Synthesis and Testing of New Antileukemic Schiff Bases of *N*-Hydroxy-*N'*-Aminoguanidine Against CCRF-CEM/0 Human Leukemia Cells *in Vitro* and Synergism Studies with Cytarabine (Ara-C)

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A series of eight new *N*-hydroxy-*N'*-aminoguanidine (HAG) Schiff bases [ArCH = NNHC(= NH)NHOH · tosylate] was synthesized as potential antitumor agents through the inhibition of the enzyme ribonucleotide reductase (EC 1.17.4.1). Five of the HAG derivatives (LK02 through LK06) were designed to contain an orthohydroxy group on the phenyl ring of ArCH = to increase the stability of the Schiff base formed. In addition, two compounds with a substituted quinoline [LK10; ArCH = (4-hydroxy-7-trifluoromethylquinolin-3-yl)methylene] or isoquinoline (LK11; ArCH = (5-nitroisoquinolin-1-yl)methylene] moiety were synthesized through multiple-step reactions involving reduction and/or oxidation. The IC_{50} values of the newly synthesized HAG Schiff bases were determined against human leukemic CCRF-CEM/0 cells in culture. The IC_{50} values of two previously reported HAG derivatives [ATL25; ArCH = (5-nitroisoquinolin-1-yl)methylene] and [LW02; ArCH = 2-hydroxy-3-allylbenzylidene)] were also determined for the first time against CCRF-CEM/0 cells. Among the compounds tested, LK11 was found to be the most potent (IC_{50}, 2.95 ± 0.1 μM) and the 4-methoxy-2-hydroxyphenyl derivative (LK02) to be the least potent (IC_{50}, 121 ± 16 μM). LK11 was about 15-fold more potent against CCRF-CEM/0 cells compared to the parent compound hydroxyguanidine sulfate (IC_{50}, 46 ± 7.1 μM) and was about 32 times more potent than LK10 (IC_{50}, 97.6 ± 0.9 μM). LK11 in combination was incubated in sequence with cytarabine (ara-C) at various molar ratios against CCRF-CEM/0 cells for 48 hr. The results were analyzed using both the isobologram and the median-effect methods. This combination at a 1:1 molar ratio was about 6-fold more potent (IC_{50}, 0.16 μM) compared to ara-C alone (IC_{50}, 1.05 μM) and about 18-fold more potent compared to LK11 alone (IC_{50}, 2.95 ± 0.1 μM). In summary, the antileukemic potency of certain HAG derivatives can be improved by the presence of orthohydroxy groups of the phenyl ring, and a 1:1 molar combination of an isoquinoline HAG compound and ara-C leads to significant synergistic antileukemic activity against CCRF-CEM/0 cells *in vitro*.

KEY WORDS: Schiff bases of hydroxyaminoguanidines; antileukemic activity; cytarabine (ara-C); synergism; median-effect plots; isobologram.

INTRODUCTION

Hydroxyurea, hydroxyguanidine, thiosemicarbazones, polyhydroxybenzohydroxamic acids, and hydroxyaminoguanidines are known to inhibit cell growth as well as the enzyme ribonucleotide reductase (EC 1.17.4.1) (RR) (1–14). Of these hydroxyurea (HU) is the only compound that is currently in clinical use. Most of the above compounds have one or several of the following disadvantages: a shorter half-life, rapid metabolic inactivation, and side effects such as gastrointestinal toxicity and/or myelosuppression. Of the hydroxyaminoguanidines synthesized, ATL25, an isoquinoline derivative, was found to be the most potent *in vitro* against L1210 cells, with an IC_{50} of 3.3 μM (7). Structure–activity studies from previous reports of hydroxybenzohydroxamic acids suggested that the orthohydroxy group could contribute to the antitumor activity of the compounds (4). In addition, the only compound that was effective against P388 solid tumors *in vivo* from a series of hydroxyaminoguanidines (7) was ATL14 [1-(3',5'-dibromo-2'-hydroxybenzylidene)-amino-3-hydroxyguanidine tosylate; NSC 371168], which contained an orthohydroxyphenyl substitution. The present work was undertaken to probe further into possible enhancement of antileukemic activity by introducing substituent groups into the orthohydroxyphenyl ring and to improve the activity even further by combination with an antileukemic agent acting by a different mechanism such as ara-C.

