New Antitumor Imidazo[2,1-*b*]thiazole Guanylhydrazones and Analogues[1]

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The synthesis of new antitumor 6-substituted imidazothiazole guanylhydrazones is described. Moreover, a series of compounds with a different basic chain at the 5 position were prepared. Finally, the replacement of the thiazole ring in the imidazothiazole system was also considered. All the new compounds prepared were submitted to the NCI cell line screen for evaluation of their antitumor activity. A few selected compounds were submitted to additional biological studies concerning effects on the cell cycle, apoptosis, and mitochondria.

Introduction

Methyl-gag (Mitoguazone) is still present in the "Cumulative List of All Orphan Designated Products" published by the FDA in October 2007. It is approved only for the treatment of diffuse non-Hodgkin's lymphoma, including AIDS-related diffuse non-Hodgkin's lymphoma. We believe that the search for new antitumor guanylhydrazones could lead to safer drugs possibly endowed with a broader range of activity. In our last paper in this field[2] and in the previous ones, the starting materials were mainly imidazothiazoles with substituents at the positions 2, 3, and 6. In this paper, we describe the search for new leads with the following rationale:

(1) Additional 6-substituted imidazothiazole guanylhydrazones. We took into consideration the thiophene ring (and its dimethyl and dichloro derivatives) connected to the 6 position of the imidazothiazole system which may be unsubstituted (**18**, **19 Aa**-**Da**, see Scheme 1) or may bear a methyl group at the 2 position (**20 Aa**-**Da**), thus being the same system present in one of the most interesting derivatives of this series, the guanylhydrazone of 6-(4-chloro-3-nitrophenyl)-2-methylimidazo[2,1-*b*]thiazole.[3]

Two additional substituents for the 6 position have been considered: the 3,5-dimethoxyphenyl group (**19Fa**) and the carboxyethyl group (**21Ea**).

(2) Variations in the basic chain at the 5 position. These have been performed in the imidazothiazoles bearing, at the 6 position, the most favorable substituents: 4-chloro-3-nitrophenyl[3] and 2,5-dimethoxy-4(or 6)-nitrophenyl.[4] In detail, while maintaining chlorine at the 2 position and the 2,5-dimethoxy-4-nitrophenyl group at the 6 position, the linear basic chain was replaced by a chain including an heterocycle such as imidazoline, pyridine, and pyrimidine (**21Hd**-**Hf**). Moreover, while maintaining the methyl group at the 2 position and the above-mentioned phenyl groups (4-chloro-3-nitrophenyl and 2,5-dimethoxy-6-nitrophenyl) at the 6 position, we considered, for the 5 position, both the introduction of a diaminoguanidine/dimethyldiaminoguanidine chain (**20Gb**, **20Ic**) and the above-described introduction of a heterocycle-containing chain (**20Gd**-**Ge**). According to the literature, the reaction of an aldehyde with diaminoguanidine may give bishydrazones.[5–7] We obtained compounds **22J** and **23H** which were submitted to antitumor tests since analogous derivatives have been reported as possible antitumor agents.[8]

(3) Replacement of the thiazole ring in the imidazothiazole system. Taking the 4-chloro-3-nitrophenyl derivatives as the lead compounds,[9,3] the thiazole ring was replaced by the following heterocycles: thiadiazole (**36**), benzothiazole (**37**), pyridine (**38**), and pyrimidine (**39**).

Chemistry

The hydrazones **18**–**21** (Scheme 1, Table 1) and **36**–**39** (Scheme 2, Table 1) were prepared by reaction between an aldehyde and the appropriate hydrazine: aminoguanidine (a), 1,3-diaminoguanidine (b, c), 2-hydrazino-2-imidazoline (d), 2-hydrazinopyridine (e), and 2-hydrazino-4-(trifluoromethyl)pyrimidine (f).

When the reaction was performed with 1,3-diaminoguanidine, compound **20Gb**, and the bishydrazones **22J**, **23H** were obtained, whereas compound **20Ic** was isolated in the presence of acetone.

The starting aldehydes **14**–**17** and **32**–**35** were obtained by means of the Vilsmeier reaction on compounds **10**–**13** and **28**–**31** prepared in turn from the appropriate 2-aminothiazoles **1**–**4** and the bromoketones **5** (to obtain the imidazo[2,1-*b*]thiazoles **10**–**13**) or from compounds **24**–**27** treated with 2-bromo-4′-chloro-3′-nitroacetophenone **5G** (for the derivatives **28**–**31**).

The IR and ^{1}H NMR spectra of the new compounds (Supporting Information, Table S1) are in agreement with the assigned structures.

Biology

As a primary screening, all the new hydrazones were submitted to the Developmental Therapeutics Program (DTP)[a]

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[a] Abbreviations: DTP, Developmental Therapeutics Program; NCI, National Cancer Institute; GI, growth inhibition; TGI, total growth inhibition; LC, letal concentration; LDH, lactate dehydrogenase; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; DClF-DA, dichlorofluorescin diacetate; PBS, phosphate-buffered saline.

