6	n.d.
Camptothecin				0.291	0.014	0.009	2.6	2.6	3.3	n.d.	n.d.	n.d.

(a) cell line MD-MBA-231; (b) cell line HT-29; (c) cell line T-24; (d) survival percentage in CRL-8799 cell line; (e) survival percentage in CRL-11233 cell line; (f) n.a. = no activity observed after 48 h incubation; (g) this compound is not symmetrical, R = 4-methoxy-5-nitroquinol-2-yl; R' = 4-methoxy-7-nitroquinol-2-yl; (h) n.d. = no data.

both carbamates and ureas possessing an aromatic central nucleus and a 4-nitrophenyl group at the terminal position (**3a**, **3c**, **4b**). The best IC_{50} were achieved for MD-MBA-231 cells in each case (14.0 μm for **3a**, 4.2 μm for **3c**, and 7.0 μm for **4b**). Cytotoxic activity decreased when the aromatic nucleus was substituted by an aliphatic chain, so, compound **3d** showed no cytotoxicity against any of the cell lines. The insertion of a naphthalene ring at the terminal position (**4d**) led to a compound showing good cytotoxic activity in all of the cell lines. However, substitution of the naphthalene ring for a heterocycle, such as quinoline or indole (not included in this publication), resulted in a complete loss of cytotoxicity. The insertion of the amine group resulted in an increase of the cytotoxicity values. The molecules that contain the amide group were not very active. Compounds **5a** and **5b** were cytotoxic against the MD-MBA-231 cell line (13.0 and 31.0 μm, respectively), while compound **5c** was active against T-24 (39.0 μm). The amines synthesized (**2a, 2b**, **6a**, **7a**, **7b**, and **7c**) displayed cytotoxic activity against at least one of the cancer cell lines. Compounds **2a** and **7b** showed good to moderate IC_{50} values in all of the cell lines. The best cytotoxicity level was achieved for compound **2b**, which exhibited an IC_{50} value of 0.63 μm in MD-MBA-231 cells.

In order to study the degree of selectivity in the cytotoxic activity of the compounds, assays using healthy cells have been carried out on some representative compounds those which showed the greatest activity in tumoral cells. The healthy cells corresponded to CLR-8799 and CLR-11233, and the survival values were between 90% and 100% for **1a**, **1c**, **2b**, **3a**, **3c**, **4a**, **4b**, **4d**, **5a**, **5b**, **5c**, and **7a**, in at least one of the tested lines. In other cases, intermediate survival values were: **1b** (79–82%), **2a** (53–64%), **3b** (80%), **4c** (86%), **6a** (42%), and **7b** (56%). Only a small fraction of the tested compounds were cytotoxic against healthy cell lines.

4.2. Apoptosis

Nine compounds showed notable activity (>1.50) in inducing apoptosis in T-24 cells, while four compounds induced significant apoptosis in MD-MBA-231 cells and six in HT-29 cells. Compounds **3c** and **4c** showed very interesting levels of apoptosis in both MD-MBA-231 and T-24 cells and compound **4d** showed this behavior in all of the cell lines. Compounds **4d** and **3c** exhibited exceptional ability in inducing apoptosis against bladder (T-24) cell line, showing apoptosis values of 25.4 and 14.3 with regard to the control cells. Furthermore, very good levels of apoptosis induction were achieved for **3a** (8.6), **4b** (3.3), and **7c** (3.8) in this cell line. Another interesting fact was that compounds **1a**, **3b**, and **5c** duplicated the apoptosis level. With regard to the HT-29 cell line, the compounds **6a**, **4d**, **7b**, **4b**, and **3c** have induced apoptosis, with the action of **6a** being very marked (13.2). These results are shown in Table 1 along with values obtained for camptothecin, used as the reference substance and known for its proapoptotic behavior.

Certain compounds, especially **1a**, **3b**, **4a**, **4b**, **4c**, and **4d** have notably provoked an increase in the caspase-3 levels in the T-24 cell line. Compounds **4b**, **6a**, and **7b** present the same effect on caspase-3 in the HT-29 cell line, and compounds **3a**, **3c**, **5a**, **5b**, and **7a** are also caspase-3 active, but to a more moderate degree. This fact is considered to be very interesting 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 converge.

5. Conclusion

Based on a general pattern inspired by the reference literature, we have completed the synthesis and biological evaluation of novel symmetrical compounds as potential cytotoxic and apoptosis inducers. These compounds were performed in a cytotoxicity in vitro assay against three cancer cell lines: mama (MD-MBA-231), colon (HT-29), and bladder (T-24). Some compounds showed good anticancer activity against all of the cell lines tested, with IC_{50} values in the low-medium micromolar range. The majority of these compounds of interest showed no significant cytotoxicity in human nontumoral cells such as CRL-8799 or CRL-11233. Compounds which showed cytotoxicity were evaluated in an apoptosis assay. Some compounds exhibited great ability in inducing apoptosis in all of the cell lines tested. The best apoptosis levels were achieved for compounds **4d** (25.4), **3c** (14.3), and **3a** (8.6) in T-24 cells; for compounds **6a** (13.2) and **4d** (9.0) in HT-29 cells; and for compounds **3c** (6.9), **4d** (6.6), and **4c** (5.8) in MD-MBA-231 cells, while for camptothecin, we obtained values between 2.6 and 3.3 for the different cell lines.

