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Short communication

Aromatic analogues of DNA minor groove binders—synthesis and biological evaluation

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Abstract

Nine carbocyclic analogues of mono- and bis-lexitropsins and two analogues of pentamidine with unsubstituted N-terminal amine group were synthesized. We have investigated the cytotoxic activity of new aromatic analogues of DNA binding ligands in MCF-7 breast cancer cells and assessed their ability to act as inhibitors of topoisomerase I and II. These studies indicate that aromatic analogues of bis-netropsin contain two identical units tethered by alkyloxyl chains are a potent catalytic inhibitor of both topoisomerases and exhibit moderate cytotoxicity in MCF-7 breast cancer cells.

Keywords: Netropsin; Bis-netropsin; DNA topoisomerase; Cytotoxicity; Minor groove binding

1. Introduction

Netropsin, Hoechst 33258 and pentamidine (Fig. 1) have long served as model compounds for biochemical and biophysical studies of drugs that bind to the DNA minor groove. Several high resolution crystal and NMR structures have been determined for these ligands bound to short oligonucleotides and provide valuable insights into the details of their interaction [1,2]. The typical binding sites for these minor groove binding drugs are four consecutive AT base pairs, where a particularly narrow groove with a floor lacking amino groups permits an optimization of van der Waals' contacts and hydrogen bonding [1,2]. Groove binding typically exerts only subtle changes in DNA conformation, and DNA remains essentially in the native form. The discovery of the details of minor groove binding of these compounds let to the synthesis of sequence-reading molecule based on them [1,2].

Recently published data suggest that DNA-binding drugs might exert their biological effects by interfering with DNA–protein interactions [1–3]. A number of minor groove binding drugs are dual inhibitors of topoisomerase I and II. These data suggest that these topological enzymes read DNA structure at least in part through the minor groove [4]. These enzymes are crucial for cellular genetic processes, such as

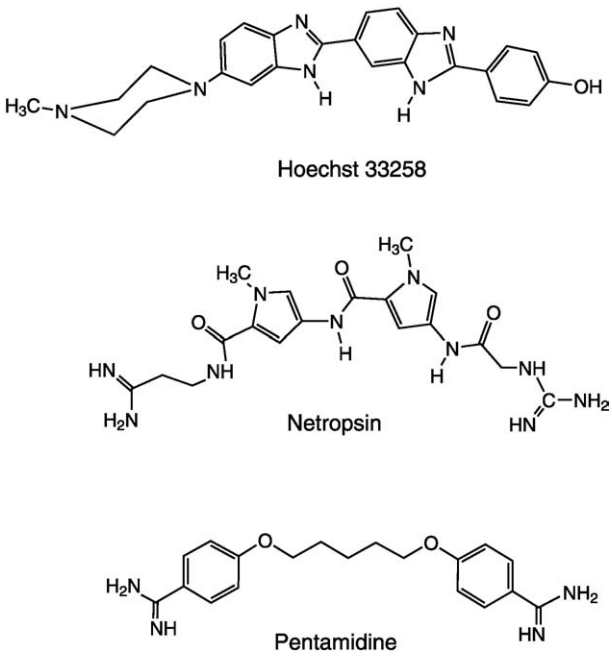

Fig. 1. Structure of Hoechst 33258, netropsin and pentamidine.

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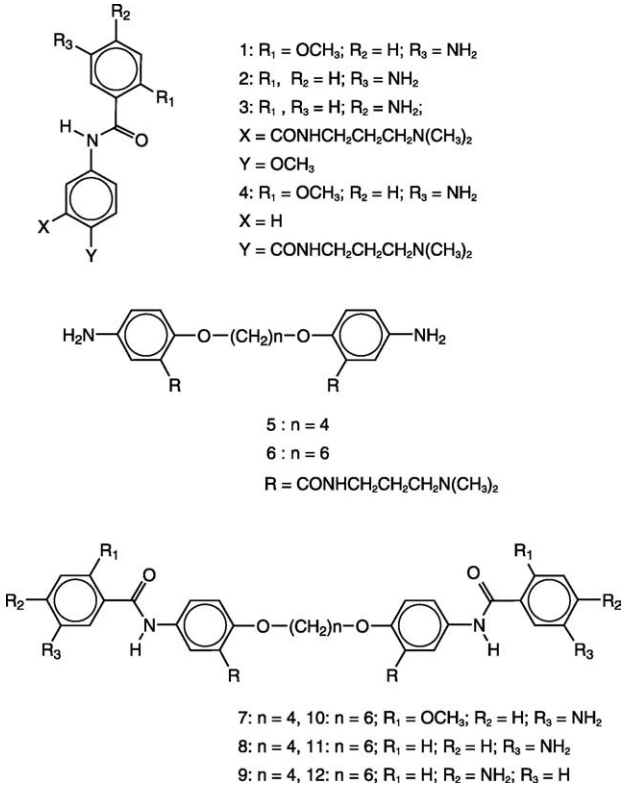

Fig. 2. Structure of compounds **1–12**.

DNA replication, transcription, recombination, and chromosome segregation at mitosis [4].