Five new compounds of the general structure [ArCH = NNHC(= NH)NHOH · tosylate] were synthesized which contained an orthohydroxyphenyl ring (LK02 through LK06). Two compounds, a quinoline derivative (LK10) and an isoquinoline derivative (LK11), were also synthesized to confirm the previous finding that the isoquinoline compound was significantly more potent than the quinoline derivative against both CCRF-CEM and L1210 cells (8,11). The 5-nitro substitution in LK11 was introduced to possibly improve the antileukemic activity of ATL25 (12,13).

The target compounds (LK02–LK07 and LK10–LK11) were tested for their antileukemic activity *in vitro* against CCRF-CEM/0 cells. In addition to these compounds, ATL25 and LW02 were also included to compare the activities of the present compounds in the CCRF-CEM/0 cell system with known RR inhibitors of hydroxyaminoguanidine origin. LW02 was the most effective of the compounds tested previously against corona virus replication (8). The most active compound from the current group was then combined with ara-C to assess any synergistic activity against CCRF-CEM/0 cells.

MATERIALS AND METHODS

Chemistry

All the aldehydes, except for those utilized in the synthesis of LK10 and LK11, were purchased commercially and used in the synthesis without further purifications. Melting points were determined using a Thomas–Hoover melting-

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point apparatus with open capillary tubes and were corrected. The elemental analyses were conducted by Galbraith Laboratories, Knoxville, TN, or by C. F. Geiger, Ontario, CA. Infrared spectra were obtained under standard operating conditions using KBr pellets and a Beckman IR-4210 spectrophotometer. Proton NMR spectra were obtained by preparing the samples in $Me_2SO\text{-}d_6$ using 5-mm NMR sample tubes (Aldrich Premium NMR sample tubes, Aldrich Chemical CO.). A Varian A-200 Gemini 200-MHz NMR spectrometer with built-in software was used to obtain the spectra.

Synthesis of 1-Amino-3-Hydroxyguanidine Derivatives

Eight new compounds were synthesized according to the general procedure reported previously (6,15). The intermediate 1-amino-3-hydroxyguanidine tosylate was prepared by the following method. Thiosemicarbazide (45.6 g, 0.5 mol) and methyl-*p*-toluene sulfonate (93.1 g, 0.5 mol) were refluxed in 500 ml of methanol for 18 hr. The resultant *S*-methylisothiosemicarbazide tosylate was recrystallized from methanol:ether (2:1). The *S*-methylisothiosemicarbazide tosylate (0.4 mol) dissolved in methanol was added to the NH_2OH at the ratio 4:5. The mixture was stirred at room temperature for 48 hr. The 1-amino-hydroxyguanidine tosylate was recrystallized in ethanol:ether (2:1) (m.p. = 139–141°C; yield = 75.0%). The final Schiff base compounds were prepared by reacting equimolar concentrations of the corresponding aldehyde and the 1-amino-3-hydroxyguanidine tosylate, except in the case of LK07, where the respective concentrations were 1:2 molar. The reaction conditions ranged from stirring at room temperature for a short time (about 10 min in the case of LK04) to refluxing for 24 hr (in the case of LK10).

Preparation of (4-Hydroxy-7-trifluoromethylquinolin-3-yl) Formaldehyde

4-Hydroxy-7-trifluoromethyl-3-quinolinecarboxylic acid (5.2 g, 20 mmol) was suspended in 200 mL of freshly distilled tetrahydrofuran (THF) and kept in an ice bath. To this, 82.5 g (2.17 mol) of lithium aluminum hydride was added slowly, in several small portions, with stirring. After stirring for 24 hr at room temperature, 25 mL of ethyl acetate was added and stirred for an additional 2 hr. After hot filtration from methanol, and concentration, 3.7 g (15 mmol) of yellow solid was collected (m.p. = 245°C; yield = 76%). In 150 mL of acetone, 2.44 g (10 mmol) of (4-hydroxy-7-trifluoromethylquinol-3-yl)methanol was dissolved and 2.5 g (28 mmol) of manganese (IV) oxide was added to this while stirring. The reaction mixture was refluxed for 0.5 hr, hot-filtered, and dried to give 2 g (8 mmol) of the product (m.p. = 245°C; yield = 85%).

