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Induction of Apoptosis by Aryl-Substituted Diamines: Role of Aromatic Group Substituents and Distance Between Nitrogens

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Abstract—A series of aromatic substituted diamines was synthesized and characterized for their cytotoxic profiles against human breast and prostate tumor cell lines. Following a structure function analysis of the effects of changes of the benzyl substituents and the distance between amino groups the most potent analogues were analyzed biologically and were shown to induce apoptosis. These compounds do not induce the enzyme SSAT or deplete intracellular polyamine levels, mechanisms demonstrated by other cytotoxic polyamine analogues. © 2002 Elsevier Science Ltd. All rights reserved.

Molecules that bind to nucleic acids have a wide variety of potent and unique biological activities.[1,2] Inhibition of transcription factor binding to the TATA box of a DNA sequence was shown by the zinc(II) complexes of a group of benzylated macrocyclic tetraamines including **1**.[3] Subsequent work by the same group has shown potent inhibition of HIV-1 TAR RNA–Tat peptide interactions by related compounds.[4] Recent reports have described the cytotoxic activities of a group of compounds related structurally by the presence of two lipophilic aromatic substituents separated by a variable length, flexible linker containing two cationic ammonium centers. Examples of the aromatic substituents include the acridines,[5,6] bis(9-methyl-phenazines),[7] anthracyclines,[8] indeno[1,2-*b*]quinolines[9] and bis (naphthalimides).[10,11] Two of these molecules, DMP 840 **2**[12] and LU 79553 **3**[11] have been reported to be undergoing clinical studies. Biochemical studies of the mechanism of action of **2** has implicated the involvement of major groove binding together with topoisomerase II poisoning.[13] Additionally, the antitumor activity of several un-substituted benzyl diamines such as dibenzylputrescine **4** has been previously reported (Fig. 1).[14,15] Due to our interest in the discovery of polyamine analogues with unique mechanisms of antitumor activities, we have synthesized a series diaminoalkanes substituted with various aromatic groups. This group of compounds was evaluated by their cytotoxic activities against human breast (MDA-MB-231) and prostate (PC-3) tumor cell lines. The more active analogues were then evaluated in detailed biological assays including the role played by

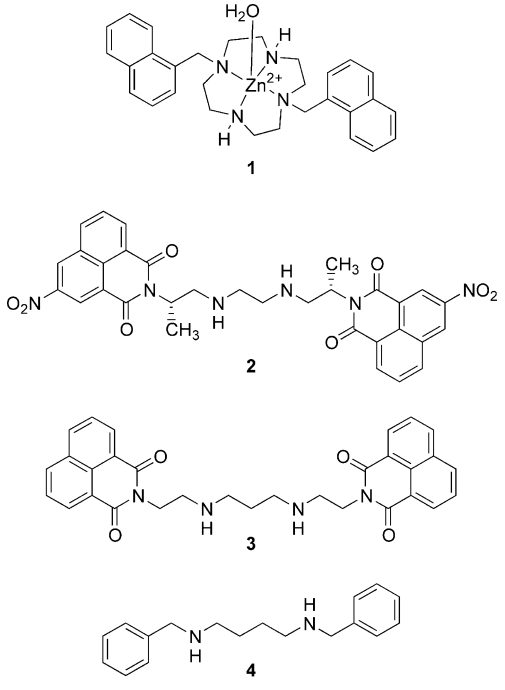

Figure 1. Aromatic-substituted diamines.

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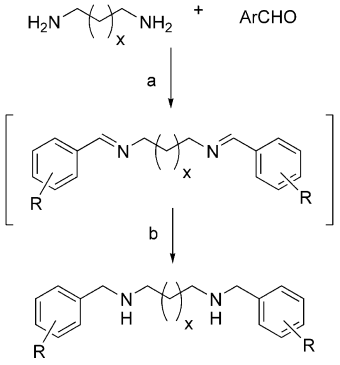
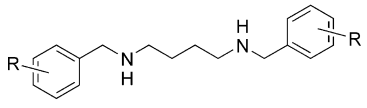
polyamine metabolism in their cytotoxic mechanism. The induction of apoptosis was demonstrated for several of the more potent analogues by staining with Annexin V/propidium iodide followed by flow cytometry analysis.