In the course of our investigations of minor groove binding drugs [5–8], we reported a synthesis and cytotoxicity of 12 aromatic analogues of DNA binding ligands **1–12** (Fig. 2) in cultured breast cancer MCF-7 cells. Compounds **1–4** are benzene derivatives of netropsin. Compounds **7–12**, in which tetra- or hexamethylene chains linked two identical units of **1, 2** or **3**, are analogues of bis-netropsin. Compounds **5** and **6** are similar to aromatic bis-amidines. We also investigated the mode of action of compounds **1–12** in a cell-free system by employing the topoisomerase I/II inhibition assay.

2. Chemistry

The benzene derivatives of lexitropsins are readily available; they can be easily modified, and are stable under most experimental conditions [9]. Compounds **2** and **3** were synthesized with good yields by standard chemical transformations described for compound **1** [5]. The starting material *N*-[5-amino-2-methoxy]benzoyl-*N*′,*N*′-dimethylpropyl-1,3-diamine dihydrochloride [5] was condensed with 3-nitro- or 4-nitrobenzoyl chloride respectively, and the obtained products were hydrogenated to aromatic amines **2** and **3**. Compound **4** was prepared from 4-nitrobenzoyl chloride by following steps: condensation with *N,N*-dimethylpropyl-1,3-diamine, hydrogenation of nitro group, condensation of obtained amine with (2-methoxy-5-phenyldiazenyl)benzoyl chloride and hydrogenation of diazenyl group. Compounds **7–12** were prepared like compounds **1–3** from 1,4-bis{4-amino-2-[(1,5-diaza-5-methylhexyl)carbonyl]phenyloxy} butane (**5**) [6] or 1,6-bis{4-amino-2-[(1,5-diaza-5-methylhexyl)carbonyl]phenyloxy}hexane (**6**). Compound **6** was obtained according to the procedure described for **5** from methyl [2-hydroxy-5-phenyldiazenyl]benzoate [6]. All compounds were prepared as hydrochloride salts.

3. Pharmacology

3.1. Cytotoxic activity

We studied the effect of compounds **1–12** and Hoechst 33258 on DNA synthesis in human MCF-7 breast cancer cells (Table 1). Hoechst 33258 was shown to possess activity against L1210 murine leukemia and several positive studies in various solid tumors led to the compound being entered into phase I clinical trials in humans, but toxicity precluded further advanced trials [10]. This compound was shown to inhibit binding of TATA-box binding protein to DNA, and also to be an effective inhibitor of mammalian DNA topoisomerase I and II [1,2]. Measurement of [^{3}H]thymidine incorporation during DNA synthesis by proliferating MCF-7 breast cancer cells showed that compounds **1–12** inhibited DNA synthesis in a dose-dependent manner. Among these derivatives, **5, 6, 8, 10, 11** and **12** proved to be slightly less potent than Hoechst 33258, with IC_{50} values of 185 ± 2 μM, 193 ± 2 μM, 57 ± 2 μM, 159 ± 2 μM, 132 ± 2 μM and 182 ± 2 μM, respectively, compared to 53 μM for Hoechst 33258. In contrast, benzene derivatives of netropsin **1–4**, and compounds **7** and **9** are clearly much less active and showed a very low level of cytotoxic potency (IC_{50} > 250 μM).

Table 1
Cytotoxic effects of **1–12** and Hoechst 33258 on the cultured breast cancer cells as measured by inhibition of [^{3}H]thymidine incorporation into DNA

Concentration	[^{3}H]Thymidine incorporation (% of control) [a]												
(μM)	Hoechst 33258	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
0	100	100	100	100	100	100	100	100	100	100	100	100	100
25	78 ± 2	98 ± 2	97 ± 2	92 ± 2	93 ± 2	84 ± 2	97 ± 2	95 ± 2	85 ± 2	88 ± 2	74 ± 2	96 ± 2	95 ± 2
50	52 ± 2	92 ± 2	91 ± 2	82 ± 2	84 ± 2	82 ± 2	90 ± 2	81 ± 2	51 ± 2	74 ± 2	63 ± 2	75 ± 2	87 ± 2
100	25 ± 2	87 ± 2	89 ± 2	78 ± 2	79 ± 2	80 ± 2	84 ± 2	79 ± 2	43 ± 2	71 ± 2	53 ± 2	66 ± 2	70 ± 2
150	11 ± 1	75 ± 2	85 ± 2	68 ± 2	74 ± 2	70 ± 2	69 ± 2	78 ± 2	35 ± 2	70 ± 2	51 ± 2	42 ± 2	60 ± 2
200	2 ± 1	68 ± 2	82 ± 2	54 ± 2	67 ± 2	49 ± 2	48 ± 2	65 ± 2	30 ± 2	64 ± 2	47 ± 2	38 ± 2	45 ± 2

[a] Mean values ± S.D. of three independent experiments done in duplicates are presented.

