Synthesis, Structure−Activity Relationships, and Antitumor Studies of 2-Benzoxazolyl Hydrazones Derived from Alpha-(*N*)-acyl Heteroaromatics

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Recently we have described the antitumor activities of 2-benzoxazolylhydrazones derived from 2-formyl and 2-acetylpyridines. In search of a more efficacious analogue, compounds in which the 2-acetylpyridine moiety has been replaced by 2-acylpyridine and α-(*N*)-acetyldiazine/quinoline groups have been synthesized. The 2-acylpyridyl hydrazones inhibited in vitro cell proliferation in the nM range, whereas the hydrazones derived from the α-(*N*)-acetyldiazines/quinolines inhibited cell growth in the μM range. Compounds tested in the NCI-60 cell assay were effective inhibitors of leukemia, colon, and ovarian cancer cells. ***E*-13k** [*N*-benzoxazol-2-yl-*N*'-(1-isoquinolin-3-yl-ethylidene)-hydrazine] inhibited the proliferation of MCF-7 breast carcinoma cells more efficiently than nontransformed MCF-10A cells. It is not transported by P-glycoprotein and a weak MRP substrate. Increased concentrations of serum or α_1-acid glycoprotein did not reduce the antiproliferative activity of the compound. In the in vivo hollow fiber assay, ***E*-13k** achieved a score of 24, with a net cell kill of OVCAR-3 (ovarian) and SF2-95 (CNS) tumor cells.

Introduction

Despite recent progress in tumor therapy, the majority of solid tumors are currently not curable by chemotherapy. Half of all cancer patients fail to respond to chemotherapy or relapse from the initial response and ultimately die from their metastatic disease.[1] Therefore, there is the urgency to develop highly active drugs that are ideally orally active. One possible improvement in tumor chemotherapy resides in continued research designed to optimize the administration of the currently available antitumor drugs or to develop derivatives of the known antitumor drugs, which are better tolerated than current ones. Another possibility is to discover novel therapeutic products by exploiting our increased understanding of tumor biology. Novel agents, for example taxanes and camptothecins that target tubulin polymerization and topoisomerase 1, respectively, are showing promising results in ovarian, breast, and colon cancers.[1] Another example is the targeting of signal transduction molecules such as bcr/abl by STI571 (Glivec).[2]

Recently we became interested in developing novel inhibitors of ribonucleotide reductase (RR), the enzyme that converts ribonucleotide diphosphates to deoxynucleotide diphosphates with the assistance of free radicals.[3] The mammalian enzyme is composed of two dissimilar subunits, M1, the large subunit containing allosteric sites and M2, the small subunit containing a binuclear iron center and a tyrosyl free radical that is essential for reductase activity.[4,5] Either unit by itself is inactive. There are three broad classes of RR inhibitors. The first class includes nucleoside analogues, which bind to the M1 subunit of the enzyme.[6] The second class is mainly made up of short chain peptides that bind at the interface binding site of both units, thereby interfering with the enzyme activity.[7] The third class of RR inhibitors bind with high affinity to the nonheme iron in the M2 subunit.[6] Potent inhibitors interfering with the M2 subunit include the α-(*N*)-hetrocyclic carboxaldehyde thiosemicarbazones (TSCs, compound type **A**, Figure 1)[8] and 2-acylpyridine-α-(*N*)-hetarylhydrazones[9] (compound type **B**, Figure 1). From these compounds, we have deduced that an N*-N*-S* or N*-N*-N* structural motif is essential for RR inhibition. Based on this hypothesis, we have synthesized 2-benzothiazolylhydrazones derived from α-(*N*)-acetylpyridines, -diazines, and -(iso)quinolines (e.g., **Ia/b**, Figure 2) as potential RR inhibitors. In an in vitro antiproliferative assay, compounds **Ia/b** were found to be 80- to 100-fold more active than the TSC analogues.[10] Based on the interesting preliminary results we have investigated the effects of the replacement of the benzothiazole ring by the isosteric heterocycles benzoxazole and benzimidazole. Compounds **IIa/b** (Figure 1) turned out to be more active than the benzothiazole analogues **Ia/b** and, moreover, were found to exhibit potent growth inhibitory activity against the proliferation of a number of cancer cell lines where effective agents are currently sought.[11,12] However, it turned out that all these compounds do not inhibit RR activity.

In a continual development effort, we have instituted a systematic structural exploration of compound **IIb**, with the goal of synthesizing a more efficacious compound or identifying a new lead for the development of second-generation compounds. Herein we report the synthesis, antiproliferative activities, and the inhibition of the in vivo tumor growth of analogues of compound **IIb**.

Chemistry. The 2-hydrazinobenzoxazoles **3a**[13] and **3b**[14] were synthesized according to reported procedures, however, avoiding the use of dioxane as a solvent in the case of compound **3a**. Reaction of 2-chlorobenzoxazole (**1**) with hydrazine hydrate (**2a**) in tetrahydrofuran (THF[a]) at −5 °C afforded **3a** in high yield. Reaction of 2-acetylpyridine and hydrazine derivative **3b** in methanol containing traces of glacial acetic acid afforded the hydrazone **4**. Compounds **5** and **6** became accessible from the condensation of 2-hydrazinobenzoxazole (**3a**) with 3-acetylpyridine or 2-acetylpyridine-*N*-oxide,[15] respectively (Scheme 1).

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[1] Abbreviations: CH_2Cl_2, dichloromethane; DIPE, diisopropyl ether; EA, ethyl acetate; EtOH, ethanol; MeOH, methanol; THF, tetrahydrofuran; and BZO, benzoxazole.

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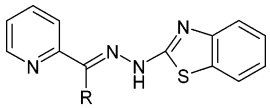
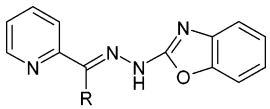
Figure 1. Proposed pharmacophore model.



Ia: R = H
Ib: R = CH_3



IIa: R = H
IIb: R = CH_3

Figure 2. Structures of lead compounds.

