Potential Antitumor Agents. 37.[1] Synthesis and Antitumor Activity of Guanylhydrazones from Imidazo[2,1-*b*]thiazoles and from the New Heterocyclic System Thiazolo[2′,3′:2,3]imidazo[4,5-*c*]quinoline

Aldo Andreani,*,† Massimiliano Granaiola,† Alberto Leoni,† Alessandra Locatelli,† Rita Morigi,† Mirella Rambaldi,† Giorgio Lenaz,‡ Romana Fato,‡ Christian Bergamini,‡ and Giovanna Farruggia‡

Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy, and Dipartimento di Biochimica "G. Moruzzi", Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy

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This paper reports synthesis and antitumor activity of new guanylhydrazones from imidazo[2,1-*b*]thiazoles and from the new heterocyclic system thiazolo[2′,3′:2,3]imidazo[4,5-*c*]quinoline. The compounds were tested as potential antitumor agents at the National Cancer Institute. The effect of the guanylhydrazone of 2-chloro-6-(2,5-dimethoxy-4-nitrophenyl)imidazo[2,1-*b*]-thiazole-5-carbaldehyde (**41**) was investigated, and it was found to be an inhibitor of Complex III of the mitochondrial respiratory chain and is able to induce apoptosis in the cell lines HT29 and HL60.

Introduction

In the previous papers[2,3] we pointed out that in a series of imidazo[2,1-*b*]thiazole guanylhydrazones, 2-, 3-, or 4-nitrophenyl at the 6 position is a suitable pharmacophoric group giving rise to some potent antitumor agents. To find congeners more active as potential chemotherapeutic agents and to study in depth the influence of the substituent in position 6, we designed new imidazothiazole guanylhydrazones bearing different dimethoxynitrophenyl groups in this position. We chose as starting material for the synthesis some dimethoxyacetophenones, which gave by nitration several nitroaryl derivatives. From these intermediates we could easily afford the guanylhydrazones bearing at the 6 position different nitroaryl groups, the feature supposed to enhance the pharmacological activity. We also prepared a few derivatives without the nitro group for comparison purpose. The substituents in position 2 and 3 of the imidazothiazole ring were the same used in the previous papers[3,4] (2- and/or 3-methyl, 2-chloro, and 2-propyl), because they demonstrated an increase in the cytotoxicity. In addition we have investigated the activity of a bis-guanylhydrazone, the correlated quinone, and two new polycyclic compounds. The synthesized compounds bear the guanidino group as other drugs: *m*-iodobenzylguanidine (MIBG), the pyridyl cyanoguanidine CHS 828, and Mitoguazone (methyl-GAG, methylglyoxal-bisguanylhydrazone, MGBG).[5] MIBG and CHS 828 have shown promising antitumor activity in preclinical tumor models, while methyl-GAG has useful clinical activity as a single agent in malignant lymphoma, carcinoma of the head, neck, and esophageal and non-small-cell lung cancer.[5] Apart from having the guanidino groups in common, there are many differences between these drugs in their structures and mechanism of action. However, even if they have distinctive biochemical properties, they all inhibit mitochondrial functions. MIBG showed to selectively inhibit Complex I (NADH-Q_1 reductase activity) in the respiratory chain,[6,7] while methyl-GAG significantly inhibits State 4 of respiration.[8] Moreover it is known that alkylguanidines such as octylguanidine are effective inhibitors of State 3 of ADP-stimulated respiration.[9] Since mitochondrial damage may be responsible, or partly so, for the antiproliferative action,[10−12] we studied the effects on the mitochondrial respiratory chain of one of the most interesting compounds synthesized, i.e., **41**, the guanylhydrazone of 2-chloro-6-(2,5-dimethoxy-4-nitrophenyl)imidazo[2,1-*b*]thiazole-5-carbaldehyde. Moreover methyl-GAG is known to induce apoptosis in different human cancer cell lines in a concentration- and time-dependent way;[13] therefore, we undertook a study to elucidate if the mechanism of action of guanylhydrazone **41** was linked to a apoptotic or necrotic pathway.