Synthesis of 5-Nitroisoquinoline Formaldehyde

To 6 mL of concentrated sulfuric acid in a small Erlenmeyer flask in an ice bath, 2.86 g (20 mmol) of 1-methylisoquinoline was added dropwise with continuous stirring. A solution of 1.5 mL each of concentrated nitric acid and sulfuric acid was added slowly so that the temperature of the flask did not exceed 35°C. The solution was poured into a 400-mL beaker containing 40 mL of H_2O and approximately 15 g of crushed ice and excess acid was neutralized using Na_2CO_3. The precipitate was dissolved in methanol, hot-filtered, and cooled to give 2.6 g (13 mmol) of 1-methyl-5-nitro-isoquinoline (yield = 65%, m.p. = 142–143°C). To 1.2 g (12 mmol) of selenium dioxide in 50 mL of 1,4-dioxane, a solution of 2 g (10 mmol) of 1-methyl-5-nitroisoquinoline in 50 mL of dioxane was added. The mixture was heated with agitation in a steam bath for 3 hr. The dark-red selenium formed was filtered off and the filtrate was concentrated *in vacuo* to give a reddish-brown gum. The residue was hot-filtered, concentrated, cooled, and recrystallized from methanol, to yield 1.1 g (5 mmol) of light yellow solid. The product was further purified by steam distillation and cooled. A white solid resulted (m.p. = 50–57°C; yield = 52%).

Biological Testing Procedures

IC_{50} Determination. The test compounds were dissolved in double-distilled water with a small amount of methanol or DMSO if necessary. The final concentrations of methanol or DMSO did not exceed 0.1%. The solutions were then filtered through 0.22 μm × 13-mm Millipore filters (Millipore GSWP). The stock solutions were made at 10^{-2} *M* and these were serially diluted to 10^{-7} *M* using the growth medium. Hydroxyguanidine sulfate was purchased from Pfaltz & Bauer Inc., Waterbury, CT. RPMI 1640 growth medium and heat-inactivated fetal calf serum were purchased from Irvine Scientific, Irvine, CA. The CCRF-CEM/0 human leukemia cell line was obtained originally from the DCT Tumor Bank, NCI, NIH, Frederick, MD, and maintained at Children's Hospital.

Sterile rectangular plastic incubator plates (4 × 6 = 24 wells per plate, 2 mL/well; Costar, Mark II, No. 3424) were filled with 0.9 mL of CCRF-CEM/0 cell suspension in RPMI. The test wells received 0.1 mL of an appropriate concentration of drug solution, while the control wells received 0.1 mL of RPMI with the final concentration being 2×10^5 cells/well (12,13,16). The drug concentrations in the test wells for the compounds ranged from 10^{-4} to 10^{-8} *M*. Each plate contained a positive control (hydroxyguanidine sulfate), which was placed in the last row. These plates were prepared in triplicate and were incubated at 37°C and supplied with 95% air/5% CO_2. After 48 hr of incubation, triplicate cell counts were made for each well. The inhibition of growth was expressed as a percentage of the growth of the untreated control wells. The dose–response curves were plotted using SIGMA plot (16,17) computer programs. The percentage inhibitions were converted into probits and the IC_{50} values were calculated (16–18).

Combination Treatment of LK11 and Cytarabine (Ara-C). Two rectangular plastic incubation plates (4 × 6 wells each) were taped together lengthwise to obtain a rectangular plate of 8 × 6 to represent the grid as described elsewhere (16). Increasing concentrations of ara-C purchased from Upjohn Co., Kalamazoo, MI, were introduced in columns on the grid and those of LK11 was filled in rows on the grid. The drug concentrations ranged from 10^{-5} to 10^{-7} *M* in half-log intervals for each drug. The third well from the left in the second row had the lowest concentration of cytarabine and LK11 combination (1:1) at 10^{-7} *M*. The seventh well from the left in the last row had the highest concentration of cyt-

arabine and LK11 combination (1:1), at 10^{-5} *M*. The diagonal wells between these two wells had the cytarabine and LK11 combination at a 1:1 molar ratio. The plates were incubated for 48 hr and triplicate cell counts were made.

Construction of an Isobologram. A 70% inhibition was chosen as the desired effect and the fractional concentrations were calculated. The construction of an isobologram was described by Berenbaum (19).