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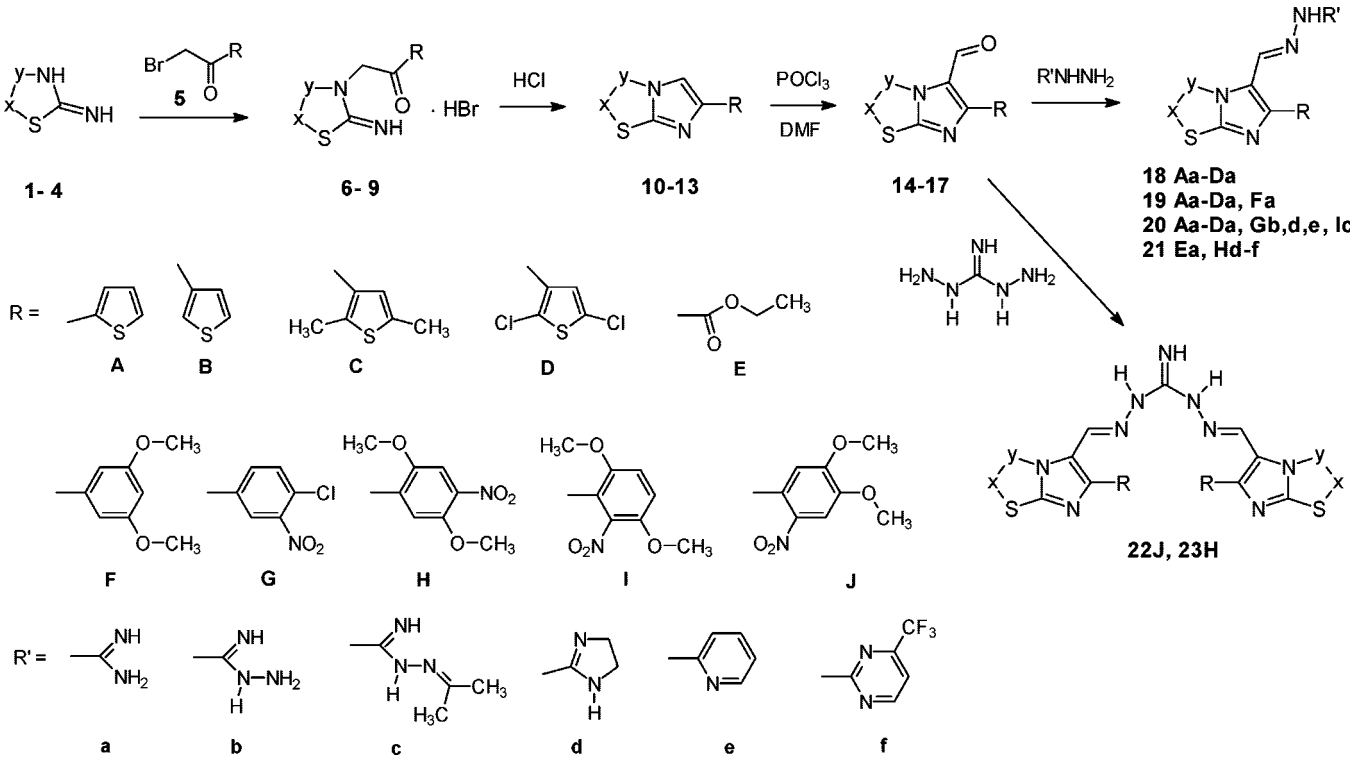
Scheme 1[a]



[a] (x−y see Table *1*).

at the National Cancer Institute (NCI) for evaluation of antitumor activity in the human cell line screen. For some insights into the biological effects of these derivatives, a few selected compounds were submitted to additional studies: **20 Da** (the most active 6-thienyl derivative), **20Gb**, **20Gd**, **21Hd** (not aminoguanidine derivatives), **36** and **37** (not imidazothiazole derivatives and in particular compound **36** showed a very potent cytotoxic activity on HL-60 leukemia, subpanel not shown).

a. Antitumor Activity. In a preliminary test at a single concentration (100 μM) against three human cell lines (NCI-H460 lung cancer, MCF7 breast cancer, and SF-268 glioma), a compound is considered active when it reduces the growth of any of the cell lines to 32% or less, and it will be passed on for evaluation in the full panel of 60 cell lines. This panel is organized into subpanels representing leukemia, melanoma, and cancers of the lung, colon, kidney, ovary, breast, prostate, and central nervous system.

The test compounds were dissolved in DMSO and evaluated using five concentrations at 10-fold dilutions, the highest being 10^{-4} M. Table 2 reports the results obtained with this test (methyl-GAG is reported for comparison purposes) expressed as −log of the molar concentration that inhibited cell growth by 50% (pGI_{50}), caused total cytostasis (pTGI = Total Growth Inhibition), or killed half of the cells (pLC_{50}). These are averages of data for nine different broad categories of the 60-cell panel: the detailed results from each cell line are reported under the Supporting Information (Table S5) for the most active compounds as well as a correlation analysis (Table S6) which provides a statistical comparison of the profiles of activity of these compounds in this screen (the highest correlation coefficients support the closest mechanistic connections between the compounds).

Among the 27 derivatives tested by the NCI, 23 were active in the primary test and were evaluated in the full panel of 60 cell lines showing growth inhibition concentrations similar to or even lower than methyl-GAG. The most interesting results obtained may be summarized as follows.

(1) 6-Substituted imidazothiazole guanylhydrazones. The variations at the 6 position of the imidazothiazole system showed that the carboxyethyl group (**21Ea**) is not suitable for this position, whereas better results were obtained with the 3,5-dimethoxyphenyl group (**19Fa**) even though the activity was not higher than that of the compounds considered in our previous papers.[9,3,4,2] The same happens with the thiophene ring and its dimethyl derivative in particular when connected to unsubstituted imidazothiazoles (**18Aa-Ca**; **19Aa-Ca**).

By contrast, in the 2-methyl-imidazothiazoles, the antitumor activity was higher (**20Aa**−**20Ca**) with mean pGI_{50} values of 5.03–5.40. For those derivatives, the pattern of selectivity is also interesting, since about half of the cell lines were more sensitive and half less sensitive than the average sensitivity of all cell lines. Very good results were obtained when the substituent at the 6 position was a dichlorothiophene: the resulting compounds (**18 Da**, **19 Da**, and **20 Da**) showed mean pGI_{50} values of 5.06, 5.79, and 5.96, respectively. This result and the previous one provide evidence[3] that a methyl group at the 2 position of the imidazothiazole system leads to enhancement of the antitumor activity, and a further confirmation is the behavior of the guanylhydrazone **20 Da** which is the most active compound of this group with a low toxicity; in fact, it showed a difference of 1.11 log units between the average concentration which caused 50% growth inhibition (pGI_{50}) and the concentration which killed 50% of the cells (pLC_{50}). Moreover, for this molecule, six of the nine subpanels were more sensitive than the average sensitivity of all cell lines.