Table 2
Viability of MCF-7 cells treated for 24 h with different concentrations of **1-12** and Hoechst 33258

Concentration (μM)	Viability of cells (% of control) [a]												
	Hoechst 33258	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
0	100	100	100	100	100	100	100	100	100	100	100	100	100
25	75 ± 2	98 ± 2	98 ± 1	98 ± 1	99 ± 2	83 ± 2	86 ± 2	92 ± 1	92 ± 1	98 ± 2	94 ± 1	86 ± 1	91 ± 1
50	67 ± 2	97 ± 2	88 ± 2	89 ± 2	92 ± 3	78 ± 1	83 ± 2	88 ± 2	78 ± 2	94 ± 1	86 ± 2	83 ± 2	83 ± 2
100	49 ± 2	94 ± 1	72 ± 1	82 ± 3	84 ± 2	76 ± 1	80 ± 1	84 ± 1	59 ± 1	91 ± 1	72 ± 3	62 ± 2	65 ± 3
150	35 ± 2	89 ± 1	70 ± 2	78 ± 2	77 ± 2	73 ± 1	79 ± 3	82 ± 2	12 ± 2	83 ± 2	63 ± 2	37 ± 1	59 ± 2
200	24 ± 1	82 ± 2	69 ± 1	72 ± 1	75 ± 1	69 ± 1	68 ± 2	78 ± 1	11 ± 1	75 ± 2	58 ± 1	10 ± 2	53 ± 1

[a] Mean values ± S.D. of 3 independent experiments done in duplicates are presented.

Cell viability of breast cancer MCF-7 cells was measured by the method of Carmichael et al. [11] using tetrazolium salt (Table 2). In terms of reduction in cell viability, the compounds rank in the order Hoechst 33258 **>8 >11**. The concentrations of **11, 8**, and Hoechst 33258 needed to 50% reduction in cell viability in breast cancer MCF-7 (IC_{50}) was found to be 124 ± 2 μM, 108 ± 2 μM, and 96 ± 2 μM, respectively.

3.2. Relaxation assay of topoisomerase I and II

The mechanism of antitumor action of compounds **7–12** may be due to their ability to inhibit the binding of some transcription factors to their consensus sequences in DNA, thereby preventing transcription. Several major forms of human cancer express high levels of topoisomerase enzymes [12]. To test whether cytotoxic properties were related to topoisomerase I/II inhibition, the aromatic analogues of DNA binding ligands **1–12** were evaluated in a cell-free system.

Purified topoisomerases I and II were incubated with increasing concentrations (range 10–100 μM) of compounds **1–12** and Hoechst 33258 in the presence of supercoiled DNA plasmid PBR322. The products of this reaction were subjected to electrophoresis in the presence of ethidium bromide in order to separate closed and open circular DNA. Comparison of **1–12** with Hoechst 33258 as inhibitors of topoisomerase I and II demonstrated that several of these compounds had similar potency (Table 3). None of the compounds **1–4** inhibited the topoisomerases mediated relaxation of supercoiled DNA at a concentration of 100 μM. As shown in Table 3 compounds **5–12** showed potent inhibitory activity on topo I and topo II with concentrations ranging from 30 to 50 μM. They exhibited similar potency to Hoechst 33258 as topoisomerase I and II inhibitors (Table 3).

4. Results and discussion

The topoisomerases induce transient cuts in DNA, which enable the strands to pass through the nicks, and then rejoin the nicked strands to DNA. During this process, covalent links are formed between topoisomerase and DNA, which are called ‘cleavable complexes’ [4]. The topo-targeting drugs can be classified as either topo poisons, which act by stabilizing enzyme-DNA cleavable complexes leading to DNA breaks, or topo catalytic inhibitors, which act at stages in the catalytic cycle of the enzyme where DNA strands remain intact and no DNA strand breaks occur [13,14]. Treatment with the classical topo I or II poison (camptothecin and etoposide, respectively) results in the production of linear DNA which demonstrates that these compounds stabilize DNA–topo covalent complexes and hence stimulate a single or double-strand cleavage by the enzymes. Conversely, with **5–12** no band corresponding to linear DNA was detected in the presence of enzyme (data not shown), implying that these compounds do not act as topo poisons. It is likely that the ability of compounds **5–12** to inhibit the activity of topo I and II that we have observed (Table 3) is simply due to blockade of the binding of these enzymes to DNA. The increasing the total size occupied by the compounds **5–12** compared to **1–4** could afford greater opportunity for inhibiting topoisomerase activity. It is possible that compounds **5–12** acts through inducing a conformational change in the DNA and hindering the formation of the cleavable complex. The flexible nature of tetra- or hexamethylene chains permits the compounds **5–12** to take an optimum conformation, thus binding effectively to double-stranded DNA.

Evaluation of the topoisomerases inhibition and cytotoxicity of **1–12** provided additional insight into the structure–activity relationships associated with these compounds. Several of compounds that were among the more potent

Table 3
Topoisomerase I/II inhibitory effect of compounds **5–12**

Enzyme	Concentration (μM) [a]								
	Hoechst 33258	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Topo I	30	50	30	30	40	40	30	30	40
Topo II	40	50	30	40	40	40	40	30	50

[a] The concentrations of the inhibitor that prevented of the supercoiled DNA from being converted into relaxed DNA were determined by averaging the data from at least three experiments.

topoisomerase I and II inhibitors did exhibit greater cytotoxicity toward human MCF-7 breast cancer cells. Compounds **1–4** were not a topoisomerase I and II inhibitors and did not possess cytotoxic activity. The fact that compounds **8** and **11** are more potent than **9** and **12** indicates that cytotoxicity in MCF-7 cultured breast cancer cells is also sensitive to the position of the terminal cationic amine groups of the compounds. The cytoxicity observed for **7** and **10** in comparison to **8** and **11** suggest that steric factors associated with methoxy groups may moderate influence the activity of similar compounds. Among the compounds evaluated, **7** and **9** were active as topoisomerase inhibitors, but did not exhibit significant cytotoxicity. It should be noted that other factors such as low penetration into cell, cellular distribution and metabolic deactivation may also influence the cytoxicity results, but they are not assessed in the present study.