The synthesis of symmetrically disubstituted derivatives utilized the straightforward one-pot route shown in Scheme 1. Following formation of the diimine intermediate by stirring the diamine with excess aromatic aldehyde, removal of drying agent and solvent gave a product that was reduced by treatment with excess $NaBH_4$ in MeOH. Standard workup and purification over silica gel gave the analogues in pure form.[16] Other diamines that were used in place of 1,4-diaminobutane included 1,3-diaminopropane (for **34** and **40**), 1,5-diaminopentane (for **35** and **41**), 2,5-diaminohexane[17] (for **37**), *m*-xylylenediamine (for **38**) and *p*-xylylenediamine (for **39**).



Scheme 1. Synthesis of arylated diamines. Reagents and conditions: (a) ArCHO (2.2 equiv), Et_3N (2.1 equiv), $MgSO_4$, CH_2Cl_2; (b) $NaBH_4$, MeOH.

Table 1. Cytotoxicity assay results for substituted aromatic compounds (**3**, **4**, and **5**–**22**)

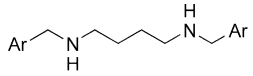


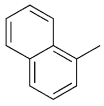
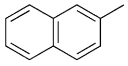
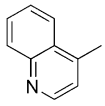
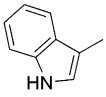
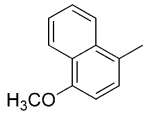
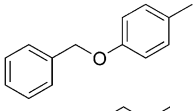
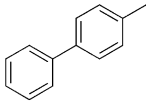
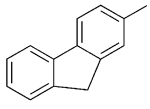
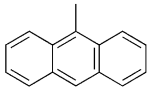
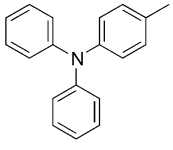
Analogue	R Group structure	MDA-MB-231 IC_{50} (μM)[a]	PC-3 IC_{50} (μM)[a]
3	LU 79553	0.06	0.07
4	H-	192	114
5[b]	(Mono)-H-	>300	211
6	2-NO_2-	62	23
7	3-NO_2-	18	9
8	4-NO_2-	205	143
9[c]	(Mono)-4-NO_2-	201	50
10	4-CH_3O-	21	6
11	3,4-Methylenedioxy-	70	56
12	3,4,5-Trimethoxy-	58	58
13	3-Cyano-	42	10
14	4-Cyano-	84	22
15	3-Azido-	19	15
16	2-NH_2-	146	98
17	3-NH_2-	>300	>300
18	4-$N(CH_3)_2$-	197	56
19	3,4-Dichloro-	65	20
20	2-PyridinylCH_2-	>300	>300
21	3-PyridinylCH_2-	>300	202
22	4-PyridinylCH_2-	577	580

[a]Values are from single experiments unless otherwise noted.
[b]Mono-aryl **5** had the structure $PhCH_2NHCH_2CH_2CH_2CH_2NH_2$.
[c]Mono-aryl **9** had the structure 4-NO_2–$ArCH_2NHCH_2CH_2CH_2CH_2NH_2$.