(2) Variations in the basic chain at the 5 position. The introduction of a basic chain different from that so far considered showed that the dimethyldiaminoguanidine chain and the pyrimidine ring lead to inactive compounds (**20Ic** and **21Hf**). Better results were obtained with the pyridine ring (**20Ge** and

Table 1. New Compounds

compd[a]	x−y	formula	MW	Mp, °C
10B	CH_2-CH_2	$C_9H_8N_2S_2$	208.29	146–151
10C	CH_2-CH_2	$C_{11}H_{12}N_2S_2$	236.36	86–87
10D	CH_2-CH_2	$C_9H_6Cl_2N_2S_2$	277.20	85–86
11C	CH=CH	$C_{11}H_{10}N_2S_2$	234.34	58–60
11D	CH=CH	$C_9H_4Cl_2N_2S_2$	275.18	110–112
12C	$H_3CC=CH$	$C_{12}H_{12}N_2S_2$	248.37	111–112
12D	$H_3CC=CH$	$C_{10}H_6Cl_2N_2S_2$	289.21	144–145
28	−	$C_{11}H_7ClN_4O_2S$	294.72	194–196
29	−	$C_{15}H_8ClN_3O_2S$	329.76	258–260
30	−	$C_{13}H_8ClN_3O_2$	273.68	192–194
31	−	$C_{12}H_7ClN_4O_2$	274.66	235–237
14A	CH_2-CH_2	$C_{10}H_8N_2OS_2$	236.32	157–158
14B	CH_2-CH_2	$C_{10}H_8N_2OS_2$	236.32	105–107
14C	CH_2-CH_2	$C_{12}H_{12}N_2OS_2$	264.37	114–115
14D	CH_2-CH_2	$C_{10}H_6Cl_2N_2OS_2$	305.21	118–120
15A	CH=CH	$C_{10}H_6N_2OS_2$	234.30	89–91
15B	CH=CH	$C_{10}H_6N_2OS_2$	234.30	150–152
15C	CH=CH	$C_{12}H_{10}N_2OS_2$	262.35	137–140
15D	CH=CH	$C_{10}H_4Cl_2N_2OS_2$	303.19	188–190
16A	$H_3CC=CH$	$C_{11}H_8N_2OS_2$	248.33	115–117
16B	$H_3CC=CH$	$C_{11}H_8N_2OS_2$	248.33	158–160
16C	$H_3CC=CH$	$C_{13}H_{12}N_2OS_2$	276.38	145–147
16D	$H_3CC=CH$	$C_{11}H_6Cl_2N_2OS_2$	317.22	165–167
17E	ClC=CH	$C_9H_7ClN_2O_3S$	258.68	150–152
32	−	$C_{12}H_7ClN_4O_3S$	322.73	205–207 dec
33	−	$C_{16}H_8ClN_3O_3S$	357.77	242–244
34	−	$C_{14}H_8ClN_3O_3$	301.69	257–259
35	−	$C_{13}H_7ClN_4O_3$	302.67	268–270
18Aa	CH_2-CH_2	$C_{11}H_{12}N_6S_2\cdot HCl$	328.85	258–260 dec
18Ba	CH_2-CH_2	$C_{11}H_{12}N_6S_2\cdot HCl$	328.85	268–270
18Ca	CH_2-CH_2	$C_{13}H_{16}N_6S_2\cdot HCl$	356.90	232–234dec
18 Da	CH_2-CH_2	$C_{11}H_{10}Cl_2N_6S_2\cdot HCl$	397.74	215–217 dec
19Aa	CH=CH	$C_{10}H_{11}N_6S_2\cdot HCl$	315.83	190–192
19Ba	CH=CH	$C_{10}H_{11}N_6S_2\cdot HCl$	315.83	211–212
19Ca	CH=CH	$C_{13}H_{14}N_6S_2\cdot HCl$	354.89	254–256
19 Da	CH=CH	$C_{11}H_8Cl_2N_6S_2\cdot HCl$	395.72	285–287 dec
19Fa	CH=CH	$C_{15}H_{16}N_6O_2S\cdot HCl$	380.86	297–300 dec
20Aa	$H_3CC=CH$	$C_{12}H_{12}N_6S_2\cdot HCl$	340.86	277–280 dec
20Ba	$H_3CC=CH$	$C_{12}H_{12}N_6S_2\cdot HCl$	340.86	314–316 dec
20Ca	$H_3CC=CH$	$C_{14}H_{16}N_6S_2\cdot HCl$	368.91	280–282 dec
20 Da	$H_3CC=CH$	$C_{11}H_{10}Cl_2N_6S_2\cdot HCl$	397.74	290–291 dec
20Gb	$H_3CC=CH$	$C_{14}H_{13}ClN_8O_2S\cdot HCl$	429.29	257–259 dec
20Gd	$H_3CC=CH$	$C_{16}H_{14}ClN_7O_2S\cdot HBr$	484.76	324–326 dec
20Ge	$H_3CC=CH$	$C_{18}H_{13}ClN_6O_2S\cdot HCl$	449.32	280–282 dec
20Ic	$H_3CC=CH$	$C_{19}H_{22}N_8O_4S\cdot HCl$	494.96	195–198 dec
21Ea	ClC=CH	$C_{10}H_{11}ClN_6O_2S\cdot HCl$	351.21	190–192 dec
21Hd	ClC=CH	$C_{17}H_{16}ClN_7O_4S\cdot HCl$	486.34	250–252
21He	ClC=CH	$C_{19}H_{15}ClN_6O_4S\cdot HCl$	495.34	225–227dec
21Hf	ClC=CH	$C_{19}H_{13}ClF_3N_7O_4S\cdot HCl$	564.33	262–264 dec
22J	CH=CH	$C_{29}H_{25}N_{11}O_8S_2\cdot HCl$	756.17	231–233dec
23H	ClC=CH	$C_{29}H_{23}Cl_2N_{11}O_8S_2\cdot HCl$	825.06	260–262 dec
36	−	$C_{13}H_{11}ClN_8O_2S\cdot HCl$	415.26	318–320dec
37	−	$C_{17}H_{12}ClN_7O_2S\cdot HCl$	450.30	288–290 dec
38	−	$C_{15}H_{12}ClN_7O_2\cdot HCl$	394.22	310–312 dec
39	−	$C_{14}H_{11}ClN_8O_2\cdot HCl$	395.20	268–270dec

[a] According to Scheme 1, the uppercase letter is related to the substituent at the 6 position (R) and the lowercase letter to the substituent at the side chain (R′).

21He), whereas the best results were obtained with the unsubstituted diaminoguanidine chain (compound **20Gb** showed mean $pGI_{50} = 5.72$) and with the imidazoline ring which is present in compounds **20Gd** and **21Hd** giving mean pGI_{50} values of 5.90 and 5.99, respectively. It is interesting to point out that **21Hd** (showing a difference of 1.36 log units between mean pGI_{50} and mean pLC_{50}) was more active than the corresponding guanylhydrazone which showed a pGI_{50} value of 5.35.[4] Moreover, compound **21Hd** is also selective toward the colon subpanel ($pGI_{50} = 6.30$).

The bishydrazones are only two compounds; nevertheless, they provide a useful suggestion for the synthesis of the next analogues since only the 2-chloro derivative **23H** showed significant antitumor activity: this compound gave a mean pGI_{50} value of 5.67 and a difference of 1.11 log units between mean pGI_{50} and mean pLC_{50}.