5. Conclusions

In this study, we have investigated the cytotoxic activity of new aromatic analogues of DNA binding ligands **1–12** in MCF-7 breast cancer cells and assessed their ability to act as inhibitors of topoisomerases I and II. Among these derivatives, compound **8** proved to be only slightly less potent than Hoechst 33258, with IC_{50} value for DNA synthesis of 57 μM, compared to 53 μM for Hoechst 33258.

Recent in vitro studies indicate that the inhibitory activity of minor groove binders as distamycin, netropsin, Hoechst 33258, etc., are rather limited to proteins, which reside within the minor groove [1–3]. We have shown in the present report that compounds **5–12** are a potent catalytic inhibitor of both topoisomerase I and II. These compounds inhibit the catalytic activity of the topoisomerase at a step prior to the formation of the topo–DNA complex. This suggests that DNA-binding may be implicated in the cytotoxicity of compounds **5–12** possibly by inhibiting interactions between topoisomerases and their DNA targets. Moreover, there might be other possible targets, such as other enzymes, involved in DNA metabolism and/or transcription factors because their activities were inhibited by some DNA minor groove binders [1–3]. Further biological evaluation is underway and these results, DNA binding studies and sequence selective protection of restriction enzyme recognition sites by described compounds will be described in due course.

6. Experimental protocols

6.1. Chemistry

Acylations of starting aromatic amines by acid chlorides were carried out at pyridine/CH_2Cl_2 solution, in catalytic presence of 4-(dimethylamino)pyridine (DMAP). The products of these reactions were purified by column chromatography (silica gel 60, Merck, 230–400 mesh), using the mixture of hexane and acetone (1:2) with 10–40% addition of methanol.

Catalytic hydrogenation of nitro or diazenyl groups was carried out in methanol (with addition of HCl_{aq}, pH 4) in the presence of Pd/C (10%). The obtained aromatic amines were purified by column chromatography using the solvent system as follow: (1) C_6H_6/C_2H_5OH 1:1; (2) CH_3OH. Acylations of amine groups, hydrogenations of nitro or diazenyl groups, and the purity of final compounds were controlled by TLC (1% NH_3 in CH_3OH). The identification of all the compounds was made by UV and by spraying of chromatograms with solution of DMAB (4-dimethylaminobenzaldehyde—1 g, 36% HCl—30 ml, EtOH—10 ml, *n*-ButOH—180 ml). Thin-layer chromatograms were prepared on precoated plates (Merck, silica gel 60 F_{254}).

The structures of all the compounds were confirmed by ^1H and ^{13}C NMR spectra recorded on Brucker AC 200F apparatus (^1H—200 MHz and ^{13}C—50 MHz) in D_6-DMSO. Melting points were determined on Büchi 535 melting-point apparatus and were uncorrected. Elemental analysis of C, H, and N was performed on a Perkin Elmer 240 analyser, while Cl was determined by Shuniger method, and satisfactory results within ±0.4% of calculated values were obtained.

6.1.1. N-*[5-(5-Amino-2-methoxy)benzamido-2-methoxy]benzoyl*-N′,N′*–dimethylpropyl-1,3-diamine dihydrochloride (**1**)*

This compound was obtained as described earlier [5].

6.1.2. N-*[5-(3-Amino)benzamido-2-methoxy]benzoyl*-N′,N′*–dimethylpropyl-1,3-diamine dihydrochloride (**2**)*

Yield 51%. M.p.: 132–133 °C. Rf = 0.34. ^1H NMR: 1.89–1.96 (m, 2H, CH_2), 2.72–2.75 (d, J = 4.3, 6H, $(CH_3)_2NH^+$), 3.06–3.08 (m, 2H, CH_2NR), 3.32–3.41 (m, 2H, $CONHCH_2$), 3.89 (s, 3H, OCH_3), 7.12–7.17 (d, J = 9.0, 1H, C-3), 7.44–7.60 (m, 2H, H-4′, H-5′), 7.79–7.93 (m, 3H, H-4, H-2′, H-6′), 8.14–8.15 (d, J = 2.63, 1H, H-6), 8.39–8.45 (t, J = 5.7, 1H, ArCONHR), 10.45 (s, 2H, ArCONHAr, H^+), ^{13}C NMR: 24.19 (CH_2), 36.32 ($CONHCH_2$), 42.00 ($(CH_3)_2NH^+$), 54.40 (CH_2NR), 56.18 (OCH_3), 112.21 (C-3), 121.07 (C-6), 122.78 (C-2′), 122.97(C-1), 124.50 (C-4), 124.67 (C-4′), 124.98 (C-3′), 129.64 (C-6′), 132.01 (C-5′), 135.37 (C-5), 136.07 (C-1′), 153.35 (C-2), 164.42 (ArCONHR), 165.09 (ArCONHAr). Anal. $C_{20}H_{26}N_4O_3{\cdot}2HCl$ (C, H, N, Cl).