Although a precise structure–activity relationship can not be defined, it is possible to point out some general tendencies observed for the active compounds, both those presented in this work and others prepared by our research team: the greatest apoptotic activity is found in compounds with a central aromatic nucleus (W) of two rings. With regard to the functional group of connection Y, the best results were obtained for the urea, carbamate, and amine groups. The most frequent R endings in compounds with apoptotic activity are aromatic, fairly voluminous, either carbocyclic or heterocyclic, with the case of the 4-nitrophenyl group standing out. In general, no direct relationship has been found between the apoptotic activity and the activation of caspase-3, thereby suggesting the existence of different mechanisms of action. The precise mechanism of action of derivatives with greater apoptotic activity is currently under study in our laboratory using microarray techniques. The ability of 10 of these synthesized compounds to provoke a caspase-3 accumulation has been taken into account before choosing this technique. The optimization of design of these compounds from a structure–activity relationship point of view and the elucidation of their mechanisms of action could very well lead to the development of a novel type of antineoplastic drugs.

6. Experimental

6.1. Synthesis

Melting points were determined with a Mettler FP82+FP80 apparatus (Greifense, Switzerland) and have not been corrected. The ^{1}H NMR spectra were recorded on either a Bruker AC-200E (Rheinstetten, Germany) or a Bruker 400 Ultrashield™ (Rheinstetten, Germany), using TMS as the internal standard (Table 2). The IR spectra were performed on a Thermo Nicolet FT-IR Nexus in KBr pellets (Table 2). Elemental microanalyses were carried out on vacuum-dried samples (Table 2) using an Elemental Analyzer (LECO, CHN-900 Elemental Analyzer). Silica gel 60 (0.040–0.063 mm) 1.09385.2500 (Merck KGaA, 64271 Darmstadt, Germany) was used for Column Chromatography and Alugram® SIL G/UV$_{254}$ (Layer: 0.2 mm) (Macherey-Nagel GmbH & Co. KG. Postfach 101352. D-52313 Düren, Germany) was used for Thin layer chromatography. Chemicals were purchased from E. Merck (Darmstadt, Germany), 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, 2440 Geel, België) and Lancaster (Bischheim-Strasbourg, France).

6.2. General procedure for compounds 1a–d

Powdered KOH (20 mmol) was added to DMSO (15 mL). After stirring for 10 min, the appropriate diphenol, 4,4′-methylenebisphenol for compounds **1a**–**c**,

Table 2. Spectroscopic data (IR and ^{1}H NMR) and elemental analysis for synthesized compounds