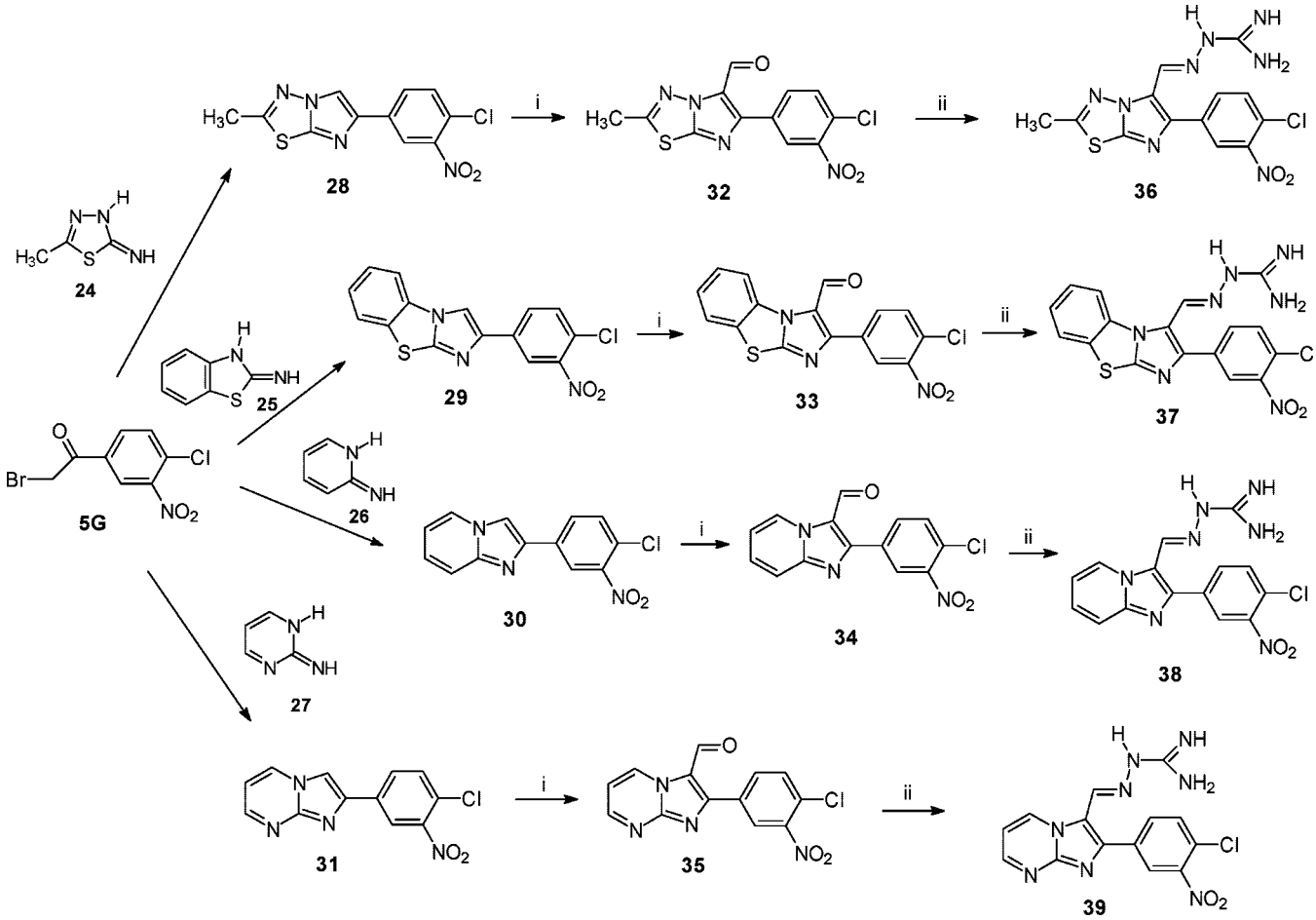
(3) Replacement of the thiazole ring in the imidazothiazole system. Replacement by a pyrimidine ring leads to an inactive compound (**39**), whereas replacement by thiadiazole (**36**), benzothiazole (**37**), and pyridine (**38**) gave interesting results with mean pGI_{50} values between 5.74 and 6.23. Noteworthy is the difference shown by these three compounds (1.10–1.59 log units) between mean pGI_{50} and mean pLC_{50}. In particular, compound **37** gave the best result with an evident selectivity toward the leukemia subpanel (mean $pGI_{50} = 6.86$); therefore, it will be considered a new lead in the design of the next series of compounds.

b. Effects on Growth and Death of HL-60 Leukemia Cells. A few compounds selected as described above were used for further assays in a biological model represented by HL-60 leukemia cells. First, we examined whether the antiproliferative effect was associated to interference in the cell cycle progression. The cells were incubated for 24 h in the presence of a relatively low concentration of the hydrazones (2 μM), and the analysis of DNA profiles was performed by flow cytometry. No significant differences were detected on cell cycle distribution (Table 3), but a decrease in cell number associated to increased cell death was observed. At the same concentration, methyl-GAG, used as a positive control, did not cause any significant effect. Only at 10 μM concentration, methyl-GAG began to cause a decrease in the cell number, accompanied with a slight change in cell cycle distribution, probably associated to the well-known inhibition of polyamine synthesis.[8] On the other hand, none of new hydrazones inhibited the activity of *S*-adenosylmethionine decarboxylase in extracts from HL60 cells in the range 1–100 μM (data not shown), and their effect was related to cytotoxicity, which was particularly evident with compounds **21Hd** and **36**.