The monobenzylated analogues **5**, **9**, and **24** were produced by similar methods via use of N^1-Boc-1,4-diaminobutane[18] as the starting amine. The anilino derivatives

Table 2. Cytotoxicity assay results for polycyclic aromatic compounds **3**, **4** and **23**–**33**

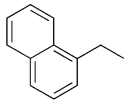
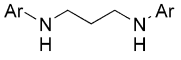
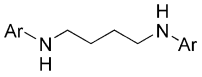
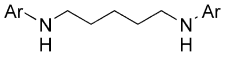
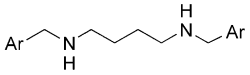
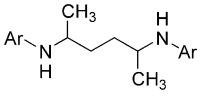
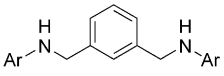
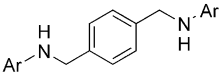


Analogue	Ar-	MDA-MB-231 IC_{50} (μM)[a]	PC-3 IC_{50} (μM)[a]
3	LU 79553	0.06	0.07
4	H-	192	114
23		9.1±2.4 (5)	10±3.0 (4)
24[b]	Mono-(1-naphthyl)-	>300	211
25		2.1	2.5
26		9.4	8.4
27		77	51
28		2.8±1.0 (4)	3.2±1.5 (4)
29		1.7 (2)	1.6 (2)
30		1.92±0.09 (3)	0.77±0.07 (3)
31		6.6±4.9 (3)	1.85±0.11 (3)
32		3.5 (2)	2.7 (2)
33		0.62±0.17 (4)	0.60±0.18 (3)

[a]Values are from single experiments unless otherwise noted by the number of repeats in parentheses. If $n=2$ average is given, if $n>2$ then ±SD is given.
[b]Mono-aryl **24** had the structure 1-napthyl$CH_2NHCH_2CH_2$-$CH_2CH_2NH_2$.

16 and **17** were produced by selective Zn/HCl reduction of the nitrobenzyl analogues **6** and **7**. The 3-azidobenzyl analogue **15** was produced from **17** using standard conditions ($NaNO_2$, 6 N HCl, NaOAc, NaN_3).[19] Analogues were analyzed by TLC and ^1H NMR. Selected analogues were further analyzed by ^{13}C NMR, HPLC and HRMS methods. All results were consistent with the proposed structures. Several compounds have been previously reported.[20a–e]

Table 3. Cytotoxicity assay results for diamine variations of analogue **23**

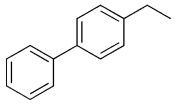
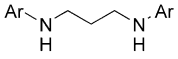
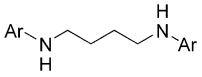
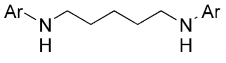
Analogue	Diamine	MDA-MB-231 IC_{50} (μM)[a]	PC-3 IC_{50} (μM)[a]
	Ar = 		
34		5.1	4.3
23		9.1 ± 2.4 (5)	10 ± 3.0 (4)
35		8.3	6.5
36[b]		25	37
37		5.0	7.0
38		19	20
39		7.0 ± 1.0 (3)	14 ± 7.0 (3)

[a]Values are from single experiments unless otherwise noted by the number of repeats in parentheses. If $n = 2$ average is given, if $n > 2$ then ± SD is given.
[b]Structure for **36** is (1-naphthyl$CH_2CH_2NHCH_2CH_2)_2$.

As shown in Table 1, the substituted benzylated diamines were evaluated by their ability to inhibit the growth of MDA-MB-231 (breast) and PC-3 (prostate) human cancer cell lines.[21] In comparison to the un-substituted benzyl analogue **4**, electron-withdrawing groups in the *meta*-position such as the 3-NO_2- analogue **7** and the 3-cyano derivative **13** had 10- and 4-fold higher activities, respectively. *para*-Substitution with the same groups gave much lower activities (compare analogues **8** and **14**). On the other hand, *para*-substitution with the electron-donating group CH_3O- gave an analogue **10** possessing 9-fold higher activity. Evaluation of the polycyclic aromatic substituted diamines shown in Table 2 showed that even greater increases in activities were possible.