Ref.	IR (cm^{-1})	^{1}H NMR (δ ppm, J in Hz)	Elemental analysis for (%) calcd/found
1a	3030, 1245, 1014, 810	[a]3.78 (s, 2H, C_6H_4–CH_2–C_6H_4); 5.05 (s, 4H, 2(CH_2–O)); 6.88–6.92 (d, J_{2-3}8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4); 7.08–7.12 (d, J_{2-3}8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4); 7.44 (br s, 8H, 2C_6H_4–Cl)	$C_{27}H_{22}Cl_2O_2$: C, 72.19/72.42; H, 4.94/4.85
1b	3030, 2934–2872, 1244, 812	[b]0.90–0.98 (t, 6H, 2CH_3); 1.43–1.50 (m, 4H, 2(CH_3–CH_2)); 1.69–1.76 (m, 4H, 2(CH_3–CH_2–CH_2)); 3.82 (s, 2H, C_6H_4–CH_2–C_6H_4); 3.87–3.93 (t, 4H, 2(O–CH_2)); 6.76–6.81 (d, J_{2-3} 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4); 7.03–7.07 (d, J_{2-3} 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4)	$C_{21}H_{28}O_2$: C, 80.77/80.54; H, 8.97/9.21
1c	3035, 2961–2881, 1243	[b]3.85 (s, 2H, C_6H_4–CH_2–C_6H_4); 5.17 (s, 4H, 2(CH_2–O)); 6.87–6.91 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4); 7.06–7.10 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4); 7.18–7.22 (d, 2H, 2$H_3C_6H_4N$); 7.49–7.53 (d, J_{3-4} = 8, 2H, 2$H_5C_6H_4N$); 7.66–7.70 (m, 2H, 2$H_4C_6H_4N$); 8.60 (d, J_{3-4} = 5, 2H, 2$H_6C_6H_4N$)	$C_{25}H_{22}N_2O_2$: C, 78.54/78.21; H, 5.75/5.47; N, 7.32/ 7.03
1d	3031, 1246, 1006	[a]3.89 (s, 2H, C_6H_3–CH_2–C_6H_3); 5.08 (s, 4H, 2(CH_2–O)); 7.04–7.08 (d, J_{3-4} = 9, 2H, H_3, $H_{3'}$ C_6H_3); 7.20 (s, 2H, H_6, $H_{6'}$ C_6H_3); 7.21–7.25 (d, J_{3-4} = 9, 2H, H_4, $H_{4'}$ C_6H_3); 7.33 (br s, 10H, 2C_6H_5)	$C_{27}H_{22}Cl_2O_2$: C, 72.19/72.10; H, 4.90/4.79
2a	3378, 1617, 811	[a]3.75 (s, 2H, C_6 H_4–CH_2–C_6H_4); 3.93 (br s, 2H, 2NH); 4.29 (s, 4H, 2(CH_2–N)); 6.53–6.57 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4); 6.95–6.99 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4); 7.29–7.35 (br s, 10H, 2C_6 H_5)	$C_{27}H_{26}N_2$: C, 85.68/85.69; H, 6.92/7.08; N, 7.40/7.39
2b	3404, 1612, 1514, 1323	[c]3.76 (s, 2H, C_6H_4–CH_2–C_6H_4); 4.24 (br s, 2H, 2NH); 4.46 (s, 4H, 2(CH_2–N)); 6.51–6.53 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4–N); 6.97–6.99 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4–N); 7.53–7.55 (d, J_{2-3} = 9, 4H, 2H_2, 2$H_6C_6H_4$–NO_2); 8.18–8.22 (d, J_{2-3} = 9, 4H, 2H_3, 2$H_5C_6H_4$–NO_2)	$C_{27}H_{24}N_4O_4$: C, 69.22/68.99; H, 5.16/5.25; N, 11.96/11.77
3a	3314, 1724, 1502, 1340	[e]3.98 (s, 2H, CH_2); 7.10–7.14 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4–O); 7.25–7.29 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4–O); 7.77–7.82 (d, J_{2-3} = 9, 4H, 2H_2, 2H_6 C_6H_4–NO_2); 8.18–8.22 (d, J_{2-3} = 9, 4H, 2H_3, 2H_5 C_6H_4–NO_2); 9.84 (s, 2H, 2NH)	$C_{27}H_{20}N_4O_8$:C, 61.38/61.58; H, 3.80/3.71; N, 10.60/10.36
3b	3320, 2979–2879, 1715	[e]3.48–3.51 (m, 4H, 2(CH_2–CH_2–Cl)); 3.64–3.67 (t, 4H, 2(CH_2–Cl)); 3.94 (s, 2H, C_6H_4–CH_2–C_6H_4); 6.99–7.03 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4); 7.18–7.22 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4); 11.13 (br s, 2H, 2NH)	$C_{19}H_{20}$ $Cl_2N_2O_4$: C, 55.47/55.22; H, 4.87/4.79; N, 6.81/6.66
3c	3318, 1758, 1510, 1330, 1010	[e]3.97 (s, 2H, CH_2); 7.21–7.24 (br s, 6H, 2C_6H_3); 7.66–7.70 (d, J_{2-3} = 9, 4H, 2H_2, 2H_6 C_6H_4–NO_2); 8.09–8.14 (d, J_{2-3} = 9, 4H, 2H_3, 2H_5 C_6H_4–NO_2); 9.90 (s, 2H, 2NH)	$C_{27}H_{18}$ $Cl_2N_4O_8$: C, 54.24/53.85; H, 3.01/3.20; N, 9.38/9.42
3d	3381, 2951–2879, 1734, 1512, 1333	[e]1.40–1.43 (m, 2H, –CH_2–CH_2–CH_2–O); 1.64–1.66 (m, 4H, 2(CH_2–CH_2–O)); 4.06–4.09 (t, 4H, 2(O–CH_2)); 7.68–7.80 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4); 8.07–8.09 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4); 9.25 (s, 2H, 2NH)	$C_{19}H_{20}N_4O_8$: C, 52.78/52.40; H, 4.63/4.42; N, 12.96/12.94
4a	3331, 2931–2881, 1636	[a]3.38–3.44 (m, 4H, 2(CH_2–CH_2–Cl)); 3.61–3.67 (t, 4H, 2(CH_2–Cl)); 3.87 (s, 2H, C_6H_4–CH_2–C_6H_4); 6.34–6.40 (t, 2H, 2(NH–CH_2)); 7.02–7.06 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4); 7.26–7.30 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4); 8.58 (s, 2H, 2(NH–C_6H_4))	$C_{19}H_{22}N_4O_2$: C, 55.65/55.69; H, 5.37/5.36; N, 13.67/13.60

Table 2 (*continued*)