Chemistry. The guanylhydrazones **29**−**41**, **50**, **51**, **56**, **58**, **61**, **66** (Schemes 1−4) were prepared from the corresponding aldehydes, whereas **44** was from the quinone **43**. The aldehyde **60** (Scheme 3) was obtained by reduction with $Na_2S_2O_4$ of the quinone **59**, prepared in turn by a convenient method of oxidative demethylation of the hydroquinone dimethyl ether **57** with ceric ammonium nitrate (CAN).[14] A versatile approach was used to afford the aldehyde **49** (Scheme 2) starting from the 6-dimethoxyphenyl imidazothiazole **46**: first it was hydrolyzed by refluxing in aqueous HBr to give **47**, then the formyl group was introduced by means of the Vilsmeier reaction. Moreover we attempted the reduction of the nitro group of the aldehydes **16**, **23**, **54**, **65** which, under the experimental conditions employed (Fe/acetic acid), did not bring to the corresponding aminoaldehydes. When the nitro group was at the 2-position (**16**, **54**) (Schemes 1, 2), the reduction was accomplished with concomitant ring closure and formation of the new heterocyclic system thiazolo[2′,3′:2,3]-imidazo[4,5-*c*]quinoline (**42**, **55**). Due to the lack of formyl group in compound **42**, the reaction with ami-

* To whom correspondence should be addressed. Tel.: +39-051-2099714, fax +39-051-2099734, e-mail: aldo.andreani@unibo.it.
† Dipartimento di Scienze Farmaceutiche.
‡ Dipartimento di Biochimica "G. Moruzzi".

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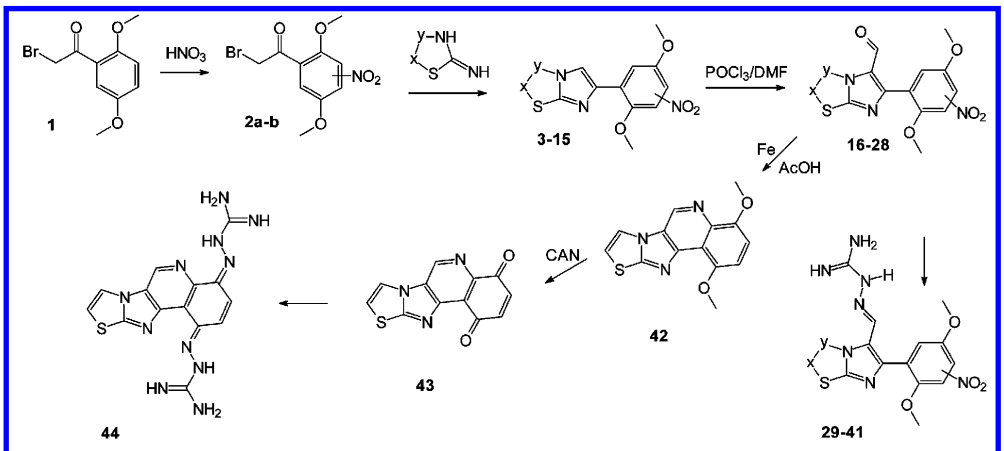
Scheme 1



Comp	x	y	NO_2 pos.	Comp	x	y	NO_2 pos.
3	-CH=	-CH=	2	**23**	-CH=	-CH=	4
4	$-CH_2-$	$-CH_2-$	2	**24**	$-CH_2-$	$-CH_2-$	4
5	$C-CH_3$	-CH=	2	**25**	$C-CH_3$	-CH=	4
6	-CH=	$C-CH_3$	2	**26**	-CH=	$C-CH_3$	4
7	$C-CH_3$	$C-CH_3$	2	**27**	$C-CH_3$	$C-CH_3$	4
8	C-Cl	-CH=	2	**28**	C-Cl	-CH=	4
9	$C-C_3H_7$	-CH=	2	**29**	-CH=	-CH=	2
10	-CH=	-CH=	4	**30**	$-CH_2-$	$-CH_2-$	2
11	$-CH_2-$	$-CH_2-$	4	**31**	$C-CH_3$	-CH=	2
12	$C-CH_3$	-CH=	4	**32**	-CH=	$C-CH_3$	2
13	-CH=	$C-CH_3$	4	**33**	$C-CH_3$	$C-CH_3$	2
14	$C-CH_3$	$C-CH_3$	4	**34**	C-Cl	-CH=	2
15	C-Cl	-CH=	4	**35**	$C-C_3H_7$	-CH=	2
16	-CH=	-CH=	2	**36**	-CH=	-CH=	4
17	$-CH_2-$	$-CH_2-$	2	**37**	$-CH_2-$	$-CH_2-$	4
18	$C-CH_3$	-CH=	2	**38**	$C-CH_3$	-CH=	4
19	-CH=	$C-CH_3$	2	**39**	-CH=	$C-CH_3$	4
20	$C-CH_3$	$C-CH_3$	2	**40**	$C-CH_3$	$C-CH_3$	4
21	C-Cl	-CH=	2	**41**	C-Cl	-CH=	4
22	$C-C_3H_7$	-CH=	2				