On the other hand, a decrease in cell number associated with increased cell death was observed, being particularly evident with compounds **21Hd** and **36**. The effects of these latter derivatives, which appear to be cytotoxic at low concentrations against HL-60 cells, were further studied. First, we determined whether these derivatives induced apoptosis. Cells were treated with 5 μM of the compounds under test, and the activation of effector caspases acting on the substrate sequence Asp-Glu-Val-Asp (DEVD) was determined. The activity of these caspase proteases, a marker of apoptotic cell death, rapidly increased in a dose-dependent mode (Figure 1A), reaching a maximum after 4 h, and thereafter declined (Figure 1B). When the assay was performed after 24 h of treatment, no caspase activation could be detected, even if cell death was largely increased, as determined by lactate dehydrogenase (LDH) release into the medium (Figure 1C). The rapid onset of apoptosis was confirmed by fluorescence microscopy analysis of treated cells. In Figure 1D, HL-60 cells stained with Hoechst 33258 after 3 h of treatment are depicted. Nuclear changes associated with hydrazones in treated cells were evident, in particular, the characteristic chromatin condensation and the presence of apoptotic bodies.

c. Effects on Mitochondria. Many guanidine-containing drugs have been reported to interfere with mitochondrial functions. Therefore, we investigated the effect of compounds **21Hd** and **36** on the mitochondrial potential ($\Delta\Psi_m$) by using the fluorescent dye $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) to measure mitochondria depolarization in intact cells. Figure 2A shows that 1 h of treatment with either **21Hd** or **36** resulted

Scheme 2[a]



[a] i = $POCl_3$/DMF; ii = aminoguanidine.

in a marked disruption of $\Delta\Psi_m$. Mitochondrial membrane depolarization has been associated with mitochondrial production of reactive oxygen species.[10] We therefore investigated whether oxidative stress increased after treatment with the hydrazones by using dichlorofluoresceine diacetate (DClF-DA), a probe for peroxide species. As shown in Figure 2B, treatment with compounds **21Hd** and **36** caused an evident increase in peroxide formation. These mitochondrial effects of the hydrazones were associated with loss of mitochondrial function and a reduction in ATP synthesis. In fact, HPLC analysis of adenine nucleotides showed a sharp decrease of cellular ATP, evidenced by the large increase in the AMP/ATP ratio (Figure 2C) that revealed a profound drop in the cellular energetic potential.

The biological studies of a few selected hydrazones have shown that the efficient antiproliferative power of compounds **21Hd** and **36** was associated with early damage to mitochondria. Actually, a marked decrease in the mitochondrial transmembrane potential was detectable as soon as 1 h after the treatment and was accompanied, as expected, with a large increase in cellular peroxides and inhibition of ATP synthesis. It appears reasonable to think that the oxidative stress and the interference with cellular energetics are major molecular mechanisms responsible for the cytotoxicity of the compounds. Mitochondrial effects of compounds **21Hd** and **36** (the effects of compound **37** were very similar to those of **36**, data not shown) were associated with an early onset of apoptosis, revealed by typical nuclear changes and by the rapid activation of caspase proteases. It is worth noting that the molecular characteristics of apoptosis are no longer evident after 24 h of treatment, suggesting an abortive form of apoptosis, possibly as a consequence of the fall in ATP, that is required to maintain the active process of apoptosis.[11]

Some of the compounds reported in this series may owe a significant part of their antitumor activity to the allylic imino nitrogen atom moiety, in which the N is part of the hydrazone link and the allylic C=C link is in the imidazole ring. This kind of allylic moiety is seen in many compounds or their metabolites that exhibit antitumor activity or a specific biological activity that can produce cancer cell death, such as angiogenesis or protein phosphorylation.[12,13] The typical allylic moiety consists of a propene sequence [−CR=CR−CH(X)−], with X signifying an imino N, amino N, or oxygen that can be an OH, a carbonyl O, or an O in ester, ether, or amide linkage. Many drugs with this structural feature also exhibit an oxygen or nitrogen bound to one or both carbon atoms of the double bond.[13] Moreover, in some of the compounds here described, metabolic oxidation of the aromatic methoxy groups may result in demethylation, followed by oxidation of the hydroxyls. This would yield a quinone, typically formed by metabolism of the catechol and hydroquinone moieties in drugs. Quinone groups are also present in unmetabolized antitumor drugs, such as the anthraquinones. Since quinones are double allylic ketones, they can be expected to contribute further to the anticancer activity. Also adding to the antitumor effect is the conjugation of the allylic double bond produced when the quinone structure is formed.[13] The effect of the nitro group in the hypothetical quinone is not known.

Table 2. Sixty Cell Panel (Growth Inhibition and Cytostatic and Cytotoxic Activity of the Compounds which Passed the Three Line Test)