The 1- and 2-substituted naphthalene derivatives **23** and **25** showed dramatic 21- and 90-fold increases in potency when compared to **4**. It is interesting to note that the mono-substituted 1-naphthyl analogue **24** showed a precipitous loss of activity. The quinoline analogue **26** gave an activity equivalent to **23** that, when compared to the 4-pyridinyl analogue **22**, again shows the significance of the polycyclic structure. Various

Table 4. Cytotoxicity assay results for diamine variations of analogue **30**

Analogue	Diamine structure	MDA-MB-231 IC_{50} (μM)[a]	PC-3 IC_{50} (μM)[a]
	Ar Structure = 		
40		2.0	0.80
30		1.92 ± 0.09 (3)	0.77 ± 0.07 (3)
41		1.3	0.48

[a]Values are from single experiments except for **30** where ± SD is given.

Table 5. Cytotoxic activities of **23**, **30**, and **33** against multiple cell types

Cell line	Tumor type	IC_{50} values (μM)[a]		
		23	**30**	**33**
MDA-231	Breast	9.1 ± 2.4 (5)	1.92 ± 0.09 (3)	0.62 ± 0.17 (4)
PC-3	Prostate	10 ± 3 (4)	0.77 ± 0.07 (3)	0.60 ± 0.18 (3)
SK-OV-3	Ovarian	8.5 (2)	0.74 (2)	0.68 (2)
A375	Melanoma	5.4 ± 0.54 (4)	0.59 ± 0.08 (3)	0.40
SK-Mel-5	Melanoma	4.6	—	0.94
T47.D	Breast	6.5	—	0.65
Mes-SA	Uterine	10.1	—	0.64
Mes-SA/Dx5	MDR Uterine	7.2	—	0.61

[a]Values are from single experiments unless otherwise noted by the number of repeats in parentheses. If $n = 2$ average is given, if $n > 2$ then ± SD is given.

other readily available substitutions on the aromatic aldehyde starting material including 4-methoxynaphthylene **28**, 4-benzyloxyphenyl **29**, 4-biphenyl **30**, 2-fluorenyl **31**, 9-anthracene **32**, and the very potent 4-diphenylamine **33** together supported the favorable activity gained through the presence of a polycyclic aromatic ring system.