Construction of Median-Effect Plots. The theory and mechanics of construction of median-effect plots were described in detail by Chou and Talalay (16,20). Again a 70% inhibition was used in obtaining the plots, where f_a (fraction affected) = 0.7 and f_u (fraction unaffected) = 0.3. The f_a vs combination index (CI) plot was constructed by representing the fraction affected (inhibited) on the *X* axis and CI values on the *Y* axis. The CI plots were drawn for both mutually exclusive and nonexclusive cases of synergism as described by Chou and Tallalay (20).

RESULTS

Table I lists the structures, melting points, and percentage yields of the eight compounds synthesized in the present study as well as the IC_{50} values of all the compounds tested against CCRF-CEM/0 cells.

Synthesis

The synthesis of 1-amino-3-hydroxyguanidine tosylate

Table I. The Yields, Melting Points, and IC_{50} Values of the Target Compounds

Ar- CH = N - NH - C (=NH) -NHOH $HSO_3-C_6H_4-CH_3$

Compound	Ar	m.p. (°C)	Yield (%)	IC_{50} μM ± SD
LK02	OH; OCH_3	165	24	121 ± 16
LK03	H_3CO; OH; OCH_3	154–156	27	4.7 ± 0.3
LK04	OH; Br	235	85	12.8 ± 0.2
LK05	OH; I; I	186–188	71	6.4 ± 0.2
LK06	OH; NO_2	229–230	76	15.0 ± 2
LK07	N	206–208	28	7.1 ± 0.1
LK10	OH; CF_3; N	221–222	30	97.6 ± 0.9
LK11	NO_2; N	156–158	32	3.0 ± 0.1
LW02[a]	OH			6.9 ± 0.1
ATL25[a]	N			3.2 ± 0.2
Hydroxyguanidine sulfate[a]				46.0 ± 7.1

[a] Compounds not synthesized in the present work.

was the key intermediate step in the synthesis of the target Schiff bases. The preparation of *S*-methylisothiosemicarbazide by reacting thiosemicarbazide and methyl-*p*-toluene sulfonate (1:1) was relatively easy, with a high yield (85%). The optimal temperature for the condensation with NH_2OH was about 0°C initially and the temperature was allowed to rise to room temperature slowly with moderate stirring. The optimal pH was found to be between 6.5 and 7.0. The yield for LK02 (4-methoxy derivative) was the lowest at 24%, whereas that of the 5-bromo derivative (LK04) was 85%. The purification of the Schiff base was simpler in some cases (LK04, LK05), with a single recrystallization step and was more difficult, requiring multiple recrystallizations in some other cases (LK06).

IC_{50} Determinations

IC_{50} values were determined for eight LK series of compounds, LW02, ATL25, and hydroxyguanidine sulfate (HG). Both ATL25 and HG served as positive controls as known inhibitors of RR. Of all the compounds, LK11 was the most potent, with an IC_{50} value of 2.95 ± 0.12 μ*M*, followed by ATL25 at 3.15 ± 0.2 μ*M*. The least active compound was LK02 (IC_{50} = 121 ± 16 μ*M*). LK05, LK07, and LW02 were also active, with IC_{50} values under 10 μ*M*. The 4, 6-dimethoxy compound (LK03) (IC_{50}, 4.7 μ*M*) was about 28-fold more potent than the 4-methoxy (LK02) compound (IC_{50}, about 121 μ*M*). It seems that the additional methoxy group has a dramatic effect on cytotoxic potency. The 3,5-diiodo compound (LK05) was twice as active as the 5-bromo compound (LK04) and about 2.5 times more active than the 5-nitro compound (LK06). LK05 was almost as active as LW02 and LK07. The isoquinoline compound, LK11, was 32 times more cytotoxic than the quinoline compound LK10 and was very close to another isoquinoline compound ATL25. LK11 was slightly more active than ATL25 (IC_{50}, 3.15 ± 0.2 μ*M*). Except for LK02 and LK10, all the compounds were clearly more effective than the parent compound, hydroxyguanidine (IC_{50}, 45.96 ± 7 μ*M*).