The alkyl-2-pyridinyl ketones **10a** (R = ethyl),[16−19] **10b** (R = *n*-propyl),[16,18] **10c** (R = isopropyl),[18] and **10d** (R = *tert*-butyl)[16,18] were synthesized by treating 2-cyanopyridine (**7**) with the corresponding alkylmagnesium halide, according to reported procedures (Scheme 1). Several of the reaction procedures were modified to maximize the yields of the ketones. Cyclopropyl 2-pyridyl ketone (**10e**)[20] was prepared in satisfactory yield by reacting 2-pyridyllithium (**9**) and cyclopropyl cyanide. The synthesis of the lithium compound **9** was accomplished by a lithium−bromine exchange reaction involving the interaction of *n*-butyllithium and 2-bromopyridine (**8**). The reaction of benzylmagnesium chloride (which is obtained from benzyl chloride and magnesium turnings) and 2-cyanopyridine (**7**) afforded 2-phenylacetylpyridine (**10f**)[21,22] in a satisfactory yield. Ketones **12a**−**k** are known compounds and, thus, were synthesized according to reported procedures (see Experimental Section). The synthesis of the hydrazone derivatives **11a**−**h** and **13a**−**k** (Scheme 1) was accomplished by reacting equimolar amounts of 2-hydrazinobenzoxazole (**3a**) with the appropriate carbonyl compounds **10a**−**h** and **12a**−**k** in methanol containing traces of glacial acetic acid (Scheme 1).

Spectroscopic Studies. The structures of all novel compounds were confirmed by IR and NMR spectroscopy, and the purity was confirmed by elemental analysis. Compounds containing the −C═N− substructure (e.g., TSCs, hydrazones, and so on) can exist in the *E*- or *Z*-form or as mixtures of *E*/*Z* isomers. Reports in the literature show that ^{1}H NMR spectroscopy can be used to differentiate between the two isomers. The most remarkable differences regarding the chemical shifts of the corresponding protons in the two isomeric forms are the resonance signals attributable to the NH protons: that is, δ (NH) = 14−15 ppm for the ***Z***-form and δ (NH) = 9−12 ppm for the ***E***-form.[23]

This conclusion is further supported by nuclear Overhauser effect (NOE) difference experiments.[24] For compounds existing in the ***E***-form, irradiation of the NH resonance leads to a positive NOE on the acetyl-CH_3 protons. Thus, considering these findings, *E*-configuration was assigned to compounds **4**−**6**, **11a**,**b**,**d**, **11f**,**g**, and **13a**−**k**. Compounds **11c** (30:70) and **11e** (10:90) were mixtures of *E*- and *Z*-isomers in the ratios indicated in brackets. In the ^{1}H NMR spectrum, the signals of the *E*/*Z*-isomers overlap except for the NH proton signals. On the other hand, compound **11h** cannot be assigned to an *E*/*Z*-isomeric form, although the signal for the NH proton appears at ∼14 ppm because of the symmetrical nature of the keto moiety.

Results

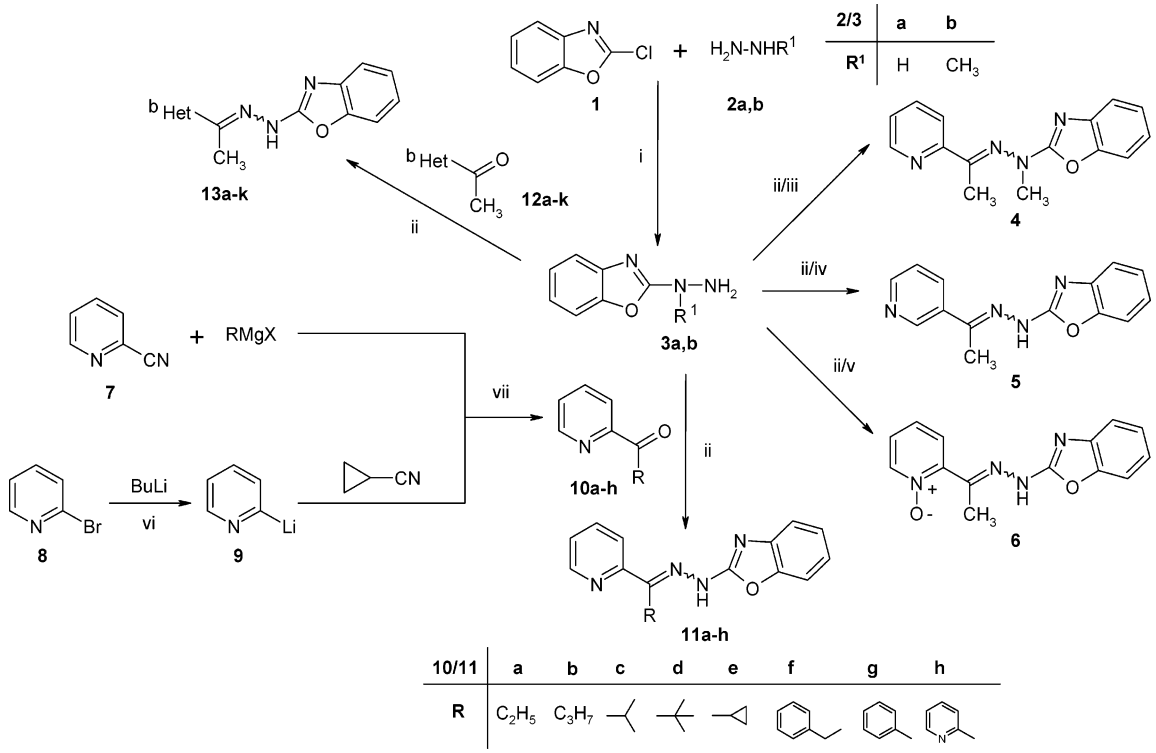
Antiproliferative Activity. The growth inhibitory activities of the new compounds were first tested in a panel of human tumor cell lines in tissue culture. This panel consists of two types of leukemia (Burkitt's lymphoma, CCRF-CEM), a cervix carcinoma (HeLa), and a colon carcinoma cell line (HT-29). Compounds ***E*-4**−**6** were synthesized to explore the importance of the N−N−N system on the antiproliferative activities of the novel hydrazones. Compared to ***E*-IIb**, the results in Table 1 show that transformation of the NH to N−CH_3 (***E*-4**) or the attachment of the hydrazone moiety beta to the hetero aromatic nitrogen (***E*-5**) results in a total loss of cytotoxic activity. However, oxidation of the hetero aromatic nitrogen to give compound ***E*-6**, which can be considered as a potential metabolite of ***E*-IIb**, results in a partial loss of activity by a factor of ∼54. Although it has been shown that ***E*-IIb** does not inhibit RR activity, it can be inferred from the results elaborated above that the N*−N*−N* structural motif is essential for the antiproliferative activities of these compounds.