compd[a]	modes	leukemia	NSCLC	colon	CNS	melanoma	ovarian	renal	prostate	breast	MG-MID[b]
18Aa	pGI_{50}	4.73	4.54	4.76	4.72	4.67	4.40	4.64	4.71	4.76	4.66
	pTGI	4.41	-	4.41	4.40	4.42	-	-	4.44	4.39	4.32
18Ba	pGI_{50}	4.72	4.54	4.64	4.60	4.66	4.84	4.55	4.76	4.49	4.62
18Ca	pGI_{50}	4.68	4.77	4.86	4.73	4.81	4.62	4.76	4.81	4.62	4.74
	pTGI	-	4.45	4.53	4.46	4.53	4.32	4.42	4.51	4.38	4.43
18Da	pGI_{50}	4.99	4.95	5.74	5.06	5.00	4.98	5.00	5.09	4.91	5.06
	pTGI	4.61	4.65	4.79	4.76	4.69	4.64	4.70	4.82	4.64	4.68
	pLC_{50}	4.32	4.43	4.52	4.47	4.45	4.41	4.46	4.55	4.38	4.43
19Aa	pGI_{50}	5.17	4.89	5.01	4.92	4.82	4.99	5.08	4.91	4.83	4.96
	pTGI	4.42	4.53	4.62	4.59	4.54	4.57	4.66	4.61	4.55	4.56
19Ba	pGI_{50}	5.16	4.82	4.85	4.82	4.77	4.81	4.83	4.83	4.76	4.85
	pTGI	4.62	4.52	4.53	4.54	4.50	4.50	4.53	4.54	4.50	4.53
19Ca	pGI_{50}	4.81	4.78	4.81	4.81	4.79	4.76	4.75	4.86	4.79	4.79
	pTGI	-	4.48	4.49	4.52	4.51	4.47	4.49	4.57	4.52	4.48
19Da	pGI_{50}	6.00	5.57	6.01	5.95	5.55	5.96	5.79	5.81	5.78	5.79
	pTGI	5.67	4.97	5.63	5.55	5.14	5.45	5.24	5.21	5.37	5.32
	pLC_{50}	5.21	4.49	4.95	4.93	4.81	4.79	4.88	4.76	4.63	4.81
19Fa	pGI_{50}	4.99	5.03	5.22	5.09	5.07	5.10	5.07	5.03	5.00	5.07
	pTGI	4.72	4.69	4.83	4.76	4.75	4.77	4.75	4.79	4.69	4.74
	pLC_{50}	4.45	4.46	4.50	4.46	4.51	4.46	4.50	4.55	4.43	4.47
20Aa	pGI_{50}	5.74	5.11	5.59	5.37	5.65	5.19	5.37	5.52	5.24	5.40
	pTGI	5.39	4.60	5.09	4.93	5.23	4.76	4.85	4.91	4.73	4.93
	pLC_{50}	4.98	-	4.64	4.48	4.86	-	4.36	4.40	-	4.46
20Ba	pGI_{50}	5.11	4.86	5.27	5.16	5.09	4.96	4.99	5.12	5.11	5.06
	pTGI	4.33	4.38	4.69	4.71	4.71	4.56	4.55	4.70	4.65	4.57
20Ca	pGI_{50}	4.32	5.01	5.43	5.08	5.13	4.92	4.85	5.32	5.35	5.03
	pTGI	-	4.60	4.93	4.74	4.82	4.48	4.49	4.72	4.87	4.65
	pLC_{50}	-	-	4.41	4.42	4.56	-	-	-	4.41	4.32
20Da	pGI_{50}	6.05	5.96	5.99	5.95	5.98	5.99	5.91	6.10	5.91	5.96
	pTGI	5.75	5.44	5.40	5.36	5.51	5.63	5.52	5.83	5.58	5.53
	pLC_{50}	5.45	4.71	4.70	-	5.11	4.71	4.85	5.56	4.40	4.85
20Gb	pGI_{50}	5.20	5.62	6.02	5.95	5.74	5.89	5.49	5.47	5.81	5.72
	pTGI	-	5.01	5.68	5.53	5.34	5.43	5.07	5.02	5.27	5.23
	pLC_{50}	-	-	4.83	5.01	4.62	4.85	4.72	4.56	4.60	4.63
20Gd	pGI_{50}	6.17	5.81	5.92	5.97	6.01	5.85	5.84	5.94	5.74	5.90
	pTGI	5.21	5.22	5.00	5.37	5.62	5.47	5.43	5.53	5.12	5.32
	pLC_{50}	-	4.55	4.68	4.88	5.03	4.32	4.83	4.82	4.69	4.71
20Ge	pGI_{50}	4.78	4.94	4.58	5.11	4.74	5.04	4.81	4.93	4.86	4.86
	pTGI	4.47	4.51	4.37	4.71	4.42	4.59	4.40	4.41	4.41	4.48
	pLC_{50}	-	4.32	4.32	4.45	4.33	4.31	4.33	-	-	4.33
21Ea	pGI_{50}	4.85	4.74	4.68	4.71	4.71	4.68	4.67	4.77	4.64	4.70
	pTGI	4.41	4.38	4.32	-	4.34	-	-	4.41	-	4.31
21Hd	pGI_{50}	5.58	6.03	6.30	6.19	5.90	6.13	5.87	6.12	5.74	5.99
	pTGI	-	5.32	5.53	5.68	5.41	5.73	5.47	5.94	4.37	5.35
	pLC_{50}	-	4.64	5.07	4.69	4.94	4.64	4.84	4.73	-	4.63
21He	pGI_{50}	5.04	4.59	4.35	4.81	4.48	4.70	4.87	4.79	4.76	4.70
	pTGI	4.45	-	-	4.52	4.33	4.48	4.51	-	4.38	4.41
	pLC_{50}	4.33	-	-	4.40	-	4.38	4.34	-	-	4.33
23H	pGI_{50}	5.63	5.69	5.65	5.79	5.72	5.74	5.66	5.71	5.48	5.67
	pTGI	-	5.40	5.15	5.59	5.29	5.42	5.31	5.42	5.17	5.30
	pLC_{50}	-	-	4.85	4.43	4.91	4.61	4.60	5.13	-	4.56
36	pGI_{50}	6.24	5.48	6.19	5.91	5.84	5.83	5.91	5.96	5.99	5.90
	pTGI	5.44	4.94	5.55	5.01	5.53	5.37	5.44	5.58	5.16	5.32
	pLC_{50}	-	-	4.79	-	4.45	4.37	4.39	5.23	-	4.37
37	pGI_{50}	6.86	6.17	6.47	6.35	6.15	5.96	6.08	5.64	6.13	6.23
	pTGI	-	5.42	5.44	5.52	5.56	5.71	5.41	4.83	5.02	5.40
	pLC_{50}	-	4.62	4.72	4.84	4.70	4.72	4.95	-	-	4.64
38	pGI_{50}	5.77	5.77	5.73	5.92	5.69	5.73	5.66	5.82	5.64	5.74
	pTGI	5.42	5.42	5.10	5.52	5.44	5.44	5.31	5.51	5.27	5.36
	pLC_{50}	-	5.20	4.57	4.84	-	5.20	4.64	5.24	4.64	4.64
methyl-GAG[c]	pGI_{50}	5.10	4.60	4.90	4.70	4.40	4.80	4.70	4.00	4.80	4.67
	pTGI	3.20	3.50	3.30	3.60	3.50	3.50	3.30	2.80	3.50	3.36
	pLC_{50}	2.60	2.80	2.90	3.00	3.00	2.80	2.70	2.60	2.80	2.80

[a] Highest conc. = 10^{-4} M. Only modes showing a value >4.30 are reported. [b] Calculated mean panel. [c] Highest conc. = $10^{-2.6}$ M.

Experimental Section (See Also Supporting Information)

1. Chemistry. The melting points are uncorrected. Bakerflex plates (silica gel IB2-F) were used for TLC. The eluent was petroleum ether/acetone in various proportions (with 0.1% of conc. NH_4OH for the analysis of the guanylhydrazones as free bases). Kieselgel 60 (Merck) was used for column chromatography. The IR spectra were recorded in nujol on a Nicolet Avatar 320 ESP spectrometer; ν_{max} is expressed in cm^{-1}. The ^1H NMR spectra were recorded in $(CD_3)_2SO$ on a Varian Gemini (300 MHz); the chemical shift (referenced to solvent signal) is expressed in δ (ppm) and J in Hz (Table S1). The EI-MS were recorded at 70 eV on a VG 7070E. Elemental analyses were performed with a Fisons Carlo Erba Instrument EA1108 and resulted within ± 0.4% of the theoretical values (Table S3).