6.1.3. N-*[5-(4-Amino)benzamido-2-methoxy]benzoyl*-N′,N′*-dimethylpropyl-1,3-diamine dihydrochloride (**3**)*

Yield 57%. M.p.: 140–142 °C, Rf = 0.32. ^1H NMR: 1.89–1.97 (m, 2H, J = 9, CH_2), 2.72–2.74 (d, J = 4.6, 6H, $(CH_3)_2NH^+$), 3.01–3.06 (m, 2H, CH_2N), 3.32–3.41 (m, 2H, $CONHCH_2$), 3.89 (s, 3H, OCH_3), 7.10–7.14 (d, J = 9, 1H, H-3), 7.23–7.27 (d, J = 8.4, 2H, H-3′, H-5′), 7.88–7.94, (dd, J = 2.6, J = 8.9, 1H, H-4), 7.97–8.01 (d, J = 8.4, 2H, H-2′, H-6′), 8.13–8.15 (d, J = 2.6, 1H, H-6), 8.37–8.42 (t, J = 5.6, 1H, ArCONHR), 10.26 (s, 1H, ArCONHAr), 10.66 (bs, 1H,

H^+). ^{13}C NMR: 24.12 (CH_2), 36.29 ($CONHCH_2$), 41.95 ($N(CH_3)_2$), 54.34 (CH_2N), 112.12 (C-3), 119.82 (C-6), 122.73 (C-1), 122.84 (C-3′, C-5′), 124.35 (C-4), 129.17 (C-2′, C-6′), 130.10 (C-5), 132.28 (C-4′), 140.36 (C-1′), 153.09 (C-2), 164.51 (ArCONHR), 165.10 (ArCONHAr). Anal. $C_{20}H_{26}N_4O_3$·2HCl (C, H, N, Cl).

6.1.4. N-*[4-(5-Amino-2-methoxybenzamido]benzoyl*-N′,N′-*dimethylpropyl-1,3-diamine dihydrochloride (**4**)*

Yield 51%. M.p.: 178–180 °C. Rf = 0.3. ^1H NMR: 1.90–1.97 (m, 2H, CH_2), 2.73 (s, 6H, $(CH_3)_2N$), 3.05–3.12 (m, 2H, CH_2N), 3.28–3.38 (m, 2H, $CONHCH_2$), 3.92 (s, 3H, OCH_3), 7.27–7.32 (d, J-8.9, 1H, H-3′), 7.52–7.58 (dd, *J* = 2.7, *J* = 8.8, 1H, H-4′), 7.66–7.68 (d, *J* = 2.7, 1H, H-6′), 7.78–7.83 (d, *J* = 8.7, 2H, H-3, H-5), 7.89–7.93 (d, *J* = 8.7, 2H, H-2, H-6), 8.68–8.74 (t, *J* = 5.8, 1H, ArCONHR), 10.43 (s, 1H, ArCONHAr), 10.62 (bs, 1H, H^+). ^{13}C NMR: 24.19 (CH_2), 36.29 ($CONHCH_2$), 41.91 ($(CH_3)_2N$), 54.43 (CH_2N), 56.43 (OCH_3) 113.29 (C-3′), 118.93 (C-3, C-5), 124.26 (C-6′), 124.78 (C-5′), 125.25 (C-1′), 126.81 (C-2, C-6), 128.08 (C-4′), 129.21 (C-1), 141.26 (C-4), 155.68 (C-2′), 163.55 (ArCONHR), 165.76 (ArCONHAr). Anal. $C_{20}H_{26}N_4O_3$·2HCl (C, H, N, Cl).

*6.1.5. 1,4-Bis{4-amino-2-[(1,5-diaza-5-methylhexyl)carbonyl]phenyloxy}butane bis(dihydrochloride) (**5**)*

This compound was obtained as described earlier [6].

*6.1.6. 1,6-Bis{4-amino-2-[(1,5-diaza-5-methylhexyl)carbonyl]phenyloxy}hexane bis(dihydrochloride) (**6**)*

Yield 45%. M.p.: 214–215 °C, Rf = 0.22. ^1H NMR: 1.47–1.59 (m, 2H, CH_2), 1.82–1.91 (m, 4H, 2XCH_2), 2.72 (s, 6H, $(CH_3)_2N$), 3.02–3.05 (m, 2H, CH_2N), 3.33–3.50 (m, 2H, $CONHCH_2$), 4.04–4.14 (m, 2H, OCH_2), 7.18–7.19 (d, *J* = 8.9, 1H, H-6), 7.45–7.51 (dd, *J* = 2.5, *J* = 8.8, 1H, H-5), 7.71–7.73 (d, *J* = 2.6, 1H, H-3), 8.31–8.37 (m, 1H, ArCONHR), 10.75 (bs, 1H, H^+). ^{13}C NMR: 24.12 (CH_2), 25.04 (CH_2), 28.19 (CH_2), 36.43 ($CONHCH_2$), 41.92 ($(CH_3)_2N$), 54.29 (CH_2N) 68.91 (OCH_2), 115.54 (C-6), 124.25 (C-4), 124.61 (C-2), 126.70 (C-3), 129.52 (C-5), 155.35 (C-1), 164.43 (ArCONHR). Anal. $C_{30}H_{48}N_6O_4$·4HCl (C, H, N, Cl).

