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Long-Chain Aminoalcohol and Diamine Derivatives Induce Apoptosis through a Caspase-3 Dependent Pathway

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Abstract—A number of long chain diamines and aminoalcohols and several of their alkyl, acyl and carbamoyl derivatives, have been synthesized and evaluated for their apoptotic activities using the Jurkat cell line. Apoptosis was measured by flow cytometry and the best results were found for the aminoalcohols displaying either a free alcohol or an amine with at least, one free hydrogen atom. The apoptotic pathway was mediated by a disruption of the mitochondria transmembrane potential and caspase-3 activation, inducing DNA fragmentation at the phase G_1/S of the cell cycle. © 2002 Elsevier Science Ltd. All rights reserved.

Introduction

Apoptosis, or programmed cell death (PCD), is a natural form of cell death controlled by a constitutively expressed machinery that induces condensation of nucleoplasm and cytoplasm, blebbing of cytoplasmic membranes and fragmentation of the cell into 'apoptotic bodies', which are rapidly recognized and eliminated by adjacent cells.[1] According to current understanding, morphological and biochemical alterations in nuclear and chromatin structures of cells undergoing apoptosis are controlled mainly by the mitochondria.[2] Thus, a breakdown of the mitochondrial transmembrane potential ($\Delta\Psi_m$) is an invariant feature of early apoptosis that precedes DNA strandbreak, regardless the cell type and the apoptotic stimuli.[3] $\Delta\Psi_m$ disrupture mediated the release of cytochrome-c and the (so-called) apoptotic inducing factor (AIF), which can mediate nuclear fragmentation.[4] The release of cytochrome-c (cyt-c) into the cytoplasm results in formation of the apoptosome, leading to activation of final executor caspases, such as caspase-3, responsible for the morphological and biochemical features of apoptosis, through the activation of multiple cytoplasmic and nuclear enzymes.[5] After the initial $\Delta\Psi_m$ dissipation, cells hyperproduce reactive oxygen species (ROS) which may contribute to the cell death process. It has been shown that these ROS are derived from the complex III of the mitochondrial respiratory chain.[6] Although less clear, there are also other ways of apoptosis described, that did not imply the mitochondria directly, for example via granzime B or the direct activation of signaling caspases, such as caspase-8, that leads to the direct activation of executor caspases without the requirement of cyt-c release and apoptosome formation, even though a $\Delta\Psi_m$ breakdown occurs secondarily.[7] Also, in some cases, ROS production can precede and cause the $\Delta\Psi_m$ breakdown and the later cell degradation. These ROS are mainly of extramitochondrial origin.[8]

Compounds inducing apoptosis in cancer cells, constitute a promising way to develop new anticancer drugs. We can find several biological activities reported in the literature for lipidic aminoalcohols an diamines. Among them, their anti-inflammatory activity,[9] their cytotoxicity for neoplastic cells[10] and their binding to DNA in concentration levels similar to that shown by doxorubicin,[11] are the most relevant aspects in relation with the research described in this paper, which attempts to establish the mechanism of cytotoxicity for this type of compounds. Thus, we now report the results of our studies on the apoptosis induced by several long chain aminoalcohol and diamine derivatives in tumoral cells. The apoptotic pathway induced by the compounds

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assayed is mediated in all the cases through the disruption of the mitochondrial permeability transition and the activation of caspase-3.

Chemistry

The synthetic pathways are summarized in Schemes 1 and 2 and the compounds prepared are shown in Tables 1–3. Details of the reactions, experimental conditions and characterization of the products, will be reported in a complete publication. As a representative preparation, Boc-aminoacid **1**, prepared from diethyl 2-acetamidomalonate and 1-bromotetradecane,[12] was transformed into a mixed anhydride, and reduced by $NaBH_4$ to give Boc-aminoalcohol[13] **2c** ($R^1 = R^3 = H$, $R^2 = Boc$). The alcohol group was protected as benzyl ether (**2b**), and the amine properly functionalized to give secondary amines. Treatment with 1.2 equivalents of ethyl bromide, *n*-hexyl bromide or ethyl bromoacetate yielded compounds **2f**, **2h** and **2l** respectively, and with one mole of glutaric or succinic anhydrides provided compounds **2n** and **2o**, respectively. The tertiary amine derivative **2q** was obtained by treatment with 2.2 equivalents of the alkylating reagent. Elimination of the benzyl group by hydrogenolysis gave the free alcohols **2a**, **2e**, **2g**, **2k**, **2m** and **2p**. Compound **2l**, by saponification with 10% KOH/MeOH, gave the free acid **2j** and its debenzylation led to the unprotected aminoalcohol **2i**.