Ref.	IR (cm^{-1})	^1H NMR (δ ppm, J in Hz)	Elemental analysis for (%) calcd/found
4b	3366, 2931–2883, 1664, 1501, 1343	[a]3.84 (s, 2H, CH_2); 7.13–7.17 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ CH_2–C_6H_4); 7.37–7.41 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ CH_2–C_6H_4); 7.65–7.69 (d, 4H, J_{2-3} = 9, $2H_2$, $2H_6NO_2$–C_6H_4); 8.16–8.20 (d, J_{2-3} = 9, 4H, $2H_3$, $2H_5$ NO_2–C_6H_4); 8.85 (s, 2H, 2(N*H*–C_6H_4–CH_2)); 9.39 (s, 2H, 2(N*H*–C_6H_4–NO_2))	$C_{27}H_{22}N_6O_6$: C, 61.61/61.22; H, 4.18/4.29; N, 15.97/16.36
4c	3321, 2932–2871, 1636	[a] 0.91–0.97 (t, 6H, $2CH_3$); 1.45–1.51 (m, 4H, 2(CH_3–CH_2)); 3.45–3.52 (m, 4H, 2(CH_2–CH_2–CH_3)); 3.85 (s, 2H, C_6H_4–CH_2–C_6H_4); 6.28–6.32 (t, 2H, 2(N*H*–CH_2)); 7.14–7.18 (d, J_{2-3} = 8, 4H, $2H_3$, $2H_5$ C_6H_4); 7.35–7.39 (d, J_{2-3} = 8, 4H, $2H_2$, $2H_6$ C_6H_4); 8.43 (s, 2H, 2(NH–C_6H_4))	$C_{21}H_{28}N_4O_2$: C, 68.45/68.22; H, 7.66/7.43; N, 15.20/15.21
4d	3281, 2931–2881, 1638	[a]3.70 (s, 2H, CH_2); 6.99–7.03 (d, J_{2-3} = 8, 4H, H_3, $H_{3'}$, H_5, $H_{5'}$ C_6H_4); 7.26–7.31 (d, J_{2-3} = 8, 4H, H_2, $H_{2'}$, H_6, $H_{6'}$ C_6H_4); 7.35–7.50 (m, 8H, $2H_3$, $2H_5$, $2H_6$, $2H_7$, $C_{10}H_7$); 7.76–7.80 (d, J_{7-8} = 7, 2H, $2H_8$ $C_{10}H_7$); 7.86–7.90 (d, J_{3-4} = 7, 2H, $2H_4$ $C_{10}H_7$); 7.95–7.99 (d, J_{2-3} = 6, 2H, $2H_2$ $C_{10}H_7$); 8.59 (s, 2H, 2NH); 8.86 (s, 2H, 2NH)	$C_{35}H_{28}N_4O_2$: C, 78.36/78.08; H, 5.22/5.43; N, 10.45/10.67
5a	3321, 3081, 2921, 2850, 1630	[c]1.25 (br s, 16H, CH_2); 1.50 (m, 4H, CH_2); 3.26 (m, 4H, CH_2); 7.49 (dd, J_{5-4} = 8, J_{5-6} = 5, 2H, H_5, $H_{5'}$); 8.15 (d, J_{4-5} = 8, 2H, H_4, $H_{4'}$); 8.62 (t, J = 5, 2H, NH, NH′); 8.68 (d, J_{6-5} = 5, 2H, H_6, $H_{6'}$); 8.96 (s, 2H, H_2, $H_{2'}$)	$C_{24}H_{34}N_4O_2$: C, 69.75/69.81; H, 8.35/8.30; N, 13.55/13.53
5b	3295, 3068, 3000–2900, 1634	[a]1.91 (q, J = 7, 2H, CH_2); 3.45 (m, 4H, CH_2); 7.68 (dd, J_{6-5} = 8, J_{6-7} = 8, 2H, H_6, $H_{6'}$); 7.86 (dd, J_{7-6} = 8, J_{7-8} = 8, 2H, H_7, $H_{7'}$); 8.07 (d, J_{5-6} = 8, 2H, H_5, $H_{5'}$); 8.07 (d, J_{8-7} = 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, $H_{2'}$)	$C_{23}H_{20}N_4O_2$: C, 71.89/71.55; H, 5.20/5.21; N, 14.57/14.57
5c	3547, 3413, 3070, 3000–2850, 1683	[d]2.05 (q, J = 7, 2H, CH_2); 3.68 (m, 4H, CH_2); 4.19 (s, 3H, $CH_{3'}$); 4.23 (s, 3H, CH_3); 7.62 (dd, J_{7-6} = 8, J_{7-8} = 8, 1H, H_7); 7.84 (s, 1H, $H_{3'}$); 7.90 (s, 1H, H_3); 8.08 (d, $J_{5'-6'}$ = 9, 1H, $H_{5'}$); 8.09 (d, J_{8-7} = 8, 1H, H_8); 8.43 (d, J_{6-7} = 8, 1H, H_6); 8.43 (d, $J_{6'-5'}$ = 9, 1H, $H_{6'}$); 8.52 (t, J=6, 1H, NH[a]); 8.61 (t, J=6, 1H, NH[b]); 9.14 (s, 1H, $H_{8'}$)	$C_{25}H_{22}N_6O_8$: C, 56.20/55.98; H, 4.12/4.22; N, 15.72/15.89
6a	3429, 3100–3000, 3000–2900, 2306	[c]1.91 (m, 4H, CH_2); 2.64 (t, J = 8 Hz, 4H, CH_2-phenyl); 3.12 (m, 4H, CH_2–CH_2–CH_2-piperaz.); 3.41 (br s, 4H, CH piperaz.); 3.74 (br s, 4H, CH piperazi.); 7.19–7.33 (m, 10H, CH phenyl); 12.03 (br s, 2H, hydrochloride)	$C_{22}H_{30}N_2 \cdot 2HCl$: C, 66.87/66.50; H, 8.09/8.21; N, 7.09/6.98
7a	3254, 3100–3000, 3000–2900	[c]3.15 (br s, 4H, CH_2–pyridine); 3.96 (br s, 4H, CH_2–N); 7.11 (br s, 1H, H_6 pyridopyrimidine); 7.19 (dd, J_{5-4} = 8, J_{5-6} = 5, 2H, H_5, $H_{5'}$ pyridine); 7.23 (d, J_{3-4} = 8, 2H, H_3, $H_{3'}$ pyridine); 7.56 (br s, 1H, H_5 pyridopyrimidine); 7.64 (dd, J_{4-3} = 8, J_{4-5} = 8, 2H, H_4, $H_{4'}$ pyridine); 8.54 (br s, 1H, H_7 pyridopyrimidine); 8.57 (d, J_{6-5} = 5, 2H, H_6, $H_{6'}$ pyridine); 7.00–7.92–8.29–8.71 (dd–br s–br s–br s, 2H, NH, NH′)	$C_{21}H_{21}N_7$: C, 67.94/67.58; H, 5.65/5.43; N, 26.40/26.49
7b	3415, 3300, 3100–3000, 3000–2900	[c]3.01 (t, 2H, CH_2[a]–indole); 3.10 (t, 2H, CH_2[c]–indole); 3.74 (m, 2H, CH_2[a]–N); 3.85 (m, 2H, CH_2[c]–N); 6.86 (dd, J_{5-4} = 8, J_{5-6} = 8, 1H, H_5 indole); 6.97 (dd, $J_{5'-4'}$ = 8, $J_{5'-6'}$ = 8, 1H, $H_{5'}$ indole); 7.03 (br s, 1H, H_6 pyridopyrimidine); 7.03 (br s, 2H, H_6, $H_{6'}$ indole); 7.14 (br s, 1H, $H_{2'}$ indole); 7.19 (br s, 1H, H_2 indole); 7.33 (d, J_{5-6} = 8, 1H, H_5 pyridopyrimidine); 7.35 (d, J_{7-6} = 8, 2H, H_7, $H_{7'}$ indole); 7.57 (d, J_{4-5} = 8, 2H, H_4, $H_{4'}$ indole); 8.63 (d, J_{7-6} = 5, 1H, H_7 pyridopyrimidine); 10.80 (br s, 2H, NH, NH′ indole); 7.96–8.40–8.52–9.55 (br s, 3H, NH, NH′, HCl)	$C_{27}H_{25}N_7 \cdot HCl$: C, 67.03/67.27; H, 5.17/5.09; N, 20.25/19.90
7c	3293, 3100–3000, 2929, 2850	[c]1.30 (br s, 8H, CH_2); 1.61 (m, 4H, CH_2); 3.51 (m, 4H, CH_2); 7.51 (dd, J_{6-5} = 8, J_{6-7} = 4, 2H, H_6, $H_{6'}$); 8.57 (s, 2H, H_2, $H_{2'}$); 8.57 (br s, 2H, NH, NH′); 8.67 (d, J_{5-6} = 8, 2H, H_5, $H_{5'}$); 8.95 (d, J_{7-6} = 4, 2H, H_7, $H_{7'}$)	$C_{21}H_{21}N_7$: C, 65.69/65.67; H, 6.46/6.35; N, 27.84/27.61