*6.1.7. 1,4-Bis{4-[(5-amino-2-methoxy)benzamido]-2-[(1,5-diaza-5-methylhexyl)-carbonyl]phenyloxy}butane bis(dihydrochloride) (**7**)*

Yield 51%. M.p.: 161–163 °C, Rf = 0.15. ^1H NMR: 1.88–1.99 (m, 4H, 2 × CH_2), 2.71 (s, 6H, $(CH_3)_2N$), 3.06–3.18 (m, 2H, CH_2N), 3.34–3.37 (m, 2H, $CONHCH_2$) 3.91 (s, 3H, OCH_3), 4.15–4.24 (m, 2H, OCH_2), 7.15–7.20 (d, *J* = 9.0, 1H, H-6), 7.26–7.31 (d, *J* = 8.75, 1H, H-3′), 7.52–7.57, (dd, *J* = 2.33, *J* = 8.75, 1H, H-5), 7.67–7.68 (d, *J* = 2.26, 1H, H-3), 7.78–7.83 (d, *J* = 8.7, 1H, H-4′), 8.02–8.03 (d, *J* = 2.28, 1H, H-6′), 8.31–8.34 (m, 1H, ArCONHR), 10.20 (s, 1H, ArCONHAr), 10.81 (bs, 1H, H^+). ^{13}C NMR: 24.19 (CH_2), 25.27 (CH_2), 36.37 ($CONHCH_2$), 41.93 ($(CH_3)_2N$), 54.36 (CH_2N), 56.46 (OCH_3), 68.53 (OCH_2), 113.25 (C-6), 113.52 (C-3′), 121.89 (C-3), 123.82 (C-2), 124.41 (C-5′), 125.42 (C-1′), 126.84 (C-6′), 127.18 (C-5), 131.83 (C-4), 145.72 (C-4′), 152.32 (C-1), 155.87 (C-2′), 163.06 (ArCONHR), 165.40 ArCONHAr). Anal. $C_{44}H_{58}N_8O_8$·4HCl (C, H, N, Cl).

*6.1.8. 1,4-Bis{4-[(3-amino)benzamido]-2-[(1,5-diaza-5-methylhexyl)carbonyl]-phenyloxy}butane bis(dihydrochloride) (**8**)*

Yield 38%. M.p.: 180–182 °C. Rf = 0.2. ^1H NMR: 1.87–2.03 (m, 4H, CH_2), 2.70 (s, 6H, $(CH_3)_2N$), 2.88–3.05 (m, 2H, CH_2N), 4.16–4.25 (m, 2H, OCH_2), 6.73–6.77 (d, *J* = 8.48, 1H, H-6), 7.11–7.18 (m, 4H, Ar′), 7.82–7.88 (dd, *J* = 2.51, *J* = 8.96, 1H, H-5), 8.08–8.09 (d, *J* = 2.57, 1H, H-3), 8.27–8.30 (t, *J* = 5.70, 1H, ArCONHR), 10.12 (s, 1H, ArCONHAr), 10.84 (bs, 1H, H^+). ^{13}C NMR: 24.19 (CH_2), 25.28 (CH_2), 36.33 ($CONHCH_2$), 41.87 ($(CH_3)_2N$), 54.31 (CH_2N), 68.45 (OCH_2), 113.03 (C-6), 113.29 (C-2′), 114.74 (C-6′), 116.79 (C-3), 122.44 (C-4′), 123.49 (C-5), 124.01 (C-2), 128.72 (C-5′), 132.46 (C-4), 135.65 (C-1′), 148.68 (C-3′), 152.06 (C-1), 165.41 (ArCONHR), 166.01 (ArCONHAr). Anal. $C_{42}H_{54}N_8O_6$·4HCl (C, H, N, Cl).

*6.1.9. 1,4-Bis{4-[(4-amino)benzamido]-2-[(1,5-diaza-5-methylhexyl)carbonyl]-phenyloxy}butane bis(dihydrochloride) (**9**)*