After establishing the essential structural elements important for growth inhibitory activity of the hydrazones, we investigated the effect of replacing the methyl function in ***E*-IIb** with various aliphatic chains and aromatic moieties on cytotoxic activity (Table 2). Compound ***E*-IIb** was highly effective in inhibiting the proliferation of Burkitt's lymphoma (IC_{50} = 0.005 μM) and CCRF-CEM (IC_{50} = 0.024 μM) cells compared to HeLa (IC_{50} = 0.77 μM) and HT-29 (IC_{50} = 1.43 μM) cell lines. Although the same trend is observed for compounds where R = ethyl (***E*-11a**), *n*-propyl (***E*-11b**), or isopropyl (***E*/*Z*-11c**), these compounds turned out to inhibit the proliferation of HeLa and HT-29 cells to a greater extent than the lead compound ***E*-IIb**. Furthermore, compounds ***E*-11d** and ***E*/*Z*-11e** bearing a highly branched aliphatic side chain showed a total loss of cytotoxic activity. Considering what is elaborated above, we can conclude that the stereochemistry around the −C═N− does not influence the growth inhibitory activities of compounds **11a**−**e**, therefore, the loss of cytotoxic activity of ***E*-11d** and ***E*/*Z*-11e** can be explained on the basis that the presence of the *tert*-butyl and cyclopropyl moieties cause a steric hindrance that restricts the proper binding of these compounds to their molecular target(s).

Of the compounds where R is aromatic (Table 2), (a) the phenyl analogue ***E*-11g** inhibited the proliferation of Burkitt's lymphoma, HeLa, and HT-29 cells to the same extent, whereas the 2-pyridyl derivative **11h** was highly active against leukemia and lymphoma cell proliferation and (**b**) replacement of the phenyl with a benzyl moiety (***E*-11f**) results in a compound with high activity against HeLa-cervix carcinoma cells. Comparison of the growth inhibitory activities of ***E*-11f**, ***E*-11g**, and ***E*-11h** gives an indication that these compounds probably induce cell death through different mechanisms of action.

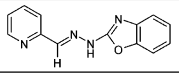
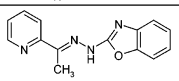
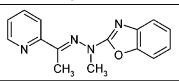
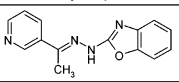
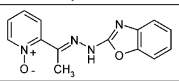
On the other hand, replacement of the 2-pyridyl moiety of ***E*-IIb** (IC_{50} = 0.005−0.024 μM) with a diazine or (iso)quinoline ring resulted in compounds with decreased activity (IC_{50} = 0.03−7.04 μM; Table 2). However, compounds ***E*-13a**−**k** were equipotent in inhibiting the proliferation of the solid tumor cell lines HeLa and HT-29 compared to the 2-acylpyridyl hydrazone analogues **11a**−**h** described above. The acetyldiazinyl hydrazone derivatives (compounds ***E*-13c**, **13d**, **13e**, **13f**, and **13h** in which Het is a 2-pyrimidine, 4-pyrimidine, or 2-pyrazine moiety) inhibited the proliferation of Burkitt's, CCRF-CEM, HeLa, and HT-29 cells in the submicromolar range. Compara-

Scheme 1[a,b]



[a] Reagents: (i) (**3a**) THF, 0−5 °C; (**3b**) EtOH, reflux; (ii) MeOH, CH_3COOH, 60−80 °C; (iii) 2-acetylpyridine; (iv) 3-acetylpyridine; (v) 2-acetylpyridine-*N*-oxide; (vi) diethyl ether, −40 to −50 °C; (vii) (a) diethyl ether/THF −15 °C; (b) 2 N HCl; (c) 2 N NaOH. [b]For structures of Het for compounds **12a**−**k**, see Experimental Section, and for compounds **13a**−**k**, see Table 2.

Table 1. Inhibition of Cell Proliferation by Hydrazones **IIa/b** and ***E*-4**−**6**[a]

		Inhibition of cell growth, IC_{50} (μM)			
Compd	**Structure**	**Burkitt**	**CCRF-CEM**	**HeLa**	**HT-29**
***E*-IIa**		0.005	0.040	0.774	1.443
***E*-IIb**		0.009	0.015	0.023	0.132
***E*-4**		> 50	> 50	> 50	> 50
***E*-5**		> 100	> 100	> 100	> 100
***E*-6**		0.027	-	0.288	4.781

[a] The mean values of at least two independent experiments in which duplicate determinations were taken within each experiment are indicated. For clearity, standard deviation has been omitted. Standard deviation is below 5%, except at low nanomolar IC_{50}s.

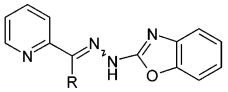
tively, the pyridazine derivatives ***E*-13a/b** were less active by a factor of 10−24. Among the acetyl-quinoline derivatives, the relative potency was in the following order: ***E*-13k** > ***E*-13j** > ***E*-13i**.