[a] DMSO-d_6; 200 MHz.
[b] $CDCl_3$; 200 MHz.
[c] DMSO-d_6; 400 MHz.
[d] $CDCl_3$; 400 MHz.
[e] $(CD_3)_2CO$; 200 MHz.

and 2,2′-methylenebis(4-chlorophenol) for compound **1d** (2.50 mmol), was added, followed by the addition of alkyl halide (10 mmol). The mixture was stirred for 24 h and then poured into water (100 mL). Extraction was carried out with dichloromethane (3 × 75 mL). The combined extracts were washed with water (3 × 75 mL) and dried over anhydrous sodium sulfate. The solvent was removed and the residue was recrystallized from ethanol and dichloromethane, unless otherwise noted.

6.3. 1,1′-Methylenebis[4-(4-chloro)benzyloxyphenyl] (1a)

From 4-chlorobenzyl chloride. Yield 55%. Mp 139–140 °C.

6.4. 1,1′-Methylenebis(4-butoxyphenyl) (1b)

From butyl bromide. Yield 33%. Mp 35–36 °C (methanol/dichloromethane).

6.5. 2,2′-[Methylenebis(1,4-phenyloxymethylene)]dipyridine (1c)

From 2-chloromethyl pyridine hydrochloride. Purified by flash-chromatography using silica gel as the solid support, and eluted with hexane:ethyl acetate (8:2 v:v) mixture. Yield 18%. Mp 78–79 °C.

6.6. 1,1′-Methylenebis(2-benzyloxy-5-chlorophenyl) (1d)

From benzyl chloride. Yield 33%. Mp 179–180 °C.

6.7. General procedure for compounds 2a–b

Molecular sieves (4 Å; 2.0 mmol) and aldehyde (15 mmol) were added to a solution of 4,4′-methylenebisaniline (5.0 mmol) in diethyl ether (30 mL). The mixture was stirred for 24 h. The solid precipitate was filtered, and then dissolved in chloroform (30 mL). The solution was filtered from the molecular sieves, which were then washed with chloroform. The solvent was removed from the filtrate and washings, and the residue was washed with diethyl ether (3 × 15 mL) and dichloromethane (3 × 15 mL), affording the intermediate imine (yield: 60–75%). $NaBH_3CN$ (1.2 mmol) was added to a solution of the imine (2.5 mmol) in methanol:HCl(c) (8:2, v:v). The reaction mixture was stirred for 24 h. The methanol was removed and the residue was taken up in water (30 mL) and extracted with diethyl ether (3 × 30 mL). The combined extracts were dried over anhydrous sodium sulfate, and the solvent was removed. The residue was purified by flash chromatography using silica gel as the solid support and then eluted with dichloromethane/methanol (95:5, v:v) mixture.