Combination Treatment with Cytarabine (Ara-C) and LK11

The results of the cytotoxic effect of LK11 in the presence of ara-C were expressed as a percentage of the cell growth from control wells. At a 10^{-7} *M* concentration a 1:1 ratio of ara-C and LK11 resulted in approximately 50% inhibition, whereas ara-C alone had 20% inhibition and LK11 alone had about 10% inhibition at 10^{-7} *M* (Fig. 1). As the concentrations approached the IC_{50} value of LK11, the synergism was less profound, albeit still evident. Probit plots were constructed from the above data and the IC_{50} of ara-C was calculated to be approximately 1 μ*M*. The IC_{50} of the combination of ara-C and LK11 (1:1 molar) was approximately 0.15 μ*M*. The combination was about six times as effective as ara-C alone and was about 18 times more cytotoxic than LK11 alone.

Isobologram Analysis

The isobologram is shown in Fig. 2. The *I* values indicate that there was synergism at five combinations ($I < 1$); there was near-additivity ($I \sim 1$) at two combinations and

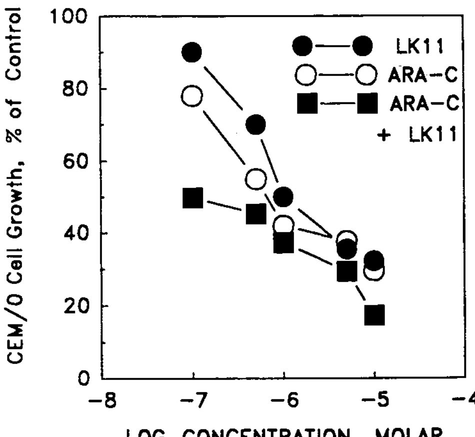

Fig. 1. Antileukemic effect of LK11 in the presence of ara-C (1:1 molar).

antagonism ($I > 1$) at three combinations. The isobologram could not accommodate all the points on a linear scale, and consequently attention was focused on synergistic aspects of this study. The datum point representing *I* value of 0.33 indicates the strongest synergism at an LK11 concentration of 5 μ*M* and an ara-C concentration of 1.4 μ*M*. When the concentrations were approximately reversed (I = 0.741, LK11 = 1.42 μ*M*, and cytarabine = 5 μ*M*), the decreased synergism seems to be approaching additivity. Even when the LK11 concentration was raised to 10 μ*M* and that of ara-C was at 0.22 μ*M*, the effect was still more of synergism (I = 0.56). There seems to be some tolerance for increases in LK11 concentrations but not in ara-C concentrations. This could be due to the nature of ara-C inhibition in the presence of LK11 or may be simply a result of its being the more potent of the two.

Median-Effect Plots

The median-effect plot of the combination treatment is shown in Fig. 3. The median-effect analysis of the data shows that ara-C is more potent than LK11 and the combination (1:1 molar) of ara-C and LK11 is approximately 6 times more potent than ara-C alone and about 18 times more potent than LK11 alone. These observations were confirmed by probit analyses. The IC_{50} values of cytarabine, LK11,

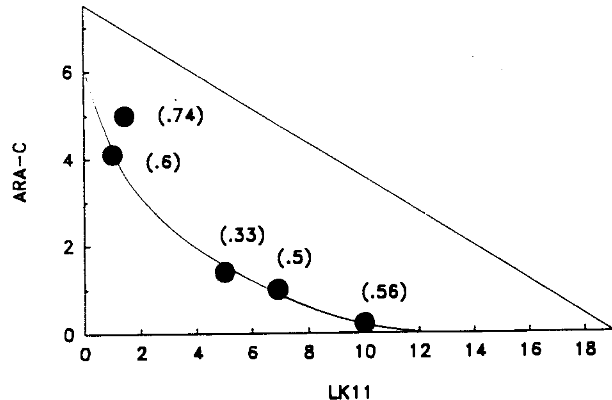

Fig. 2. Isobologram of LK11 and ara-C.

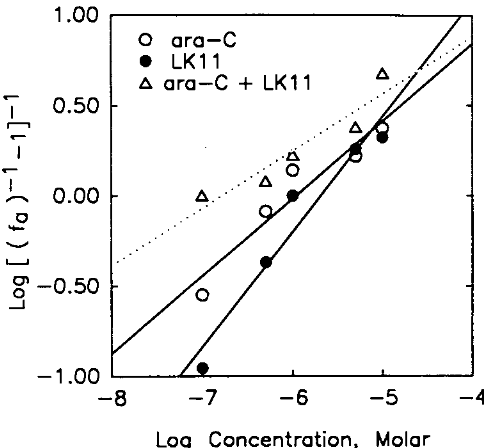

Fig. 3. Median-effect plot of ara-C, LK11, and a 1:1 molar combination of ara-C and LK11.

and their combination match well with the D_m values calculated from the median-effect plots. The r values of the median-effect plots are well over 0.9 and indicate that the data are suitable for this type of analysis.