Yield 41%. M.p.: 167–169 °C, Rf = 0.2. ^1H NMR: 1.91–2.15 (m, 4H, 2 × CH_2), 2.70–2.72 (d, *J* = 4.55, 6H, $(CH_3)_2NH^+$), 3.06–3.16 (m, 2H, CH_2N), 3.35–3.38 (m, 2H, $CONHCH_2$), 4.07–4.32 (m, 2H, OCH_2), 7.16–7.20 (d, *J* = 8.32, 3H, H-6, H-3′, H-5′), 7.87–7.94 (m, 3H, H-5, H-2′, H-6′), 8.09–8.11 (d, *J* = 2.6, 1H, H-3), 8.27–8.32 (t, *J* = 5.9, 1H, ArCONHR), 10.22 (s, 1H, ArCONHAr), 10.72 (bs, 1H, H^+).^{13}C NMR: 24.19 (CH_2), 25.27 (CH_2), 36.29 ($CONHCH_2$), 41.88 ($(CH_3)_2N$), 54.33 (CH_2N) 68.45 (OCH_2), 113.29 (C-6), 119.08 (C-3), 122.52 (C-3′, C-5′), 123.45 (C-2), 124.10 (C-5), 129.04 (C-4) 129,16 (C-2′, C-6′), 132.37 (C-4′), 141.50 (C-1′), 152.09 (C-1), 163.53 (ArCONHR), 165.38 (ArCONHAr). Anal. $C_{42}H_{54}N_8O_6$·4HCl (C, H, N, Cl).

*6.1.10. 1,6-Bis{4-[(5-amino-2-methoxy)benzamido]-2-[(1,5-diaza-5-methylhexyl)-carbonyl]phenyloxy}hexane bis (dihydrochloride) (**10**)*

Yield 44%. M.p.: 165–167 °C, Rf = 0.19. ^1H NMR: 1.42–1.52 (m, 2H, CH_2), 1.83–1.91 (m, 4H, 2 × CH_2), 2.73 (s, 6H, $(CH_3)_2N$), 3.06–3.18 (m, 2H, CH_2N), 3.91 (s, 3H, OCH_3), 4.11–4.18 (m, 2H, OCH_2), 6.95–6.99 (d, *J* = 8.81, 1H, H-6), 7.13–7.17 (d, *J* = 8.99, 1H, H-3′), 7.48–7.54, (m, 1H, H-5), 7.63–7.65 (d, *J* = 2.6, 1H, H-3), 7.78–7.83 (m, 1H, H-4′), 8.04–8.06 (d, *J* = 2.53, 1H, H-6′), 8.31–8.40 (m, 1H, ArCONHR), 10.19 (s, 1H, ArCONHAr), 10.58 (bs, 1H, H^+). ^{13}C NMR: 24.26 (CH_2), 25.17 (CH_2), 28.43 (CH_2), 36.31 ($CONHCH_2$), 41.95 ($(CH_3)_2N$), 54.37 (CH_2N), 56.43 (OCH_3), 68.76 (OCH_2), 113.26 (C-6), 114.13 (C-3′), 122.38 (C-3), 123.89 (C-2), 124.26 (C-5′), 124.58 (C-1′), 125.01 (C-6′), 127.42 (C-5), 131.81 (C-4), 139.72 (C-4′), 152.41

(C-1), 155.68 (C-2′), 163.02 (ArCONHR), 165.29 ArCONHAr). Anal. $C_{46}H_{62}N_8O_8 \cdot 4HCl$ (C, H, N, Cl).

*6.1.11. 1,6-Bis{4-(3-amino)benzamido-2-[(1,5-diaza-5-methylhexyl)carbonyl]-phenyloxy}hexane bis(dihydrochloride) (**11**)*

Yield 35%. M.p.: 182–184 °C. Rf = 0.25. ^{1}H NMR: 1.45–1.60 (m, 2H, CH_2), 1.88–1.92 (m, 4H, CH_2), 2.72–2.74 (d, J = 3.43, 6H, $(CH_3)_2NH^+$), 3.05–3.18 (m, 2H, $CONHCH_2$), 3.35–3.38 (m, 2H, CH_2N), 4.05–4.17 (m, 2H, OCH_2), 7.13–7.18 (d, J = 9.03, 1H, H-6), 7.33–7.37 (m, 1H, H-4′), 7.45–7.53 (m, 1H, H-5′), 7.70 (s, 1H, H-2′), 7.77–7.81 (m, 1H, H-6′), 7.88–7.92 (dd, J = 2.57, J = 8.91, 1H, H-5), 8.18–8.28 (d, J = 2.61, 1H, H-3), 8.29–8.34 (t, J = 5.58, 1H, ArCONHR), 10.41 (s, 1H, ArCONHAr), 10.84 (bs, 1H, H^+). ^{13}C NMR: 24.19 (CH_2), 25.28 (CH_2), 28.41 (CH_2), 36.32 ($CONHCH_2$), 41.94 ($(CH_3)_2N$), 54.35 (CH_2N), 68.72 (OCH_2), 113.28 (C-6), 119.89 (C-2′), 123.17 (C-6′), 123.42 (C-3), 123.47 (C-4′), 124.33 (C-5), 124.44 (C-2), 129.45 (C-5′), 132.06 (C-4), 135.96 (C-1′), 137.48 (C-3′), 152.45 (C-1), 164.64 (ArCONHR), 165.27 (ArCONHAr). Anal. $C_{44}H_{58}N_8O_6 \cdot 4HCl$ (C, H, N, Cl).