6.8. 4,4′-Methylenebis(*N*-benzylaniline) (2a)

From benzaldehyde. Yield 18%. Mp 112–113 °C.

6.9. 4,4′-Methylenebis[*N*-(4-nitrobenzyl)aniline](2b)

From 4-nitrobenzaldehyde. Yield 22%. Mp 152–153 °C.

6.10. General procedure for compounds 3a–d

The corresponding isocyanate (5.0 mmol) was added to a heterogeneous mixture of the appropriate phenol, 4,4′-methylenebisphenol for compounds **3a**–**b** and 2,2′-methylenebis(4-chlorophenol) for compound **3c**, or alcohol, 1,5-pentanediol for compound **3d**, (2.5 mmol), copper(I) chloride (0.50 g), and dry dioxane (20 mL) under stirring. After stirring for 5 h, the reaction mixture was diluted with diethyl ether (40 mL), washed with brine (3 × 40 mL) and water (3 × 40 mL), and then dried over anhydrous sodium sulfate. The solvent was removed and the residue was recrystallized from a suitable solvent.

6.11. Methylenebis[(4,1-phenylene)-*N*-(4-nitrophenyl)carbamate] (3a)

From 4-nitrophenyl isocyanate. A pure compound was afforded after washing with diethyl ether (3 × 15 mL) and absolute ethanol (3 × 15 mL). Yield 34%. Mp 232–233 °C.

6.12. Methylenebis[(4,1-phenylene)-*N*-(2-chloroethyl)carbamate] (3b)

From 2-chloroethyl isocyanate. Yield 27%. Mp 175–176 °C (ethanol).

6.13. Methylenebis[(4-chloro-2,1-phenylene)-*N*-(4-nitrophenyl)carbamate] (3c)

From 4-nitro phenylisocyanate. A pure compound was afforded after washing with diethyl ether (3 × 15 mL) and absolute ethanol (3 × 15 mL). Yield 25%. Mp 131–132 °C.

6.14. Pentane-1,5-diylbis[*N*-(4-nitrophenyl)carbamate] (3d)

From 4-nitrophenylisocyanate. Yield 37%. Mp 200–201 °C (methanol/dichloromethane).

6.15. General procedure for compounds 4a–c

A solution of the appropriate isocyanate (5.0 mmol) in diethyl ether (10 mL) was added dropwise to a solution of 4,4′-methylenebisaniline in diethyl ether (25 mL). The reaction mixture was stirred for 30 min. The solid precipitate was filtered, washed with diethyl ether (3 × 15 mL) and boiling ethanol (3 × 15 mL) and then dried, unless otherwise noted.

6.16. Methylenebis[*N*-(4,1-phenylene)-*N*′-(2-chloroethyl)urea] (4a)

From 2-chloro ethylisocyanate. Yield 82%. Mp 228–229 °C.

6.17. Methylenebis[*N*-(4,1-phenylene)-*N*′-(4-nitrophenyl)urea] (4b)

From 4-nitro phenylisocyanate. Yield 21%. Mp > 300 °C.

6.18. Methylenebis[*N*-(4,1-phenylene)-*N*′-(*n*-propyl)urea] (4c)

From propyl isocyanate. Yield 85%. Mp 218–219 °C.

6.19. Methylenebis[*N*-(4,1-phenylene)-*N*′-(naphth-1-yl)urea] (4d)

4,4′-Methylenebis(phenylisocyanate) (2.0 mmol) was suspended in diethyl ether (30 mL) and a solution of

1-amino naphthalene (4.2 mmol) in diethyl ether (15 mL) was added dropwise. The reaction mixture was stirred for 5 h. The solid precipitate was filtered, washed with methanol (3 × 10 mL), and boiling acetone (3 × 10 mL) and then dried, unless otherwise noted. Yield 46%. Mp > 300 °C.

6.20. *N*,*N*′-Dodecane-1,12-diyldinicotinamide (5a)

A solution of nicotinic acid (4.00 g, 32.5 mmol) in thionyl chloride (15–20 mL) was stirred 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). This solution was added dropwise at room temperature for 1 h to a mixture of the 1,12-dodecanediamine (3.25 g, 16.2 mmol) and triethylamine (4.52 mL, 32.5 mmol) in dry chloroform. After said addition, the mixture was heated under reflux for 5 h. The solvent was removed and the resulting residue was suspended in water (100 mL), filtered, washed with water, and recrystallized. Yield 30%. Mp 152–153 °C (ethanol).