Organ-Specific Tumor Cell Lines. Selected compounds were submitted for testing in the organ-specific panel of human tumor cell lines at the National Cancer Institute, Bethesda, MD. This screen utilizes 60 human tumor cell lines, representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, kidney, prostate, and breast.[25] Advantages of the NCI-60 cell line in vitro assay and microarray data are that the results can be used to determine the uniqueness of a new lead for which the molecular target(s) might not be known. The GI_{50} values obtained along with the mean graph midpoint (MG MID) values are summarized in Table 3. The MG MID value is the average of the GI_{50} values for all the cell lines tested for drug concentrations in the range of 10^{-4}−10^{-8} M.[26]

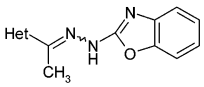
As can be seen, all the compounds exhibited high growth inhibitory activity across the cell lines tested, with an MG MID of 0.036−10.20 μM. All the compounds tested at the NCI inhibited the proliferation of colon (mean GI_{50} = 0.012−3.9 μM), ovarian (mean GI_{50} = 0.018−10.47 μM), and leukemia (mean GI_{50} = 0.012−5.13 μM) cell growth at lower concentrations compared to the MG-MID values given in Table 3. It should be mentioned that the compounds exhibited striking sensitivity to these cancers inhibiting six out of seven colon, five out of six leukemic, and four out of six ovarian cell lines. On the other hand, breast and prostate cancer cells are insensitive to the inhibitory activities of the novel hydrazones described. Only compounds ***E*-11f**, ***E*-11h**, ***E*-13d/e**, and ***E*-13k** inhibited the growth of nonsmall cell lung (NSCL) cancer cells at GI_{50} values equal to or below the MG-MID values. Furthermore, compounds ***E*-11f**, ***E*-11h**, ***E*-13c**, ***E*-13d**, ***E*-13e**, ***E*-13f**, and ***E*-13k** were also highly effective in renal cancer cells (mean GI_{50} = 0.018−0.199 μM), whereas only ***E*-11h** and ***E*-13f** inhibited the growth of melanoma cells above the MG-MID values.

The results are encouraging because colorectal, ovarian, and renal cancers are one of the leading causes of cancer mortality worldwide, and current chemotherapeutic agents administered alone or in combination, are frequently compromised by the development of drug resistance or dose-limiting toxicities. In view of the above-elaborated negatives of cytotoxics in current use, we conducted further studies to evaluate the potential usefulness of these agents. Compound ***E*-13k** was chosen as a reference substance for the following studies.

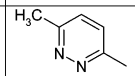
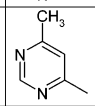
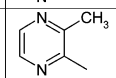
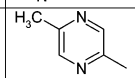
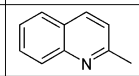
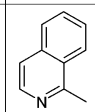
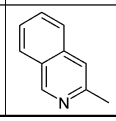
Table 2. Physical Data, Yields and Antiproliferative Activity of 2-benzoxazolylhydrazones **11a**−**h** and **13a**−**k**[a]



11a-h



13a-k

Compd	R	% yield	mp, °C	Inhibition of cell growth, IC_{50} (μM)			
				Burkitt	CCRF-CEM	HeLa	HT-29
***E*-IIb**	CH_3	-	-	0.005	0.024	0.774	1.43
***E*-11a**	CH_2CH_3	70	128-130	0.002	0.004	0.052	0.409
***E*-11b**	$CH_2CH_2CH_3$	65	132-134	0.006	0.006	0.153	0.851
***E/Z*-11c**		45	269-271	0.014	0.021	0.185	0.645
***E*-11d**		55	127-129	> 10	> 10	> 10	> 10
***E/Z*-11e**		60	290-292	0.185	-	4.49	20.59
***E*-11f**		65	208-210	> 10	> 10	0.042	0.219
***E*-11g**		70	158-160	0.120	0.078	0.043	0.044
11h		80	159-163	0.012	0.044	0.156	6.98
Compd	**Het**						
***E*-13a**		80	219-202	1.30	0.72	1.18	2.64
***E*-13b**		70	211-213	6.27	2.55	3.39	6.77
***E*-13c**		84	98-99	0.03	0.13	0.18	0.27
***E*-13d**		80	213-215	0.144	0.36	0.50	0.19
***E*-13e**		90	214-216	0.042	0.23	0.21	0.11
***E*-13f**		80	218-221	0.28	0.43	0.69	0.34
***E*-13g**		48	188-190	0.90	1.30	1.86	2.14
***E*-13h**		80	233-235	0.15	0.62	0.37	0.29
***E*-13i**		75	254-256	5.49	7.04	4.53	7.76
***E*-13j**		70	177-180	0.20	0.35	0.34	1.93
***E*-13k**		80	203-206	0.03	0.14	0.10	0.04

[a] The mean values of at least two independent experiments in which duplicate determinations were taken within each experiment are indicated. For clearity, standard deviation has been omitted. Standard deviation is below 5%, except at low nanomolar IC_{50}s.

Inhibition of Tumor versus Nontumor Cells. To explain whether tumor cells are inhibited more than nontumor cells, ***E*-13k** was tested in MCF-7 breast adenocarcinoma cells versus the nontumorigenic breast epithelial cell line MCF-10A. As shown in Figure 3, the proliferation of the normal MCF-10A cells was inhibited less than that of the transformed MCF-7 cells. This is an indication that the compound may exhibit less antiproliferative activity in normal cells compared to tumor cells. A comparison with hydroxyurea implicates a better therapeutic window for ***E*-13k**.