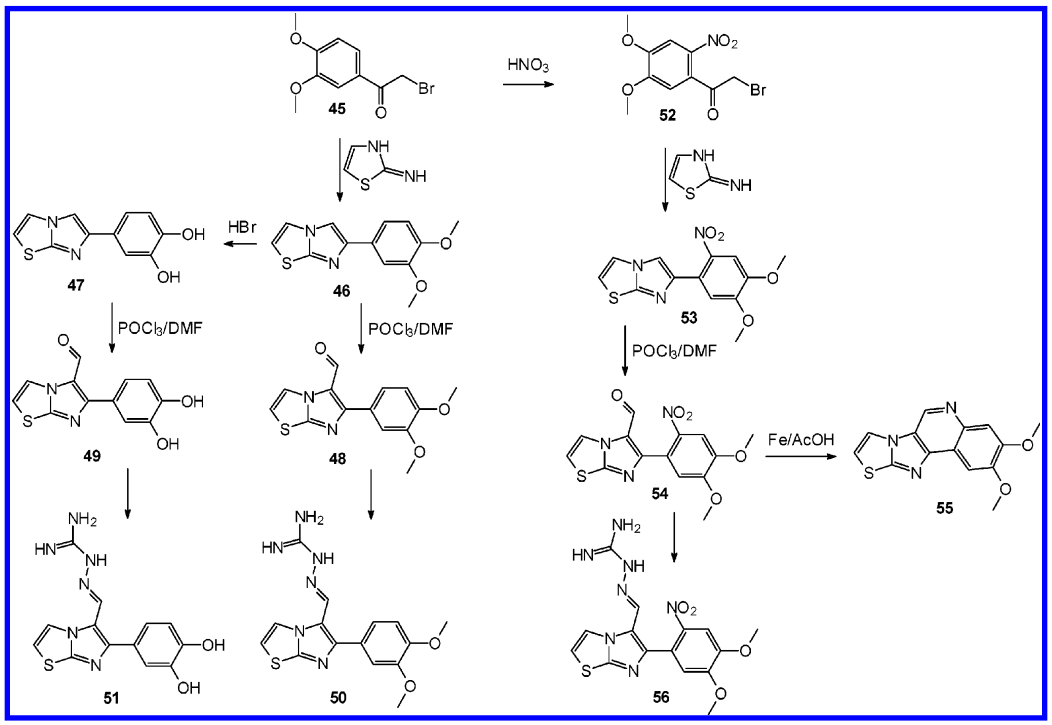
noguanidine was performed at the carbonyl groups of the corresponding *p*-quinone **43**, prepared in turn by oxidative demethylation with CAN. Under the same experimental conditions, compound **55** did not furnish the analogue *o*-quinone.

Biological Results and Discussion

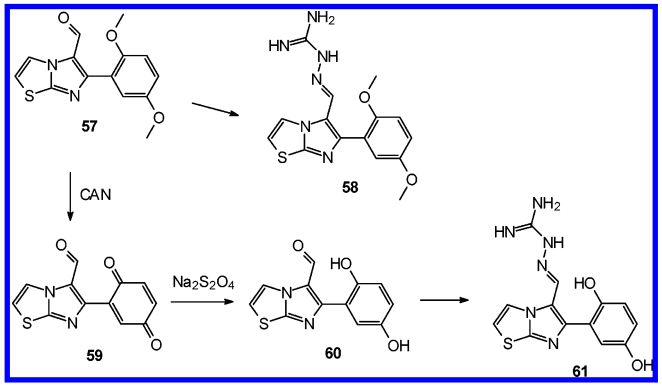
(a) In Vitro Growth Inhibition and Cytotoxicity.[15] The test was performed by the National Cancer Institute (NCI, Bethesda, MD) as in our previous papers.[2,3] According to a primary screening, the guanylhydrazones **29**−**41**, **50**, **51**, **56**, **58**, **61**, **66**, the bisguanylhydrazone **44**, and the new heterocycles **42**, **43**, and **55** were evaluated for their cytotoxic activity on three human cell lines. Compounds **29**, **31**, **33**−**38**, **40**, **41**, **43**, **44**, **50**, **55**, **58**, **61** were active on the three cell line test and therefore were evaluated in the 60 cell lines, using five concentrations at 10-fold dilutions, the highest being 10^{-4}M and the others 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M. The results are expressed as log, taking into consideration the growth inhibitory power (GI_{50}), the cytostatic effect (TGI), and the cytotoxic effect (LC_{50}). The values of log GI_{50} range from −4.04 to −6.08, log TGI from −4.01 to −5.65, and log LC_{50} from −4.01 to −5.01 (see Supporting Information). The order of magnitude for the activity of methyl-GAG[16] and the tested compounds is similar. Compound **41** is the most active of the series showing the following log GI_{50} values (panel/cell line): −5.81 (colon/COLO 205), −6.23 (ovarian/OVCAR-3), −5.84 (renal/RXF 393), and −5.72 (leukemia/K-562) and is under review by BEC (Biological Evaluation Committee of the NCI). The result obtained from all the compounds tested shows that those bearing the nitro group in ortho or meta position are weakly active (**29**, **31**, **33**−**35**) or inactive (**30**, **32**, **66**).