6.21. *N*,*N*′-Propane-1,3-diyldiquinoline-3-carboxamide (5b)

A solution of 3-quinolinic acid (2.00 g, 11.5 mmol), and thionyl chloride (15–20 mL) in dry chloroform (50 mL) was stirred under reflux for 2 h. The solvent was removed and the residue was suspended in dry chloroform (30 mL). Triethylamine was then added (1.61 mL, 11.6 mmol). This solution was added dropwise to a mixture of 1,3-propanediamine (0.48 mL, 5.8 mmol) and triethylamine (1.61 mL, 11.6 mmol) in dry chloroform at room temperature for 1 h. The mixture was then heated under reflux for 4 h and the solvent was removed. The residue was suspended in water (100 mL) and filtered off. The resulting solid was washed with boiling water (4 × 25 mL) and recrystallized from *n*-hexane/ethanol. Yield 66%. Mp 200–201 °C.

6.22. 4-Methoxy-*N*-(3-{[(4-methoxy-7-nitroquinolin-2-yl)carbonyl]amino}propyl)-5-nitro quinoline-2-carboxamide (5c)

A solution of 4-methoxy-2-quinolinic acid (2.50 g, 11.7 mmol) in H_2SO_4 (c) (15 mL) and HNO_3 (c) (5 mL) was stirred and heated to 60 °C for 4 h. The mixture was cooled to 0 °C. Next, while under stirring, NaOH 20 N (30 mL) was added until pH 6–7. The solution was filtered and the solid was dissolved in boiling methanol. After cooling to room temperature, the solution was filtered, and the methanol of the filtrate was removed under low pressure. The residue obtained was the nitrated 4-methoxy-2-quinolinic acid (1.10 g, 4.43 mmol). A solution of nitrated 4-methoxy-2-quinolinic acid (1.10 g, 4.43 mmol) and thionyl chloride (10 mL) in dry chloroform (50 mL) was stirred under reflux for 2 h. The solvent was removed and the residue was suspended in dry chloroform (30 mL). Triethylamine was then added (0.61 mL, 4.43 mmol). This solution was added dropwise at room temperature for 1 h to the mixture of 1,3-propanediamine (0.18 mL, 2.2 mmol) and triethylamine (0.61 mL, 4.43 mmol) in dry chloroform. The mixture was then heated under reflux for 4 h. The solvent was removed. The resulting residue was suspended in water (100 mL), filtered, washed with water (4 × 25 mL), and purified by flash chromatography, using silica gel as the solid support and with *n*-hexane/ethyl acetate (1:1, v:v) as the eluent. Compounds **5c** (yield 26%. Mp 224–225 °C) and *N*,*N*′-propane-1,3-diylbis(4-methoxy-5-nitroquinoline-2-carboxamide) (yield 19%. Mp 273–274 °C) were obtained.

6.23. 1,4-Bis(3-phenylpropyl)piperazine dihydrochloride (6a)

A solution of piperazine (0.65 g, 7.5 mmol) and triethylamine (2.10 g, 15.1 mmol) in chloroform (50 mL) was stirred under reflux. A solution of 3-phenylpropane bromide (2.29 mL, 15.1 mmol) in chloroform (20 mL) was added dropwise for 2 h. The mixture was stirred under reflux for 48 h. The solvent was removed, and the resulting residue was dissolved in water (50 mL) and extracted with dichloromethane (3 × 50 mL). The combined extracts were washed with water (2 × 25 mL) and dried over anhydrous sodium sulfate. The solvent was removed and HCl 1.5 N (15 mL) was added. The mixture was extracted with dichloromethane (3 × 30 mL). The solution was stirred vigorously after each addition, and the precipitate that appeared was filtered. The combined solids were recrystallized. Yield 39%. Mp 226–227 °C (ethanol).

6.24. General procedure for compounds 7a and 7b

(a) Preparation of pyrido[2,3-*d*]pyrimidin-2,4-diol: a mixture of 2-aminonicotinic acid (5.00 g, 36.2 mmol) and urea (9.00 g, 150 mmol) was pulverized and heated to 210 °C (15 min at this temperature). After cooling to room temperature, NaOH 2 N (50 mL) was added. The solution was heated until complete dissolution, and the mixture was added to solid CO_2. The solid was filtered, washed with cool water (2 × 10 mL), and suspended in boiling acetic acid. The solution was filtered and washed with diethyl ether. Pyrido[2,3-*d*]pyrimidin-2,4-diol was obtained. Yield 62.3%. IR: 3406, 3176, 3100–3000, 2850, 2754, 1720, 1671 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6, δ): 6.76 (dd, J_{6-5} = 8 Hz, J_{6-7} = 5 Hz, 1H, H_6); 7.99 (d, J_{5-6} = 8 Hz, 1H, H_5); 8.35 (d, J_{7-6} = 5 Hz, 1H, H_7); 11.8 (br s, 2H, OH). Anal. Calcd for $C_7H_5N_3O_2$ (%): C, 51.55; H, 3.06; N, 25.75. Found (%): C, 51.55; H, 3.07; N, 25.72. (b) Preparation of 2,4-dichloropyrido[2,3-*d*]pyrimidine: a solution of pyrido[2,3-*d*]pyrimidin-2,4-diol (0.60 g, 3.7 mmol) and *N*,*N*-dimethylformamide (two drops) in phosphorus oxychloride (40 mL) was stirred under reflux for 24 h. The solvent was removed and ice (50 g) was added to the resulting residue. The solution was extracted with chloroform (5 × 50 mL). The combined extracts were washed with water (2 × 50 mL) and dried over anhydrous sodium sulfate. The solvent was then removed and 2,4-dichloropyrido[2,3-*d*]pyrimidine (0.74 g) was obtained and used immediately without further purification.