Drug Resistance and Drug Inactivation. Resistance to anticancer therapy is a major problem in the treatment of cancer patients. Resistance is, among others, frequently caused by the expression of the mdr1 (multi-drug resistance gene 1, ABCB1) or MRPs (multi-drug resistance associated proteins). Therefore, there is the view that new agents that are not transportable by these drug transporters can go a long way to alleviate some of the pitfalls in cancer chemotherapy.[27,28]

The antiproliferative activity of ***E*-13k** was examined in multidrug resistant HeLa−mdr1 cells in which P-glycoprotein is overexpressed due to transfection of the mdr1 gene.[27] Vinblastine was included as a positive control. The antiproliferative activity of vinblastine, a drug transported by the P-glycoprotein (P-gp), is strongly enhanced by 5 μM verapamil (Figure 4). However, 5 μM verapamil does not show this effect when combined with ***E*-13k**, indicating that mdr1 does not transport ***E*-13k**. An interesting observation is that some of the novel hydrazones inhibit the proliferation of mdr1, overexpressing cancer cells at lower IC_{50} values compared to the corresponding none P-gp overexpressing cells (results not shown).

A comparison of the MRP (ABCC1) expressing HL60/AR with the parental sensitive HL60 cell line shows a slight resistance to ***E*-13k** (Figure 5). The resistance ratio of the IC_{50} values in HL60/AR versus HL60 wild-type cells is approximately 1.7.

The binding of drugs to plasma proteins can have important implications on the pharmacodynamics/pharmacokenetics of such agents. The most important drug binding proteins in plasma include human serum albumin and alpha-1-acid glycoprotein.[29,30]

Therefore, we investigated whether the antiproliferative activity of ***E*-13k** is reduced if the serum in the medium is increased. In medium containing 30% fetal calf serum, the antiproliferative activity of ***E*-13k** was not reduced compared to that of a 10% serum (Figure 6). In a medium containing 0.1% α-1-acid glycoprotein, no difference to the standard medium was observed (Figure 7). Antitumor drugs may be applied intravenously, which may require heat sterilization. As shown in Figure 8, the same IC_{50} is obtained for both heat-treated and nonheat-treated samples of ***E*-13k**. This indicates that the novel hydrazones are stable to heat sterilization.

Hollow Fiber Assay. On the basis of the in vitro cytotoxic results elaborated in Table 3, compounds ***E*-13c**, ***E*-13e**, ***E*-13f**, and ***E*-13k** were selected for further evaluation as anticancer agents in an in vivo hollow fiber assay.[31] It has been suggested that this assay may well be suited for the prioritization of compounds for more advanced stages of in vivo xenograft drug evaluation.

An arbitrary score of 2 is assigned each time a compound produced a 50% or greater reduction in viable cell mass compared to the vehicle-treated controls. A drug is considered successful if it has a combined IP+SC score of ≥ 20, an SC score ≥ 8, or a net cell kill of one or more cell lines that can be observed. The results, listed in Table 4, show that of the compounds tested, only ***E*-13k** (NSC 693638) fulfilled one or more of the scoring criteria, with a combined score of 24 (IP = 20 and SC = 4). Furthermore, ***E*-13k** showed a net cell kill of OVCAR-3 (ovarian) as well as SF-295 (CNS) cancer cells.

Discussion

The in vitro result presented here implies that, the novel hydrazones may be interesting candidates that can find applica-

Table 3. Inhibitory Concentration (GI_{50} in μM) Values and Histological Cancer Types for the Novel Hydrazones ***E*-5**, ***E*-11f/h**, ***E*-13c−f**, and ***E*-13k**

	compound (mean GI_{50} μM)							
cancer type[a]	***E*-6**	***E*-11f**	***E*-11h**	***E*-13c**	***E*-13d**	***E*-13e**	***E*-13f**	***E*-13k**
leucemia	5.13	0.026	0.025	0.012	0.054	0.023	0.182	0.017
NSCL	12.30	0.059	0.049	0.051	0.148	0.044	0.251	0.026
colon	3.39	0.020	0.023	0.031	0.087	0.023	0.129	0.012
CNS	7.24	0.11	0.112	0.166	0.275	0.112	0.407	0.089
melanoma	16.98	0.10	0.028	0.050	0.224	0.117	0.174	0.037
ovarian	10.47	0.020	0.022	0.018	0.107	0.039	0.199	0.022
renal	11.20	0.066	0.069	0.032	0.219	0.022	0.173	0.015
prostrate	12.30	0.062	0.060	0.048	1.51	0.138	0.199	0.144
breast	22.90	0.148	0.234	0.079	0.302	0.123	0.447	0.068
MG-MID	10.20	0.059	0.050	0.042	0.170	0.069	0.204	0.036

[a] Each cancer type represents the arithmetic mean of five to eight cancer cell lines (NSCL = nonsmall cell lung; CNS = central nervous system).

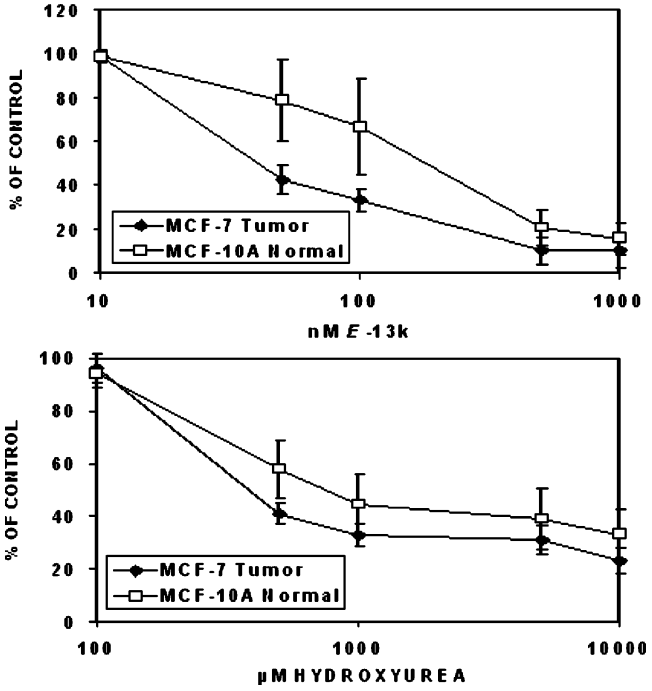


Figure 3. Effects of ***E*-13k** on the antiproliferative activity of nontransformed MCF-10A and transformed MCF-7 breast carcinoma cells. The cells were treated with the indicated concentrations of the drugs for 72 h. The mean (±SD) of two independent experiments, in which two samples were taken within each experiment, is indicated.