Diamines **3** were prepared from the aminoalcohol **2c** by mesylation, substitution with sodium azide and reduction with $NaBH_4$ in the presence of Pd/C, to give the Boc-diamine **3b**,[14] which through alkylation/acylation as above indicated, followed (or not) by Boc-deprotection, gave the diamines and diamine derivatives **3**. Acylation of compound **2c** with heptanoyl chloride or palmitoyl chloride provided the corresponding ester derivatives, which after Boc deprotection and new acylation gave compounds **2r**, **2s** and **2t**.

Aminoalcohols **4**, were obtained from 1-hexadecene by epoxidation with *m*-chloroperbenzoic acid and subsequent opening of the oxirane ring by ammonium hydroxide, or cyclohexyl or benzylamines.

Biological Assays

Jurkat cells (T-lymphoma cells) were used for all biological determinations.[15] The percentage of cells undergoing apoptosis was determined by flow cytofluorimetric analysis of the cell cycle.[16]

To evaluate $\Delta\psi_m$ and ROS generation cells were incubated with 20 nM $DiOC_6(3)$,[17] which accumulates in the mitochondria as a function of its potential and 2 μM dihydroethidine (HE), fluorescent after oxidation followed by cytofluorimetric analysis.

To determine both DNA strand breaks (apoptosis) and cell cycle, cells were subjected to paraformaldehyde fixation and TUNEL staining, followed by counterstain with propidium iodide (PI) and a treatment with RNAse as described.[18] Caspase-3 activity was studied by incubating 30 μg of cytoplasmic proteins from cell lysates with 15 μg of the specific caspase-3 substrate Ac-DEVD-AMC, following the manufacturer's indications.[19]

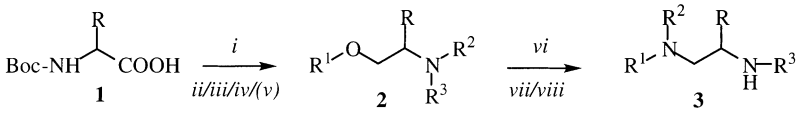
Results and Discussion

In preliminary experiments, using combined cytofluorimetric techniques to discriminate both necrosis and apoptosis (data not shown), we determined that cell death induced in Jurkat cells by the compounds tested was due to apoptosis, in the range of the concentrations studied.

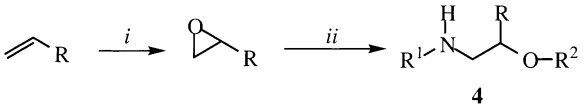
To study structure–activity relationships for our compounds, the apoptosis induced by them in this cell line after 24 h of stimulation was measured by cell cycle analysis after PI staining of DNA in untreated or treated cells. The percentage of hypodiploidy was used as indicator of cell death by apoptosis (cells with fragmented DNA). The results are indicated in Tables 1–3 as IC_{50} values, calculated from experiments with increased concentrations of the compounds from 0.1 to 100 μM.

It can be seen from data in Table 1, that all three types of compounds, primary (**2**) and secondary (**4**) aminoalcohols and ethylenediamines (**3**), contain representative substances inducing apoptosis.