*6.1.12. 1,6-Bis{4-(4-amino)benzamido-2-[(1,5-diaza-5-methylhexyl)carbonyl]-phenyloxy}hexane bis(dihydrochloride) (**12**)*

Yield 40%. M.p.: 178–180 °C, Rf = 0.3. ^{1}H NMR: 1.41–1.59 (m, 2H, CH_2), 1.83–1.92 (m, 4H, 2 × CH_2), 2.72 (s, 6H, $(CH_3)_2N$), 3.06–3.19 (m, 2H, CH_2N), 3.35–3.40 (m, 2H, $CONHCH_2$), 4.05–4.20 (m, 2H, OCH_2), 7.11–7.15 (d, J = 8.6, 1H, H-6), 7.24–7.28 (d, J = 8.0, 2H, H-3′, H-5′), 7.87–7.91 (d, J = 8.7, 1H, H-5) 7.97–8.01 (d, J = 8.0, 2H, H-2′, H-6′), 8.11 (s, 1H, H-3), 8.22–8.26 (m, 1H, ArCONHR), 10.28 (s, 1H, ArCONHAr), 10.69 (bs, 1H, ArCONHAr).^{13}C NMR: 24.21 (CH_2), 25.12 (CH_2), 28.38 (CH_2), 36.29 ($CONHCH_2$), 41.91 ($(CH_3)_2N$), 54.34 (CH_2N), 68.71 (OCH_2), 113.21 (C-6), 120.01 (C-3), 122.66 (C-3′, C-5′), 123.20 (C-2), 124.25 (C-5), 129.16 (C-2′, C-6′), 130.18 (C-4), 132.23 (C-4′), 139.99 (C-1′), 152.27 (C-1), 164.46 (ArCONHR), 165.29 (ArCONHAr). Anal. $C_{44}H_{58}N_8O_6 \cdot 4HCl$ (C, H, N, Cl).

6.2. Pharmacology

6.2.1. Materials

Hoechst 33258, topoisomerase I, ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (USA). Topoisomerase II was purchased from Amersham Pharmacia Biotech. Stock cultures of breast cancer MCF-7 were purchased from the American Type Culture Collection, Rockville, MD. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in cell culture were products of Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (USA). [^{3}H]Thymidine (6.7 Ci/mmol) was the product of NEN (USA).

6.2.2. MCF-7 cell culture

Stock cultures of breast cancer MCF-7 cells were maintained in continuously exponential growth by weekly passage in DMEM supplemented with 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin at 37 °C in humid atmosphere containing 5% CO_2 in an incubator. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin, 0.02% EDTA in calcium-free phosphate-buffered saline. The study was carried out using cells from passages three to seven, growing as monolayer in 6-well plates (Nunc) (5×10^6 cells per well).

6.2.3. DNA synthesis assay

To examine the effect of studied compounds on cells proliferation MCF-7 cells were seeded in 6-well plates and grown as described above. Cells culture were incubated with varying concentrations of compounds **1–12**, Hoechst 33258 and 0.5 μC of [^{3}H]thymidine for 24 h at 37 °C. The cells were then harvested by trypsinization and washed (with cold phosphate-buffered saline) with centrifugation for 10 min at 1500 g several times (4–5) until the dpm in the washes were similar to the reagent control. Radioactivity was determined by liquid scintillation counting. [^{3}H]Thymidine uptake was expressed as dpm/well.

6.2.4. Cell viability assay

The assay was performed according to the method of Carmichael using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [11]. Confluent cells, cultured for 24 h with various concentrations of studied compounds in 6-well plates were washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37 °C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 mol/l HCI in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of breast cancer MCF-7 cells cultured in the presence of ligands was calculated as a percent of control cells.

6.2.5. Relaxation assay of topoisomerase I

PBR322 plasmid DNA (0.083 μg) was incubated with 1 unit of topoisomerase I in a standard reaction buffer (50 mM Tris–HCI (pH 7.9),1 mM EDTA, 0.5 M NaCI, 1 mM dithiothreitol) in the presence of the test compound at concentrations ranging from 10 to 100 μM. The mixture was incubated at 37 °C for 1 h and the reaction was terminated by addition of 2 μl of 10% SDS and 2 μl of proteinase K (1 mg/ml). The reaction mixture was subjected to electrophoresis through a 0.8% agarose gel containing 0.5 mg/ml ethidium bromide in TBE buffer (90 mM Tris–borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light using UVI-KS4000i gel documentation and analysis system (SyngenBiotech, San Carlos, CA, USA).

6.2.6. Relaxation assay of topoisomerase II

PBR322 plasmid DNA (0.083 μg) was incubated with 1 unit of topoisomerase II in a standard reaction buffer (10 mM Tris–HCI (pH 7.9), 1 mM ATP, 50 mM KCl, 5 mM $MgCI_2$, 50 mM NaCI, 0.1 mM EDTA, and 15 μg/ml bovine serum albumin) in the presence of the test compound at concentrations ranging from 10 to 100 μM. The mixture was incubated at 37 °C for 1 h and the reaction was terminated by addition of 2 μl of 10% SDS and 2 μl of proteinase K (1 mg/ml). The reaction mixture was subjected to electrophoresis through a 0.8% agarose gel containing 0.5 mg/ml ethidium bromide in TBE buffer (90 mM Tris–borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light using UVI-KS4000i gel documentation and analysis system (SyngenBiotech, San Carlos, CA, USA).

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