An increase in activity is obtained with a para nitro group (**36**−**38**, **40**, **41**) and in particular when a substituent is also present in position 2 of the imidazothiazole (**38**, **41**). Moreover in the derivatives without the nitro group (**50**, **61**) it is evident a flattening of the values (MG-MID = 10^{-4} M) and a general decrease of cytotoxic effect. These data confirm the importance of

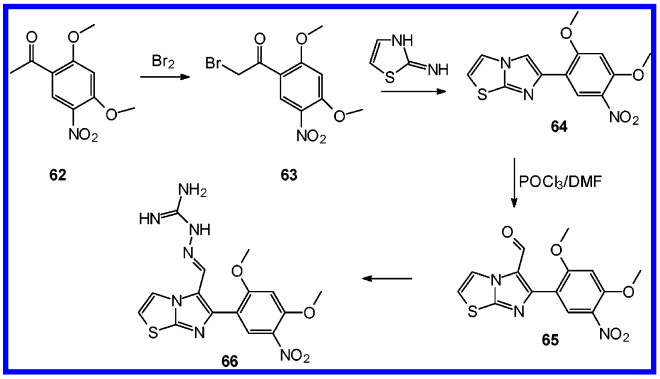
Scheme 2



Scheme 3



Scheme 4



the nitro group to increase the inhibition of cell proliferation. SARs are not possible at present for compounds **43**, **44**, and **55** since they are derivatives of the new heterocyclic system thiazolo[2′,3′:2,3]imidazo[4,5-*c*]quinoline. They did not show a potent antitumor activity, but since **43** and **55** have an appreciable activity on leukemia and renal cancer respectively, this new heterocyclic system will be considered as a possible new lead.

(b) Effects on Respiration of Rat Liver Mitochondria. The experiments were conducted testing methyl-GAG and **41** at the concentration of 35 μM and measuring the inhibitory effect on mitochondrial respiration in coupled rat liver mitochondria. A polarographyc apparatus (Clark electrode) detected the oxygen consumption, and the reaction was started adding a saturating amount of the appropriate substrate: glutamate/malate for NADH$-O_2$ activity, succinate for succinate-O_2 activity, and ascorbate/*N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) to test Complex IV (Cytochrome *c* oxidase enzyme) activity. The respiratory rates were expressed as ngA_{oxigen}/min/mg of protein. As shown by data reported in Table 1, methyl-GAG induces only a slight decrease in the oxygen consumption rate when the substrate used is ascorbate/TMPD. This is in disagreement with earlier findings [8,11] suggesting that the significant inhibition observed in the literature on State 4 respiration could be an artifact due to the very high concentration of methyl-GAG used in those experiments (6 mM). On the