Within the series of primary aminoalcohols **2**, it can be noted, that the acylation of the amino group (**2a**, **2b**/**2m**, **2n**) and the diacylation of both the amino and the alcohol functions (**2r**, **2s**, **2t**) either decreased or abolished the apoptotic activity, respectively. On the other hand, monoalkylation of the amino group left the potency practically unchanged (**2a**/**2e**), while dialkylation (**2a**/**2p**) led to the most potent compound of this series. Related to the benzylation of the hydroxyl group, it influences differently the apoptotic capacity of these compounds depending on the degree of substitution of the amine. A small increase of the potency or no influence is observed

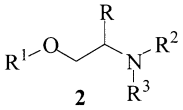


Scheme 1. Synthesis of lipidic aminoalcohols and diamines and their derivatives (**2** and **3**). $R = (CH_2)_{13}CH_3$; (i) (a) EtOOCCl/*N*-methylmorpholine/THF; (b) $NaBH_4$/MeOH; (c) BnCl/NaH/DMF; (d) HCl/THF/argon; (ii) EtBr (1.1 or 2.2 equiv) or $EtOOCCH_2Br$ (1.1 equiv)/Et_3N/DMF; (iii) glutaric anhydride/EtOAc; (iv) H_2/Pd/C/AcOH; (v) 10% KOH/MeOH; (vi) (a) MsCl/Et_3N/CH_2Cl_2; (b) NaN_3/DMF; (c) $NaBH_4$/MeOH and Pd/C/AcOH; (vii) EtBr (1.1 or 2.2 equiv) or $EtOOCCH_2Br$ (1.1 or 2.2 equiv)/Et_3N/DMF; (viii) *n*-$C_6H_{13}COCl$ or *n*-$C_{15}H_{31}COCl$/Et_2O.



Scheme 2. Synthesis of lipidic aminoalcohols **4**: R = $(CH_2)_{13}CH_3$; (i) *m*-CPBA/CH_2Cl_2/$NaHCO_3$; (ii) H_2N-R^1.

Table 1. Structures of aminoalcohols **2** and their apoptosis induction values



2

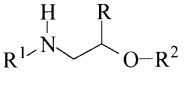
2	R^1	R^2	R^3	IC_{50} μM
a	H	H	H	7
b	Bn	H	H	3
c	H	Boc	H	28
d	Bn	Boc	H	nt
e	H	Et	H	6
f	Bn	Et	H	6
g	H	Hex	H	6
h	Bn	Hex	H	nt
i	H	CH_2COOH	H	nt
j	Bn	CH_2COOH	H	3
k	H	CH_2COOEt	H	nt
l	Bn	CH_2COOEt	H	nt
m	H	$CO(CH_2)_3COOH$	H	24
n	Bn	$CO(CH_2)_3COOH$	H	>50
o	Bn	$CO(CH_2)_2COOH$	H	30
p	H	Et	Et	1
q	Bn	Et	Et	20
r	$C_6H_{13}CO$	$C_6H_{13}CO$	H	na
s	$C_6H_{13}CO$	$C_{14}H_{29}CO$	H	na
t	$C_{14}H_{29}CO$	$C_{14}H_{29}CO$	H	na

R = –$(CH_2)_{13}CH_3$; nt = not tested; na = not active.

in the cases of the free amine (**2a**/**2b**) or monoalkylamine (**2e**/**2f**), respectively, but a 20-fold decrease is provoked in the case of dialkylamine (**2p**/**2q**). These results mean that, at least, one of the three labile exchangeable hydrogen atoms of the molecule should be retained to maintain the apoptotic activity.

In the cases of secondary aminoalcohols **4** and diamines **3** (Tables 2 and 3) the number of structural variations covered are fairly lesser than those that could be possible by changing the substituents on the two heteroatoms, thus a complete structure–activity analysis cannot be performed. Nevertheless, both types of compounds seem to display levels of activity similar to those of primary aminoalcohols and also to share a similar behaviour.

Table 2. Structures of aminoalcohols **4** and their apoptosis induction values



4

4	R^1	R^2	IC_{50} μM
a	H	H	nt
b	c-C_6H_{11}	H	6
c	Bn	H	27

R = –$(CH_2)_{13}CH_3$; nt, not tested; na, not active.

The trisubstituted diamines are practically inactive (**3m**, **3q**) and the most potent apoptotic compounds contain one (**3b**) or two (**3d**, **3k**) substituents on the nitrogen atoms. Interestingly and, in contrast with the case of aminoalcohols, carbamoylation (acylation) of the amino group attached to a secondary carbon atom in the aliphatic chain, seems to enhance (**3a**/**3b**) rather than decrease (**2a**/**2c**) or remove (**2r**, **2s**, **2t**) the activity.