The starting compounds **1**–**5** and **24**–**27** are commercially available. The imidazothiazoles **10A**; **11A**,**B**,**F**,**J**; **12A**,**B**,**G**,**I**; and

Table 3. Effects of Some Derivatives on Cell Cycle, Growth, and Death of HL-60 Leukemia Cells

	cell cycle phase[b]			cell no.	Cell Death
compd[a]	G0/G1	S	G2/M	cells/mL (x10^{-3})	propidium iodide positive cells (%)
Ctrl	34.7	51.0	14.3	850 ± 72	4.4 ± 1.8
20 Da	35.3	50.0	14.7	750 ± 23	7.4 ± 1.6
20Gb	35.0	51.9	13.1	760 ± 11	7.8 ± 4.3
20Gd	31.3	53.7	15.0	780 ± 12	11.9 ± 2.7
21Hd	33.1	51.9	15.1	485 ± 10	22.0 ± 1.5
36	29.9	53.6	16.5	410 ± 64	31.0 ± 1.7
methyl-GAG	43.6	40.9	15.5	702 ± 26	5.2 ± 2.0

[a] 2 μM concentration except methyl-GAG which was 10 μM. [b] Percentage of cells in the different phases of the cell cycle.

13E,**H** and the aldehydes **15F**,**J**; **16G**,**I**; and **17H** were prepared according to the literature.[14–16,3,4,17]

1a. Synthesis of the Imidazo[2,1-*b*]thiazoles 10B−D; 11C,D; and 12C,D (Scheme 1). The appropriate 2-aminothiazole or 2-aminothiazoline **1**–**4** (20 mmol) was dissolved in 100 mL of acetone and treated with the equivalent of the appropriate 2-bromoacetylthiophene **5B**−**D**. The reaction mixture was refluxed for 2–3 h (according to a TLC test), and the resulting salt (**6**–**9**) was separated by filtration and used without further purification ($\nu_{C=O}$ absorption was confirmed around 1700 cm^{-1}) in the subsequent step. It was refluxed for 1 h with 200 mL of 2 N HCl, and before complete cooling, the solution was cautiously basified by dropwise addition of 15% NH_4OH. The resulting base was collected by filtration and crystallized from petroleum ether (**10C**,**D**, **11C**,**D**, **12C**,**D**) or from acetone/petroleum ether (**10B**), with a yield of 70–80% (**10C**; **11C**,**D**; **12C**,**D**) and 45% (**10B**,**D**).

2a. Synthesis of Compounds 28−31 (Scheme 2). Compounds **28**−**31** were prepared with the same procedure described for the synthesis of the imidazo[2,1-*b*]thiazoles, using the appropriate 2-amino derivative (**24**−**27**) which was treated with the 2-bromoacetophenone **5G**. The crude products were purified by crystallization from ethanol with a yield of 60–70%.

3a. Synthesis of the Aldehydes 14A−D, 15A−D, 16A−D, 17E (Scheme 1), and 32–35 (Scheme 2). The Vilsmeier reagent was prepared at 0–5 °C by dropping $POCl_3$ (54 mmol) into a stirred solution of DMF (65 mmol) in $CHCl_3$ (5 mL). The appropriate condensed imidazole system (**10**–**13**, **28**–**31**, 5 mmol) was suspended in $CHCl_3$ (20 mL). The mixture thus obtained was dropped into the Vilsmeier reagent while maintaining stirring and cooling. The reaction mixture was kept for 3 h at room temperature and under reflux for 1–24 h (according to a TLC test). Chloroform was removed under reduced pressure, and the resulting oil was poured onto ice.

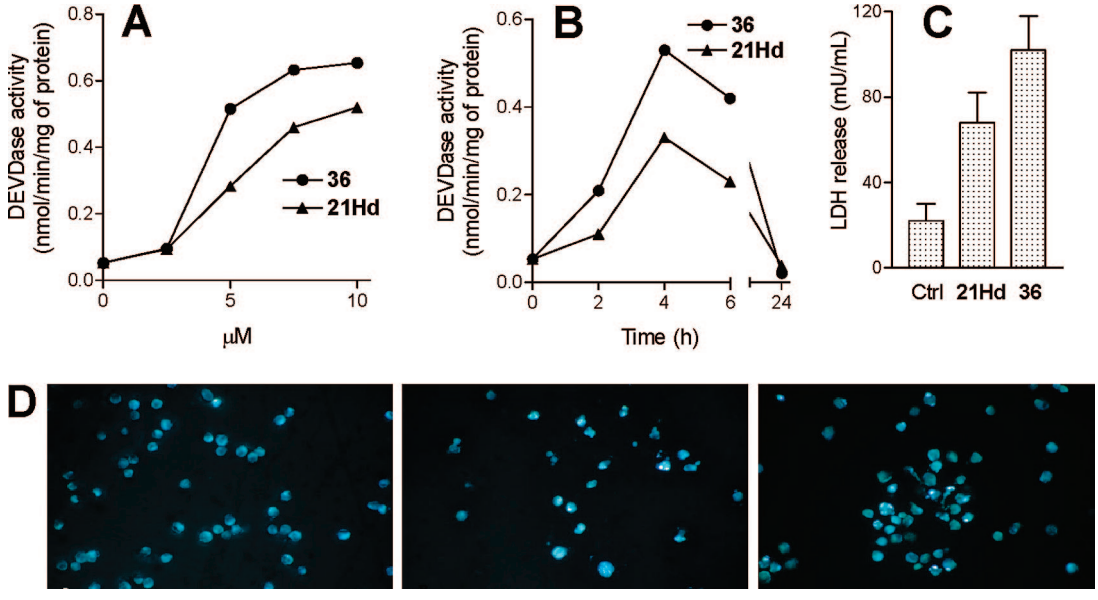


Figure 1. Induction of apoptosis in HL-60 cells by derivatives **21Hd** and **36**. (A) The activity of caspase proteases cutting the peptide sequence DEVD (DEVDase activity) was measured in cells treated for 4 h with the indicated concentration of the compounds. (B) DEVDase activity in cells treated with 5 μM of compounds **21Hd** and **36** was measured at the indicated time. (C) LDH release into the medium following 24 h in the presence of 5 μM of **21Hd** and **36**. (D) Morphological evaluation of nuclei stained with Hoechst 33258 obtained from control cells (left), cells treated for 3 h with 5 μM of compounds **21Hd** (central panel) or **36** (right). Each panel represents the results obtained in one typical experiment repeated at least twice with comparable results.