Table 1. Effect of methyl-GAG and Compound **41** on Respiration in Coupled Isolated RLM[a] (State 4 and State 3) and on DNP-Uncoupled Respiration

compd (concn 35 μM)[b]	State 4	State 3	ICR[c]	DNP
Glutamate-Malate ngA_{oxigen}/min/mg of RLM				
control	17.4 ± 3.8	90.0 ± 5.5	5.3 ± 0.8	110.5 ± 21.0
methyl-GAG	17.9 ± 2.8	80.0 ± 4.0	4.9 ± 1.2	106.2 ± 14.2
41	23.1 ± 5.2	47.7 ± 23.6*	2.3 ± 1.2**	74.2 ± 19.3
Succinate ngA_{oxigen}/min/mg of RLM				
control	40.6 ± 2.8	134.5 ± 13.1	3.3 ± 0.3	191.0 ± 35.8
methyl-GAG	39.2 ± 1.9	141.1 ± 26.1	3.7 ± 0.7	168.7 ± 19.8
41	33.4 ± 4.3	54.9 ± 10.7**	1.7 ± 0.4**	81.7 ± 20.9
Ascorbate-TMPD ngA_{oxigen}/min/mg of RLM				
control	83.1 ± 14.6	125.9 ± 22.1	1.5 ± 0.2	141.9 ± 20.6
methyl-GAG	91.1 ± 15.1	128.2 ± 13.7	1.7 ± 0.4	155.5 ± 9.9
41	98.0 ± 8.3	141.8 ± 15.9	1.5 ± 0.2	158.3 ± 10.1

[a] RML: rat liver mitochondria. [b] Mean of five and eight independent measures. [c] ICR: respiratory control index; * $P < 0.05$ ** $P < 0.001$.

other hand, compound **41** has a more pronounced inhibitory effect on the oxygen consumption induced by both glutamate-malate/succinate, whereas it has no effect on the respiration supported by ascorbate/TMPD as described in Table 1. Beside an uncoupling effect, compound **41** inhibits ADP-stimulated respiration (State 3) as well as uncoupled respiration, obtained by 2,4-dinitrophenol (DNP) addition: it seems therefore reasonable to assume a direct effect on one of the respiratory complexes. The lack of effect on ascorbate/TMPD respiration excludes Complex IV as the target of compound **41**. Measurements of separate enzyme complexes of the respiratory chain in the presence of **41** revealed that it is not capable of inhibition on Complex I and on Complex II (succinate-DCIP reductase activity) (data not shown). These results suggest that Complex III (ubiquinol−cytochrome *c* reductase) should be the target of this compound. To test this hypothesis we have measured the oxygen consumption induced by saturating amounts of CoQ_1H_2. The inhibition of **41** on $CoQ_1H_2-O_2$ activity is dose dependent (see Supporting Information) but it was not possible to reach a 100% inhibition (60 μM of **41** induces about 80% of inhibition). It should be reminded that this compound has a very low water-solubility, and it was impossible to test higher concentrations. Moreover the low water-solubility of compound **41** may induce aggregation that can prevent its efficient partition in the lipid phase where the target enzyme is located.

(c) Growth Inhibition and Effects on Cell Viability and Mitochondrial Potential. To investigate the mechanism of action of these compounds, the antiproliferative effects of compound **41** were studied by monitoring them in two tumor cell lines: HT29 (colon adenocarcinoma) and HL60 (promyelocytic leukemia). These cell lines were chosen considering the interesting activity reported by NCI's assays on colon cancer and leukemia panels. We have investigated the effects of treatments on cell growth in the range of concentrations between 10^{-8}M and 10^{-4} M (see Supporting Information). It is evident that no detrimental effect was detectable after 24 h at doses up to 10^{-5} M; only after 48 h compound **41**, just at the 10^{-6} M concentration, affected the proliferation in HT29 cells. However, after 24 h in both the cell lines, the higher doses caused a marked cytotoxic effect, leading to death about 75% of the cell populations at the concentration 10^{-5} M and of the whole cultures at 10^{-4} M. Moreover in HT29 cells a growth inhibitory effect was observed after 48 h of treatment at the 10^{-6} M concentration, but in this case no increase in cell death was detectable. Taking into account the activity shown by compound **41** on Complex III and to assess if it could interfere with mitochondrial function in intact viable cells as well as on isolated mitochondria and in order to discriminate if the observed cytotoxicity was due to an apoptotic or a necrotic effect, the HT29 and HL60 cells were treated with compound **41** at the10^{-5} M concentration. Five, seven, and twenty-four hours after treatment, cells were analyzed by flow cytometry for cell death, mitochondrial membrane depolarization, and cell dimensions by using propidium iodide (PI) exclusion for cell viability, the potentiometric fluorescent dye $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) to measure mitochondrial membrane depolarization in intact viable cells,[17,18] and forward-angle light scattering (FS) signals to evaluate variation in the cell size. In fact, in most cases, apoptosis can be distinguished from necrosis on the basis of the scatter parameters:[19] in the initial stages of apoptosis the cell shrinks, while the membrane remains intact; during necrosis, cell swelling occurs as a result of early failure of the membrane integrity. As a consequence of these cellular changes, FS decreases during the initial phases of apoptosis, whereas during necrosis, FS increases immediately.[20]