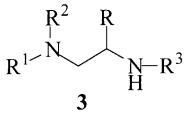
To investigate the apoptotic pathway induced by these compounds, we selected for further studies one representative compound from each group, namely the aminoalcohols **2a** and **4b** and the diamine **3a**. Jurkat cells were treated with increasing concentrations of these compounds for 24 h and the percentage of subdiploid cells (DNA fragmented cells) determined by PI staining. Values are means ± SE of three independent experiments (Fig. 1).

Next, we investigated the possible role of the mitochondria in the apoptotic pathway induced by these compounds. Jurkat cells were treated for 6 h with a dose of each compound that represents the IC_{50} for apoptosis induction, and the loss of $\Delta\psi_m$ detected by flow cytometry. We show in Figure 2A a representative experiment carried out with compound **2a**, inducing a clear loss in the mitochondrial transmembrane potential (53% of the cells) compared to untreated cells (<1%). Interestingly, 22% of the cells having low $DiOC_6(3)$ staining, presented also a significant increase in the amounts of intracellular ROS.

Taking into account that the dissipation of mitochondria transmembrane potential precedes the DNA fragmentation (data not shown), we could assume that the mitochondria plays a key role in the apoptotic pathway induced by aminoalcohols and diamines. Thus, pretreatment of Jurkat cells with rotenone, a good inhibitor of the complex I of the mitochondrial respiratory chain,[22] clearly reduced the percentage of $(HE\rightarrow Eth)^{high}$ cells, showing that the ROS generation induced by this compounds are from a mitochondrial source (Fig. 2A). These results are in agreement with observations indicating that, in most cases of apoptosis, ROS are originated at complex III of the mitochondria.[6] It seems likely that the mechanism of the pre-apoptotic $\Delta\Psi_m$ dissipation is mediated by so-called 'permeability transition (PT) pores', a step that can be transiently blocked by Cyclosporin A (CsA), which binds to cyclophillin-D present in the mitochondria pores and abrogates the PT opening mediated by some apoptotic stimuli.[23] This holds true also for the $\Delta\Psi_m$ breakdown induced by the compound **2a**, since, as it is shown in Figure 2A, pretreatment of Jurkat cells with CsA prevented the aminoalcohol-induced $\Delta\Psi_m$ disruption.

The caspase family consists of postaspartate-cleaving cysteine proteases that have been shown to be required for apoptosis in a number of experimental systems. The loss of $\Delta\Psi_m$ is followed by the release of cyt-c from the mitochondria, formation of the apoptosome and activation of the executor caspase-3. To study the activation of this caspase in a direct way, we incubated the

Table 3. Structures of diamines **3** and their apoptosis induction values



3

3	R^1	R^2	R^3	IC_{50} μM
a	H	H	H	55
b	H	H	Boc	6
c	H	Et	H	nt
d	H	Et	Boc	6
e	H	CH_2COOH	H	31
f	H	CH_2COOH	Boc	nt
g	H	CH_2COOEt	H	na
h	H	CH_2COOEt	Boc	28
i	H	$CH_2(CH_2)_3COOH$	H	55
j	H	$CH_2(CH_2)_3COOH$	Boc	18
k	Et	Et	H	7
l	Et	Et	Boc	21
m	Et	Et	$CH_2(CH_2)_3COOH$	na
n	CH_2COOH	CH_2COOH	H	nt
o	CH_2COOH	CH_2COOH	Boc	nt
p	CH_2COOEt	CH_2COOEt	H	32
q	CH_2COOEt	CH_2COOEt	Boc	>60

R = $-(CH_2)_{13}CH_3$; nt, not tested; na, not active.