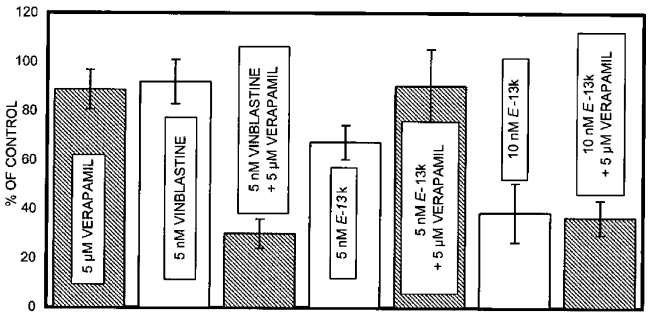


Figure 4. Combination of ***E*-13k** with verapamil. Multidrug resistant HeLa−mdr1 cells were treated with the indicated drugs for 72 h. Inhibition of cell proliferation compared to untreated controls was evaluated by the SRB assay. The mean (±SD) of two independent experiments in which two samples were taken within each experiment is indicated.

tion, especially for the treatment of colon, ovarian, and renal cancers. Moreover, it was found that the compounds exhibited preferential activity also against transformed cells over normal cells and were active under conditions that otherwise render a standard class of antitumor agents ineffective.

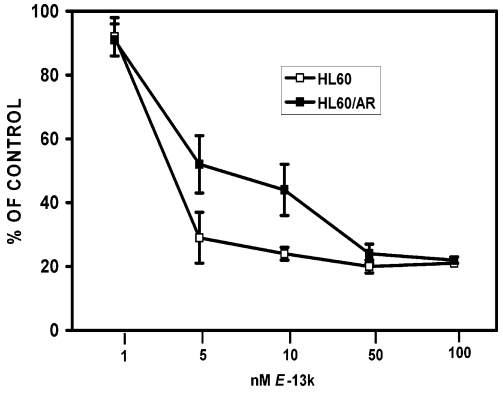


Figure 5. Inhibition of cell proliferation in sensitive HL60 and MRP (ABCC1) expressing HL60/AR cells. The mean (±SD) of two independent experiments in which four samples were taken within each experiment is indicated.

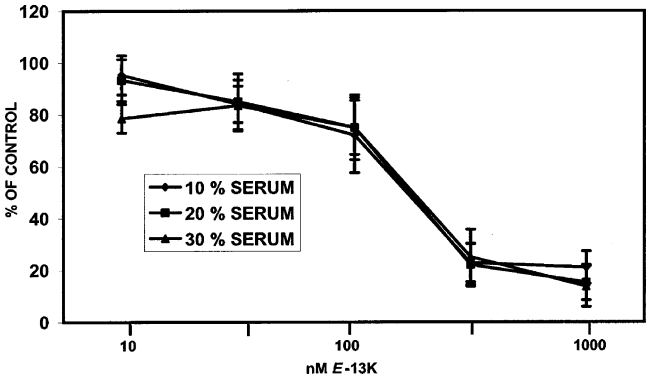


Figure 6. Influence of serum on the inhibition of cell proliferation by ***E*-13k**. HT-29 colon carcinoma cells were treated for 72 h with ***E*-13k** in medium containing the indicated concentrations of fetal calf serum. The mean (±SD) of two independent experiments in which two samples were taken within each experiment is indicated.

Compounds showing tissue specific profiles in the NCI disease oriented in vitro antiproliferative assay were advanced to the in vivo hollow fiber assay. Of the compounds tested in the in vivo hollow fiber assay, ***E*-13k** was found to be effective and also induced a net cell kill in cancer cells. This is important because it indicates that ***E*-13k** is not only cytostatic, but it really can kill cancer cells.

An important issue in cancer treatment is antitumor drug resistance. Verapamil does not increase the activity of ***E*-13k** in HeLa−mdr1 cells (Figure 4), and there is only a small difference between HL60 and HL60/AR cells (Figure 5). These are indications that the expression of mdr1 or MRP does not significantly influence the activity of these compounds.

To date, the putative mechanism(s) by which the novel hydrazones induce cell death is lacking. Patterns of drug activity

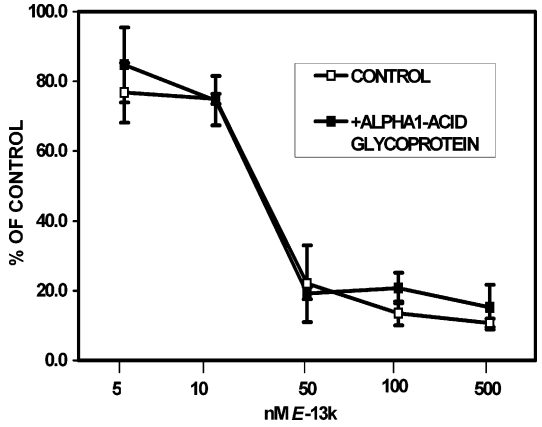


Figure 7. Inhibition of cell proliferation of Burkitt's lymphoma cells in normal medium (control) and in medium containing 0.1% α_1-acid glycoprotein. The mean (±SD) of two independent experiments in which two samples were taken within each experiment is indicated.