Cytofluorimetric analysis showed that compound **41** was able to induce a rapid cell death in HT29 cells: the percentage of dead cells increased from 7 ± 5% in controls to 18 ± 8% after 5 h of treatments and to 32 ± 5% after 7 h. In parallel, an increase in cells characterized by a low mitochondria potential and reduced size was observed: this population represented the 0.8% ± 0.6% in control cells and was respectively the 6 ± 2%, 12 ± 4%, and 37 ± 8% after 5, 7, and 24 h. Furthermore, a clear decrease in mitochondrial potential of viable cells with normal size was detectable after 7 h of treatment. The reduction in mitochondrial potential preceded the cell shrinkage and obviously the alteration in plasma membrane permeability, evidenced by the PI uptake: this supported the hypothesis of induction of apoptosis by mitochondrial perturbation, which produces the characteristic morphological changes of apoptosis culminating, at the end of the process, with the loss of membrane integrity.

Interestingly, HL60 cells treated with this dose (10^{-5} M) showed a very high cytotoxic effect even after 3 h, while 10^{-6} M did not show any effect. An intermediate dose (5×10^{-6} M) had to be used to induce effects similar to those achieved in HT29 with the dose 10^{-5} M (data not shown). Methyl-GAG, at the same concentrations, showed a faint effect on cell proliferation; only the 10^{-4} M dose is able to decrease HT29 and HL60 growth respectively to 24 ± 6% and to 32 ± 4% of the controls after 24 h of treatment. Moreover no effect was detectable on mitochondrial membrane depolarization with methyl-GAG treatment.

Conclusion

In general the new derivatives did not show a very high potency, but some of them had interesting activities in selected cell lines. In particular compound **41** had good features as potential antitumor agent; therefore, its mechanism of action was studied. In the past decades mitochondria have become the target of increased interest because many studies have demonstrated that they have an essential role on the regulation of cell death.[21−24] Moreover one mechanism by which tumor cells survive in the presence of chemotherapeutic agents is the increase of antiapoptotic activities.[25] Development of cytotoxic drugs that target mitochondria may provide a new strategy to induce apoptosis in tumor cells. Since drugs bearing the guanidino groups influence the mitochondrial functions at different levels, we studied compound **41** by focusing our attention on these biological organelles. Compound **41** was able to inhibit Complex III and consequently produce a decrease in the mitochondrial potential; moreover it was able to induce at 10^{-5} M concentration, a time dependent increase of

cells showing dull $DiOC_6(3)$ fluorescence and reduced FS signals, thus suggesting that compound **41** is able to induce apoptosis. The relationships between mitochondrial respiratory inhibitors and cell death are unknown; nevertheless a decrease of total electron flow through the respiratory chain may be responsible for enhanced production of reactive oxygen species[26] that on the other hand may generate apoptosis.[27,28] In any case it is reported that other Complex III inhibitors[29,30] induce apoptosis probably as a consequence of enhanced ROS (reactive oxygen species) production due to the impairment of the mitochondrial respiratory chain. By the collected data we may assume that the action on the mitochondria is fundamental or at least of principal importance to induce the antiproliferative effect. The molecular skeleton of these guanhylhidrazones allows structural modifications that we will consider in the development of new antitumor agents with this original mechanism of action.

Experimental Section

General Procedure for the Synthesis of the Guanylhydrazones 29−41, 44, 50, 51, 56, 58, 61, 66. The appropriate aldehyde (**16**−**28**, **48**, **49**, **54**, **57**, **60**, **65**) or quinone **43** (10 mmol) was dissolved in ethanol and treated with the calculated amount of aminoguanidine hydrochloride, prepared in turn from an ethanol suspension of aminoguanidine bicarbonate and an excess of 37% HCl. The reaction mixture was refluxed for 5−12 h, and after cooling, the resulting precipitate was collected by filtration. In the cases of compounds **29**, **30**, **33**, **34** the precipitate was favored by addition of ethyl ether to the ethanol solution.

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Supporting Information Available: Additional experimental procedures, C, H, N analytical data, ^{1}H NMR and IR spectroscopy, and tables and figures related to the biological assays. This material is available free of charge via Internet at http://pubs.acs.org.

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