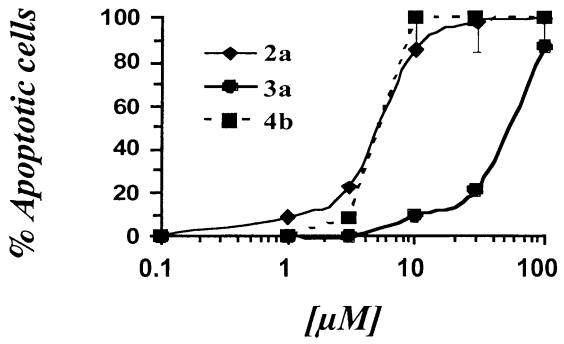


Figure 1. Induction of apoptosis by aminoalcohols **2a** and **3a** and diamine **4b**.

cells with the representative compounds **2a**, **3a** and **4b** for 6 h, a time at which the $\Delta\Psi_m$ dissipation is evident (Fig. 2A), and the activity of caspase-3 was determined by a highly specific and sensitive fluorometric method. Fig. 2B shows that the treatment of Jurkat cells with these compounds led to the activation of this caspase in a specific manner, since this activation was inhibited by the caspase-3 specific inhibitor zDEVD.fmk as it is shown for compound **2a**.

Finally, to examine whether the aminoalcohol-induced apoptosis in Jurkat cells is dependent on a specific phase of the cell cycle, we performed double staining experiments with PI and FITC-dUTP as described above. With this technique the phase of the cell cycle when

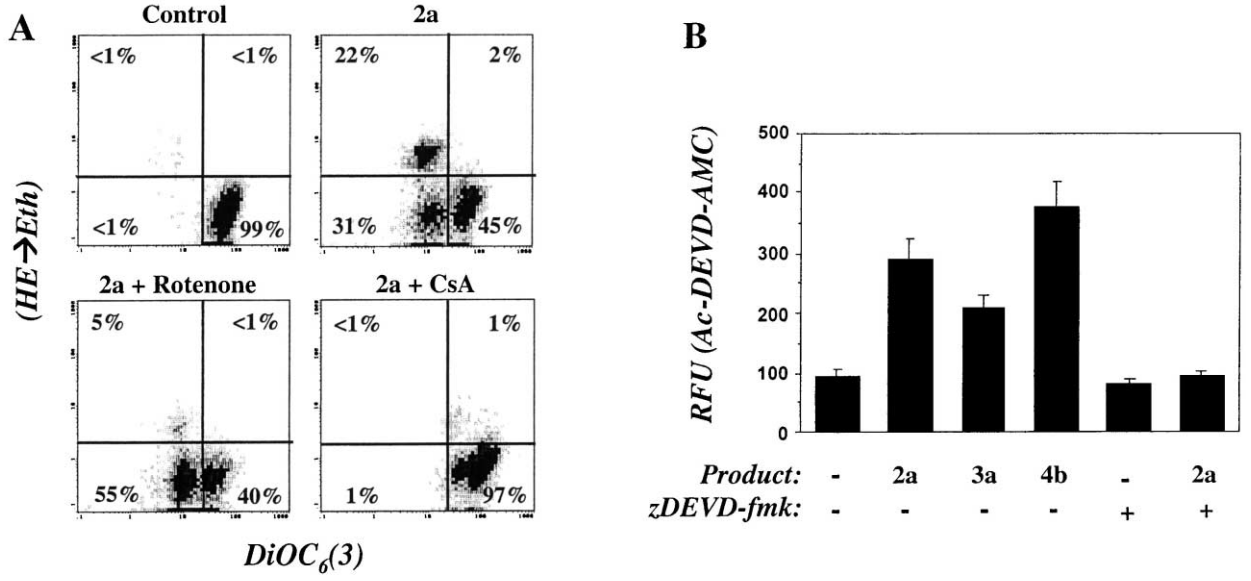


Figure 2. (A) Aminoalcohol **2a** induce $\Delta\Psi_m$ disruption and ROS generation in Jurkat cells.[20] (B) Aminoalcohols and diamines activate caspase-3 in Jurkat cells.[21]