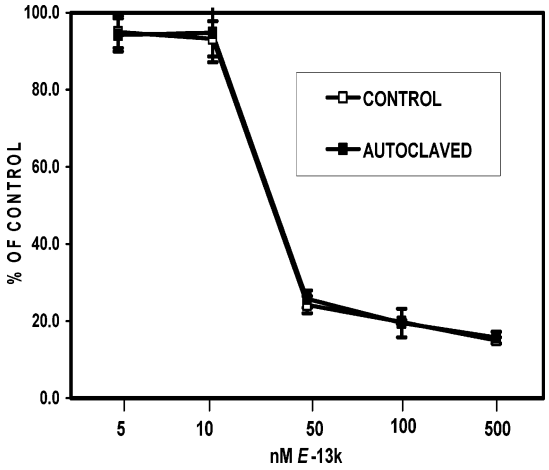


Figure 8. Inhibition of cell proliferation of Burkitt's lymphoma cells by normal (control) and autoclaved (15 min, 121 °C) ***E*-13k**. The mean (±SD) of two independent experiments in which two samples were taken within each experiment is indicated.

Table 4. Anticancer Activities of the Hydrazones ***E*-13c**, ***E*-13e**, ***E*-13f**, and ***E*-13k** in the in Vivo Hollow Fiber Assay

compound no.		IP score	SC score	total score	cell kill
***E*-13c**	**NSC 693639**	8	2	10	N
***E*-13e**	**NSC 693637**	2	6	8	N
***E*-13f**	**NSC 703100**	10	2	12	N
***E*-13k**	**NSC 693638**	20	4	24	Y

across the NCI-60 human cancer cell lines have been shown to contain detailed information that can be used to delineate the mechanism of action of new antitumor agents.[32,33] Antitumor agents with identical mechanisms of action possess identical or nearly identical cytotoxicity patterns. A Pearson correlation coefficient (PCC) with the COMPARE algorithm of >0.5 between an unknown and a known compound shows a similar mechanism of action. Using ***E*-13k** (NSC 693638) as a seed, a COMPARE analysis showed a positive correlation coefficient of >0.6 with a number of hydrazones of this class of compounds and a negative correlation with antitumor agents of known mechanism of action. This indicates that the hydrazones described represent a novel class of antitumor agents with a novel mechanism(s) of action. Currently, an intense study is underway to elucidate the biochemical mode of action of these compounds, and the results will be published soon.

In summary, one of the main goals of the present research, which was to design and synthesize compounds that show high efficacy compared to compound ***E*-IIb** or to obtain a suitable lead compound(s) for further chemical manipulation, has largely been met.

Experimental Section

Chemistry. Infrared spectra (IR) were recorded from KBr pellets on a Mattson Galaxy Series FTIR 3000 spectrophotometer. ^{1}H NMR spectra were recorded from DMSO-d_6 solutions on a Varian Gemini 2000 (^{1}H, 199.98 MHz; ^{13}C, 49.95 MHz) spectrometer. The center of the solvent signal was used as internal standard, which was related to TMS with δ 2.49 ppm (^{1}H) and 39.50 (^{13}C). Assignments are based on chemical shift considerations as well as homonuclear NOE difference experiments. Melting points were determined on a Reichert Thermovar hot stage microscope and are uncorrected. Elemental analyses were performed at the "Institut für Physikalische Chemie", University of Vienna, Austria, and the data for C, H, N are within ±0.4% of the calculated values. Reactions were monitored by TLC using Polygram SIL G/UV$_{254}$ (Macherey−Nagel) plastic backed plates (0.25 mm layer thickness) and visualized using a UV lamp. Column chromatography was performed using Kieselgel 60 (0.040−0.063 mm).

The following ketones utilized as starting materials were obtained according to the literature cited: methyl 3-pyridazinyl ketone (**12a**),[34] methyl 3-(6-methylpyridazinyl) ketone (**12b**),[35] methyl 2-pyrimidinyl ketone (**12c**),[36] methyl 4-pyrimidinyl ketone (**12d**),[35] methyl 4-(6-methylpyrimidinyl) ketone (**12e**),[37] methyl 2-(5-methylpyrazinyl) ketone (**12h**),[38] 2-acetylquinoline (**12i**),[39] 1-acetylisoquinoline (**12j**),[10] and 3-acetylisoquinoline (**12k**).[40] Methyl 2-pyrazinyl ketone (**12f**) and methyl 2-(3-methylpyrazinyl) ketone (**12g**) were purchased from Maybridge Co. 2-Benzoylpyridine (**10g**) and di-(pyridin-2-yl)-methanone (**10h**) were purchased from Sigma-Aldrich Co.

***E*-*N*-Benzoxazolyl-2-yl-*N*-methyl-*N*′-[1-pyridin-2-yl-ethylidine]-hydrazine (4).** A mixture of 2-acetylpyridine (0.67 g, 5.58 mmol) and **3** (0.92 g, 5.58 mmol) in MeOH (25 mL) containing 5 to 10 drops glacial acetic acid was heated at 60 °C, and the reaction was followed by TLC (CH_2Cl_2/EA 7:3). After completion (18 h), the reaction mixture was evaporated to dryness to give a dark brown oil. The compound was purified by column chromatography, eluting with CH_2Cl_2/EA (7:3) to give 0.85 g (53%) of a yellow oil. ^{1}H NMR (δ ppm) 2.51 (s, 3H, CH_3), 3.49 (s, 3H, N-CH_3), 7.08−7.28 (m, 2H, BZO-H5/6), 7.44−7.58 (m, 3H, BZO-H4/7, pyri-H5), 7.59 (dt, J = 1.8 Hz, J = 7.6 Hz, 1H, pyri-H4), 8.15 (d, J = 8.0 Hz, 1H, pyri-H3), 8.72 (dd, J = 1.2 Hz, J = 4.8 Hz, 1H, pyri-H6). Anal. ($C_{15}H_{14}N_4O$) C, H, N.