6.25. *N*,*N*′-Bis(2-pyridin-2-ylethyl)pyrido[2,3-*d*]pyrimidine-2,4-diamine (7a)

A solution of 2,4-dichloropyrido[2,3-*d*]pyrimidine (0.74 g, 3.7 mmol), triethylamine (1.0 mL, 7.4 mmol) and 2-(2-aminoethyl)pyridine (0.95 g, 7.4 mmol) in ethanol (50 mL) was stirred under reflux for 24 h. The solvent was removed, and the resulting residue was dissolved in HCl 1 N (25 mL) and washed with chloroform (3 × 25 mL). NaOH 10% was added until pH 9–10, and the solution was extracted with chloroform (4 × 25 mL). The combined extracts were washed with water (2 × 25 mL) and dried over anhydrous sodium sulfate. The solvent was removed and ethyl acetate/isopropanol (100 mL, 1:1, v:v) was added to the resultant oil. The residue was then filtered and recrystallized. Yield 15%. Mp 156–157 °C (ethyl acetate/2-propanol).

6.26. *N*,*N*′-Bis[2-(1*H*-indol-3-yl)ethyl]pyrido[2,3-*d*]pyrimidine-2,4-diamine (7b)

A solution of 2,4-dichloropyrido[2,3-*d*]pyrimidine (0.74 g, 3.7 mmol) and 3-(2-aminoethyl)indole (2.38 g, 14.8 mmol) in ethanol (50 mL) was stirred under reflux for 24 h. The solvent was removed, and water (25 mL) and chloroform (25 mL) were added to the resulting residue. The mixture was stirred and filtered. The solid was dissolved in boiling isopropanol and the solvent was removed. The resultant residue was purified by flash chromatography, using silica gel as the solid support and with dichloromethane/methanol (95:5, v:v) as the eluent. Once the solvent was removed, the residue was recrystallized. Yield 10%. Mp 162–163 °C (*n*-hexane/methanol).

6.27. Procedure for compound *N*,*N*′-dipyrido[2,3-*d*]pyrimidin-4-yloctane-1,8-diamine (7c)

(a) 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 to 170 °C (2 h at this temperature). After cooling to room temperature, water (50 mL) was added. The solution was then filtered and washed with water (2 × 10 mL). The solid was recrystallized from water and pyrido[2,3-*d*]pyrimidin-4-ol was obtained. Yield 55%. IR: 3422, 3100–3000, 3000–2900. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.56 (dd, J_{6-5} = 8 Hz, J_{6-7} = 5 Hz, 1H, H_6); 8.32 (s, 1H, H_2); 8.51 (d, J_{5-6} = 8 Hz, 1H, H_5); 8.95 (d, J_{7-6} = 5 Hz, 2H, H_7); 12.55 (br s, 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. (b) 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 under reflux for 1 h. The solvent was removed under low 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.

A solution of 4-chloropyrido[2,3-*d*]pyrimidine (0.84 g, 5.1 mmol), triethylamine (0.70 mL, 5.1 mmol) and 1,8-octanediamine (0.37 g, 2.5 mmol) in chloroform (50 mL) was stirred under reflux for 48 h. The solvent was removed under low pressure. The resulting residue was suspended in water (50 mL), filtered, washed with water (3 × 20 mL), and recrystallized. Yield 20%. Mp 195–196 °C (ethyl acetate/ethanol).

6.28. Evaluation of cytotoxic potential against human cancer cell lines

An assay using neutral red[48] 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/streptomicin. For this assay, cells were obtained from 80–90% confluent T-75 flasks by detaching the cells with PBS/EDTA. Cells (100 μm) were seeded at a density of 20×10^3/well in 96-well plates, but only the 60 inner wells were used in order to avoid possible border effects. Before adding the compounds, cells were allowed to attach to the bottom of the wells for 12 h. 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 min at room temperature and then read using a plate reader at 540 nm absorbance. Data was calculated as a percentage of total absorbance found for cells in nondrug-treated wells.

6.29. Evaluation of potential selectivity

Cytotoxic activity was determined by performing the aforementioned neutral red assay. However, this time, the cell lines used were CRL-8799 from mama epithelium and CLR-11233 from liver. The test concentration used was the highest IC_{50} determined for each compound in the three cultures in tumor cell lines.

6.30. Evaluation of apoptosis induction in human cancer cell lines

Apoptosis was quantified using a detection kit called the *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.[49,50] 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 the IC_{50} values determined in the previous cytotoxicity assay for each one of the cell lines as the test concentrations. The apoptosis measurements were taken after 48 h of incu-

bation. As previously indicated, a relative value of 1 was attributed to the apoptosis detected in the control cultures in which the test compound is not present.

6.31. Evaluation of caspase-3 activation

Detection was carried out by means of flow cytometry, using *the Active-Caspase-3 FITC Mab apoptosis kit* from Pharmingen, which 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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