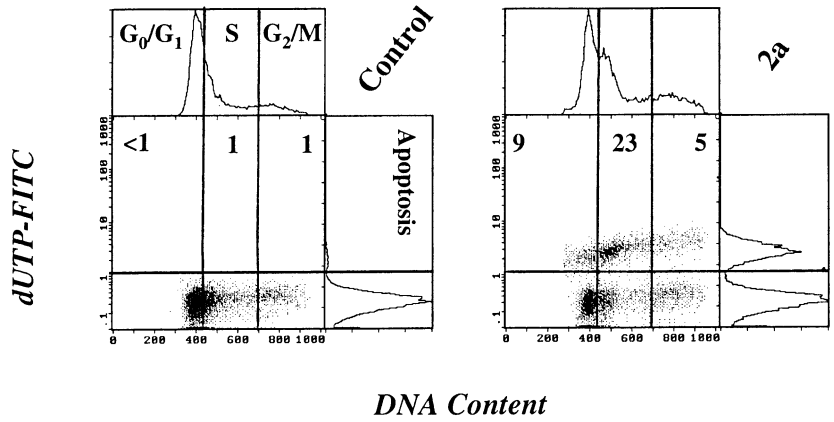


Figure 3. Cell cycle dependency for apoptosis induced by aminoalcohol **2a** (10 μM) on Jurkat cells.[24]

DNA fragmentation occurs can be established. Figure 3 shows that in Jurkat cells treated for 24 h with compound **2a**, most of the DNA fragmentation occurs in the transition of the G_1/S phase of the cell cycle. Thus, apoptosis induced by this compound will be primarily directed to growing cells, a fact which converts these type of structures into interesting lead compounds for the development of new anticancer drugs.

Acknowledgements

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15. Otherwise indicated, all reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Flow cytofluorimetric analyses were carried out on an Epics XL Analyzer (Coulter, Hialeah, FL, USA).
16. For that, ethanol fixed cells were subjected to RNA digestion and PI staining. With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining allows the determination of the percentage of subdiploid cells (sub-G_0/G_1 fraction).
17. 3,3′-Dihexyloxacarbocyanine iodide, (Molecular Probes, Eugene, OR, USA).
18. In this method, fixation in formaldehyde prevents extraction of low molecular weight DNA from apoptotic cells and thus the cell cycle distribution estimates both apoptotic and non-apoptotic cells.
19. The AMC liberated was measured using a spectrofluorometer (Hitachi F-2500 model, Hitachi Ltd., Tokyo, Japan) with an excitation wavelength of 380 nm and an emission

wavelength range of 400–550 nm. Data were collected as the integer of relative fluorescence intensity minus the background fluorescence. As a control, the cell-permeable caspase-3 inhibitor zDEVD-fmk (Bachem, Bubendorf, Switzerland) was used.
20. Cells were pretreated or not for 1 h with either 10 μM cyclosporin A (CsA) or 100 μM rotenone, followed by incubation with 10 μM of compound **2a** for 6 h, and then stained with the potential sensitive dye $DiOC_6(3)$ and the ROS-oxidable probe HE. Results are shown as the percentage of cells obtained in biparametric histograms delimited by four compartments, namely, $\Delta\Psi_m^{high}$ (normal cells, bottom-right compartment); $\Delta\Psi_m^{low}$ (bottom-left); $(HE->Eth)^{high}$ (ROS generating cells, top-right), and $(HE->Eth)^{high}/\Delta\Psi_m^{hlow}$ (pre-apoptotic cells, top-left compartment). Results are representative of five independent experiments.
21. Cells were treated with the IC_{50} apoptotic concentrations of compounds **2a**, **3a**, and **4b** for 6 h, lysed and 30 μg of protein from cell extracts were incubated with the specific caspase-3 substrate Ac-DEVD-AMC for 1 h at 37 °C. Results represent the integer of relative fluorescence intensity from the AMC liberated from reaction minus the background fluorescence. The activation of caspase-3 by compounds **2a** was inhibited in the presence of zDEVD-fmk (20 μM). Values are means ± SE of three independent experiments.
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24. Jurkat cell stimulated by compound **2a** (10 μM) for 24 h, then cell cycle: *X*-axis, G_0/G_1, S and G_2/M phases separated by vertical lines and the DNA strand breaks (*Y*-axis) analysed by flow cytometry. Results are representative of three different experiments.