***E*-*N*-Benzoxazolyl-2-yl-*N*′-[1-pyridin-3-yl-ethylidine]hydrazine (5).** A mixture of 3-acetylpyridine (0.50 g, 4.13 mmol) and 2-hydrazinobenzoxazole (**2**; 0.62 g, 4.13 mmol) in MeOH (25 mL) containing 5 to 10 drops glacial acetic acid was heated at 70 °C. The reaction was complete after 18 h (TLC, CH_2Cl_2/EA, 7:3). After cooling, the mixture was evaporated to dryness, and the residue was recrystallized from EA/EtOH to give light yellow needles (75%): mp 197−199 °C; IR ν_{max} 3126, 2750, 1640, 1578, 1457, 1237, 990, 737 cm^{-1}; ^{1}H NMR (δ ppm) 2.37 (s, 3H, CH_3), 7.03−7.25 (m, 3H, BZO-H5/6, pyri-H5), 7.40−7.47 (m, 2H, BZO-H4/7), 8.22 (dt, J = 2.2 Hz, J = 8.0 Hz, 1H, pyri-H4), 8.57 (dd, J = 1.8 Hz, J = 4.8 Hz, 1H, pyri-H6), 9.10 (s, 1H, pyri-H2). Anal. ($C_{14}H_{12}N_4O$) C, H, N.

***E*-2-[1-(Benzoxazol-2-yl-hydrazono)-ethyl]-pyridin-1-oxide (6).** A mixture of 2-acetylpyridine-*N*-oxide (0.76 g, 5.58 mmol) and 2-hydrazinobenzoxazole (**2**; 0.83 g, 5.58 mmol) in MeOH (25 mL) containing 5 to 10 drops glacial acetic acid was heated at 60 °C. The reaction was followed by TLC (CH_2Cl_2/EA 7:3), and a voluminous light precipitate formed after 3 h. The mixture was cooled overnight, and the precipitate was collected by filtration and recrystallized from EtOH to give light yellow crystals (70%): mp 248−252 °C; IR ν_{max} 3006, 1657, 1586, 1458, 1238, 849, 727 cm^{-1}; ^{1}H NMR (δ ppm) 2.28 (s, 3H, CH_3), 7.04−7.27 (m, 3H, BZO-H5/6, pyri-H5), 7.40−7.50 (m, 3H, BZO-H4/7, pyri-H3), 7.57 (dd, J = 2.6 Hz, J = 6.6 Hz, 1H, pyri-H4), 8.28 (dd, J = 1.2 Hz, J = 5.8 Hz, 1H, pyri-H6). Anal. ($C_{14}H_{12}N_4O_2$) C, H, N.

General Procedure for the Preparation of the Target Hydrazones 11a−h and 13a−k. A mixture of 3.35 mmol of the appropriate carbonyl compound (**10a−h** and **12a−k**) and 2-hydrazinobenzoxazole (**2**; 3.3 mmol) in methanol (25 mL) containing 5 to 10 drops glacial acetic acid was heated at 80 °C, and the reaction was followed by TLC (CH_2Cl_2/EA 7:3). After completion, the reaction mixture was placed in a fridge overnight. The precipitates that separated out were filtered. Where there was no precipitate formed, the reaction mixture was evaporated to dryness, the residue was treated with DIPE, and the solid product that separated out was filtered. The compounds were recrystallized from the appropriate solvents.

Biological Methods. (a) Cytotoxicity Assays. Burkitt's lymphoma (CA 46, ATCC CRL 1648), CCRF-CEM (acute lymphoblastic leukemia, ATCC CCL 119), HeLa (epitheloid cervix carcinoma, ATCC CCL 2), multidrug resistant HeLa−mdr1 (= HeLa cells transfected with a wild-type multi-drug resistance gene 1, mdr1 = ABCB1[41]), HL60, and HL60/AR cells (expressing MRP = ABCC1[42]) were grown in RPMI 1640 medium. To HL60/AR cells was added 100 nM daunomycin and to HeLa−mdr1 cells was added 100 nM vinblastine every other week. HT-29 (colon adenocarcinoma, ATCC HTB 38) were grown in McCoy's 5A medium. MCF-7 (ATTC HTB-22) and MCF-10A (ATTC CRL-10317) were grown in Eagle's minimum essential medium, Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 0.01 mg/mL insulin. The media were supplemented with 10% fetal calf serum (except Burkitt's lymphoma with 15% and HL60 and HL60/AR with 20%), 2 mM glutamine, and 50 μg gentamycin/mL.

Detection of Cellular Proliferation. Inhibition of cell proliferation of HeLa, HeLa−mdr1, HT-29, KB-3-1, KB-C1, KB-HU, MCF-7, and MCF-10A cells was detected by the SRB assay.[43] Dose−response curves for Burkitt's lymphoma, CCRF-CEM, HL60, and HL60/AR cells were detected by an MTT assay[44] (Roche, Vienna, Austria). Approximately 10 000 cells per well were seeded in 96-well plates. After an initial incubation of 4 h, various drug concentrations were added to the cells and exposed continuously at 37° C in a humidified atmosphere of 95% air and 5% CO_2 for 72 h. The drugs were dissolved in dimethyl sulfoxide (DMSO). The concentration of DMSO was 0.5% and was also added to controls and not toxic. Subsequently, the samples were processed, and the absorption was detected by a microplate reader (Model 3550, Bio-Rad, Hercules, CA). Experiments with different concentrations of serum or addition of 0.1% α_1-acid glycoprotein and controls were counted with an electronic cell counter (Casy1, Schaerfe System, Reutlingen, Germany).

NCI Growth Inhibitory Determination. Cell culture and drug application procedures have been described previously.[25] Briefly, cell lines are inoculated into a series of 96-well microtiter plates, with varied seeding densities, depending on the growth characteristics of each cell line. Following a 24-h drug-free incubation, test agents were added at five 10-fold dilutions, with a maximum concentration of 100 μM. Cellular protein levels were determined after 48 h of drug exposure by sulforhodamine B colorimetry.

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Supporting Information Available: Experimental details on the preparation and ^{1}H NMR data of 2-acylpyridines **10a−5** and the novel hydrazones **11a−h** and **13a−k** and the microanalytical data (C, H, N) for compounds **4−6**, **11a−h**, and **13a−k**. This material is available free of charge via the Internet at http://pubs.acs.org.

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