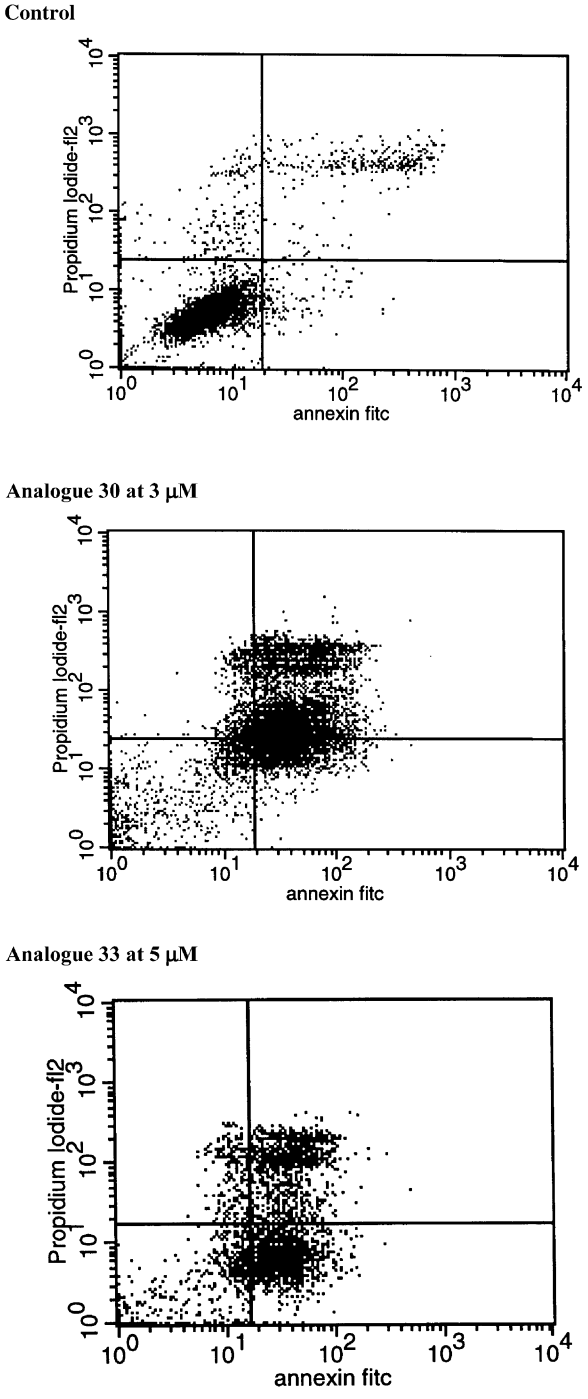


Figure 2. Annexin/propidium iodide assays were performed using A375 melanoma cells following 3 days of drug treatment. Apoptotic cell death was assessed by annexin V-fluorescein isothiocyanate binding and propidium iodide co-staining according the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Analysis by flow cytometry used a Becton Dickinson FACScan analyzer.

Following the demonstration of the improvement in activity shown by the polycyclic aromatics in comparison to the benzyl analogues, the effect of changes in the diamine structure was explored. As shown by analogues **34**, **23**, and **35** in Table 3, variation in the length of the aliphatic chain linker had minor effect on the activities. Compound **36**, with an extra methylene between the naphthalene group and the amine had a reduced activity in comparison with **23**. Addition of methyl groups on the α-carbon of the diamine gave an analogue **37** with slightly better activity when compared to **23**. Substitution of either a 3- or 4-xylylenediamine (**38** and **39**) resulted in analogues with activities similar to **23**. These data show that the structure of the linker between the nitrogen atoms has a minor role in activity.

Table 4 shows the lack of effect from alteration in the distance between the amino groups of analogue **30** had on its cytotoxic activity. Both the three and five methylene group containing analogues **40** and **41** gave similar results to those shown by **30**.

We next explored the effects of several of the most active analogues on a variety of human cancer cell lines. These results are shown in Table 5. Noteworthy data include the retention of the effects of analogues **23** and **33** on both the wild-type (Mes-SA) and multidrug resistant (Mes-SA/Dx5) uterine carcinoma cell types.

Results from an Annexin V/propidium iodide fluorescent cell-staining assay[22] showed a significant population of **30** and **33** treated A375 melanoma cells were undergoing apoptosis following a 3-day treatment with drug. Lesser amounts of **23** treated cells were induced to undergo an apoptotic cell death cascade. As shown by two representative examples in Figure 2, treatment with **30** or **33** at 3 and 5 μM concentrations, respectively, resulted in the appearance of 61 and 77% of cells with apoptotic characteristics.

We have explored the mechanism of action of the most active analogues based on their structural similarity to putrescine. We have tested the ability of **23** and **33** to influence the levels of the natural polyamines in treated MDA-MB-231 cells and saw no changes in the levels of putrescine, spermidine, or spermine (data not shown). Compound **30** induced a slight 9 ± 5-fold increase in the level of SSAT activity in SK-MEL-28 cells.[23] A Co-incubation with a large excess of putrescine (50-fold higher than drug concentration) had no effect on the cytotoxicity of **23** or **33** on MDA-MB-231 cells. Co-incubation with the polyamine biosynthesis inhibitor α-difluoromethylornithine (DFMO) had no effect on the IC_{50} values of these two compounds. Based on these observations we have concluded that these compounds act via a mechanism(s) distinct from those determined for other cytotoxic polyamine analogues.[24]

An alternative hypothesis is that these analogues may induce their biological effects through interactions with DNA. The presence of two ammonium centers in these molecules may enable their targeting to the anionic phosphodiester backbone of nucleic acids, followed by

the aromatic portion of the molecules increasing their affinity through either a hydrophobic or intercalative mechanism. Initial indications of the drug's ability to interact with nucleic acids were assessed by a DNA/ethidium bromide dye displacement assay.[25] Results supported the moderate ability of these molecules to interact with DNA. A DC_{50} of 8.7, 8.7 and 9.4 μM for polydAdT were found for **23**, **30**, and **33**, respectively. Distamycin A gave a DC_{50} value of 44 μM using the same conditions.