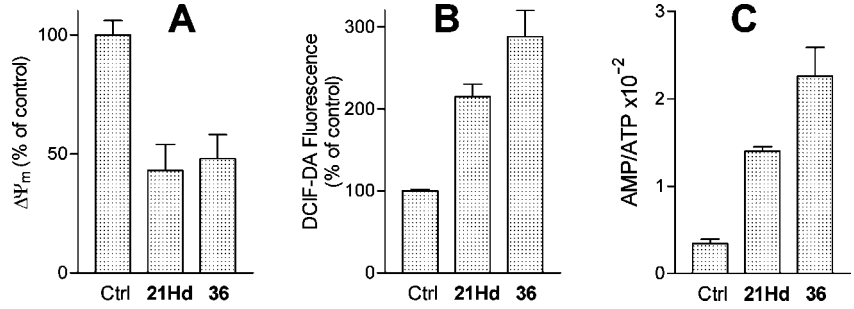


Figure 2. Effects of compounds **21Hd** and **36** on mitochondria. HL-60 cells were treated with 5 μM of the derivatives. (A) The mitochondrial membrane potential $\Delta\Psi_m$ was determined by flow cytometry after 1 h. (B) Intracellular peroxides were detected by flow cytometry following a 3 h incubation. (C) The AMP/ATP ratio was calculated following the measurement of adenine nucleotide levels in cells treated for 3 h. All the results are mean ± SEM of triplicate determinations.

The crude aldehydes **14A**,**D**; **15A**−**D**; **16A**−**D**; **17E**; and **32**−**35** were collected by filtration, whereas compounds **14B**,**C** precipitated when the solution was basified by addition of NH_4OH.

The crude aldehydes were crystallized from ethanol with a yield of 70–90%.

4a. Synthesis of the Hydrazones 18–23 (Scheme 1) and 36–39 (Scheme 2). The appropriate aldehyde (10 mmol) was dissolved in ethanol and treated with the equivalent of the appropriate hydrazine hydrochloride: aminoguanidine (for compounds **18Aa-Da**, **19Aa-Da**,**Fa**, **20Aa-Da**, **21Ea**, and **36**–**39**), 1,3-diaminoguanidine (for compounds **20Gb**, **20Ic**, **22J**, and **23H**), 2-hydrazinopyridine (for compounds **20Ge** and **21He**), 2-hydrazino-4-(trifluoromethyl)pyrimidine (for compound **21Hf**).

In the case of compounds **20Gd** and **21Hd**, the aldehyde was treated with 2-hydrazino-2-imidazoline hydrobromide and hydrochloride.

The reaction mixture was refluxed for 5–30 h according to a TLC test, and the resulting precipitate was collected by filtration with a yield of 25–30% (**21Hd**, **22J**, and **23H**), 50–60% (**19Ca**, **19 Da**, **20Gb**, **20Gd**, **20Ic**, **21Hf**, and **37**), and 80–90% (**18Aa-Da**, **19Aa**, **19Ba**, **19Fa**, **20Aa**, **20Ba**, **20Ca**, **20 Da**, **20Ge**, **21Ea**, **21He**, **36**, **38**, and **39**).

Compound **20Ic** was obtained by refluxing the reaction mixture in the presence of acetone.

2. Biology. 2a. Antitumor Activity. The antitumor tests were performed by the National Cancer Institute (NCI, Bethesda, Md) as in our previous papers.[18] The test compounds were dissolved in DMSO and diluted 1:400 in complete culture medium resulting in a final DMSO concentration of 0.25%.

2b. Effects on HL-60 Leukemia Cells. Cell Culture. HL-60 (human promyelocytic leukemic) cells were maintained in exponential growth at 37 °C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% Foetal Bovine Serum and 2 mM glutamine. Under these growth conditions, cell doubling time was about 24 h. Cell growth was assessed by cell counting using Coulter Counter model Z1 (Florida, USA). All the compounds under test were dissolved in dimethylsulfoxide and diluted to the required concentration in medium.

Flow Cytometry. The test was performed using a BioRad Brite HS cytometer with excitation and emission settings at 488, 530, and 605 nm, respectively. At least 10 000 cells were collected, and the data analysis was performed using the WinMDI software (J. Trotter, Scripps Resarch Institute, S.Diego, USA).

Cell Cycle. The cells were counted, harvested for centrifugation at 250*g* for 5 min, washed twice with PBS (phosphate-buffered saline), fixed in cold 70% ethanol, and stored at −20 °C. The cells were then centrifuged at 250*g* for 5 min, washed twice with PBS, resuspended in DNA staining solution [50 μg/mL of Propidium Iodide (PI) and 10 μg/mL of RNAase in PBS] for 30 min at a concentration of 1×10^6 cells/mL. Following flow cytometric analysis, the percentage of the cells in the different phases of the cell cycle was calculated using the software ModFit (Verity, USA).

Peroxide Levels. Intracellular peroxide levels were assessed cytofluorometrically.[19] Briefly, cells were incubated with 5 μM DClF-DA (Molecular Probes, Leiden, The Netherlands), made as a 10 mM stock in DMSO, for 30 min at 37 °C.

Mitochondrial Membrane Potential ($\Delta\Psi_m$). To measure $\Delta\Psi_m$, mitochondria were selectively probed with potential-sensitive $DiOC_6$.[4] After treatment, cells were incubated with medium containing 40 nM $DiOC_6$ for 40 min at a cell concentration of 1×10^6 cell/mL at 37 °C in the dark. Fluorescence was detected by flow cytometry.

Fluorescence Microscopy. For microscopical evaluation, cells grown on a glass coverslip were fixed with 4% paraformaldehyde (Sigma, USA) in PBS, stained with Hoechst 33432 (Sigma USA) 0.1 μg/mL, and analyzed by fluorescence microscopy as described.[20]

Cell Death. Cell viability was assessed by flow cytometry: cells were counterstained by using PI (5μg/mL) to assess plasma membrane integrity and cell viability. In some experiments, cell death was estimated by measurement of LDH efflux from damaged cells into the medium. LDH release was monitored by collecting aliquots of medium from each flask; these aliquots were then analyzed spectrophotometrically for LDH activity by measuring NADH levels at 340 nm.[21]

Caspase Activity. The activity of caspase enzymes hydrolyzing the peptide sequence DEVD (Asp-Glu-Val-Asp), indicated as DEVDase activity, was measured in cell extracts by a fluorimetric assay.[2]

Adenine Nucleotide Level. The cellular content of adenine nucleotides was determined by HPLC after extraction in perchloric acid and conversion into fluorescent etheno-derivatives.[22]

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Supporting Information Available: IR and ^1H NMR of the new compounds (Table S1), NSC numbers (Table S2), elemental analyses (Table S3), mass spectra (Table S4), detailed results from NCI for the most active compounds (Table S5), and correlation analysis of the profiles of activity of these compounds (Table S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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