In conclusion, we have obtained nearly a 300-fold improvement in the cytotoxic properties of a series of compounds through optimization of the aromatic constituent attached to a central diamine core of the molecule. Large polycyclic aromatic substitution led to the working hypothesis that these molecules may be interacting with nucleic acids via a combination of ionic and either hydrophobic or intercalating interactions. The observed ability of several of these compounds to induce apoptosis provided an alternative suggestion that interaction with apoptosis pathway components may be playing a role in their cytotoxic activities.[26]

References and Notes

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16. The synthesis of **23** is given as a representative example. All reagents were used as received from Aldrich in their purest and driest forms available. The mixture produced by addition of 2.38 g (27 mmol) of 1,4-diaminobutane, 9.37 g (60 mmol, 2.2 equiv) of 1-naphthaldehyde, 8.0 mL (57.4 mmol, 2.1 equiv) of Et_3N and 6.0 g of anhyd $MgSO_4$ in 100 mL of CH_2Cl_2 was stirred at 25 °C for 2 h. Filtration and evaporation gave a yellow oil as the crude diimine. This was suspended in 300 mL of CH_3OH and 5.46 g (144 mmol, 5.3 equiv) of solid $NaBH_4$ was carefully added portion-wise. The resulting solution was stirred at 25 °C for 20 h when the reaction was quenched by the careful addition of 6 N HCl until strongly acidic (~35 mL). The resulting mixture was diluted by the addition of 300 mL of H_2O and 300 mL CH_2Cl_2. The aqueous layer was made basic by the addition of excess 4 N NaOH. Two additional washes of the basic aqueous layer with equal portions of CH_2Cl_2 were performed. The combined organic layers were washed with brine, dried and evaporated to give 10.38 g yellow oil as the crude product. Column chromatography using $CHCl_3$/^{i}PrOH/concd NH_4OH 88:10:2 gave 8.42 g (84%) white foam. The analytical sample was crystallized from EtOH. ^1H NMR $CHCl_3$, ppm from internal $Si(CH_3)_4$ δ 8.22 (d, 2H), 7.96 (d, 2H), 7.84 (d, 2H), 7.55 (m, 8H), 4.32 (s, 4H), 2.84 (m, 4H), 1.71 (m, 4H), 1.42 (br s, 2H); ^{13}C NMR ($CHCl_3$, ppm from residual solvent signal): δ 135.9, 133.6, 131.6, 128.5, 127.5, 125.9, 125.7, 125.4, 125.2, 123.4, 51.6, 49.8, 27.9. HRMS by MALDI-FTMS: calcd for $C_{26}H_{28}N_2Na^+$ 391.2150. Obsd: 391.2155. Samples were analyzed for purity by HPLC over a C-18 column using a 40 min gradient of 5–100% CH_3CN in H_2O both containing 0.1% TFA.
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21. Cells were plated in 96-well plates in the respective media (MDA-MB-231 and A375 used DMEM while PC-3 used F12K. SK-OV-3, Mes-SA and Mes-SA/Dx5 cells were cultured in McCoy's 5A media. SK-Mel-5 cells used EMEM while T47.D cells used RPMI 1640 media. All media contained 1 mM aminoguanidine, 50 U/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum. A375 and T47.D used 1 mM sodium pyruvate while A375, T47.D and MDA-MB-231 had 2 mM L-glutamine. Additionally, T47.D cells had 0.4% insulin). After 24 h, drug was added and cells were allowed to grow for an additional 72 h. Cell number was determined by MTS assay used as described by manufacturer (Promega).
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