DISCUSSION

The Schiff base formation is a nucleophilic substitution of the bimolecular (S_N2) type, where the amine is the nucleophile and the rate-limiting step is the loss of water from carbinolamine. The yields were consistent with the predictions based on the reaction mechanism. The reactions in the syntheses of intermediates of the aldehydes of LK10 and LK11 proceeded smoothly and as predicted.

The IC_{50} value of HG [as determined by Cory *et al.* (21) in L1210 cell system] at 38 μM compares well with the IC_{50} value from the present work at 46 μM, indicating that the results from the L1210 system and CCRF-CEM/0 system are slightly different. This also suggests that the L1210 system is slightly more sensitive of the two to HG. Cory *et al.* also found that L1210 cells, in general, are more sensitive to HAG derivatives than other cell types such as HT-29 (22). This generalization could be confirmed by similarly comparing the IC_{50} values of 5-nitro (LK6) and 4-methoxy (LK2) compounds from Cory and co-worker's data to this work. Similarly the 4,6-dimethoxy (LK3) compound was more potent in the L1210 cell type than in the CCRF-CEM/0 cell system.

Cory *et al.* (21) reported that the 5-nitro compound (LK6) was found to be the most potent among other nitro-substituted HAG derivatives in the L1210 and HT-29 cell lines. The orthohydroxy group seemed to enhance the activity, probably by providing more stability through several possible structural conformations resulting from intramolecular hydrogen bond formations. Such hydrogen bonds could occur among the oxygen, hydrogen, and nitrogen atoms present on the ring and in the hydroxyguanidine chain. This hypothesis could be further strengthened by the observation that LK6 (2-hydroxy-5-nitro compound) was approximately 10 times more potent than compound IIIA, which lacked only the orthohydroxy group. Cory *et al.* (14,21,22) expanded upon the work of T'ang *et al.* (7) and pointed out that the isoquinoline derivative of HAG was approximately 10 times more potent than the quinoline derivative in the L1210 cell lines. The present report confirms this observation. In fact the difference in potencies between these two types of compounds (LK11, a 5-nitroisoquinoline; and LK10, a quinoline) in this report was approximately 30-fold. But the IC_{50} value of LK11 (2.95 μM approximately) is only slightly better than the IC_{50} value (3.15 μM) of unsubstituted quinoline derivative (ATL25). Even though the substitutions on LK10 and LK11 were not identical for making direct comparisons, the basic difference in potencies between these compounds should be attributed mainly to the quinoline and isoquinoline rings. At that structural level, they differ in the electron density on the ring as well as the geometry and the relative position of the ring nitrogen to the hydroxyaminoguanidine moiety. Molecular models made in this laboratory have suggested that intramolecular hydrogen bonding leads to different conformations for quinoline and isoquinoline compounds. Molecular models of orthohydroxy compounds have also shown various possible conformers due to intramolecular hydrogen bonding (12). The fact that the orthohydroxy compounds were obtained in higher yields in the present [compared to some previously synthesized compounds in this laboratory that lacked the orthohydroxy group (6–8)] suggests that these compounds were more stable, most probably because of intramolecular hydrogen bonding facilitated by the orthohydroxy group. More elaborate studies are needed to identify which of these conformations is the most stable for each compound and if that conformation indeed contributes to cytotoxicity.

The results of the combination effect of isoquinoline-HAG (LK11) with cytarabine suggest synergistic activity at certain concentrations. Since cytarabine and LK11 are presumed to have different mechanisms of action, such a synergism is called mutually nonexclusive synergism and is expected. The synergism shown in the present experiment is moderately strong, since the 1:1 combination of cytarabine and LK11 has an IC_{50} of 0.15 μM, which is approximately one-sixth of the IC_{50} value of ara-C. But some combinations resulted in mere additivity, and some even in antagonism. One reason for this could be that the scheduling of combination was not optimal, or LK11 might have additional mechanisms of action that interfere in some way with the action of ara-C. The importance of proper scheduling of combinations in cancer studies has been documented (23). More detailed studies into the mechanism and sites of action of LK11 and on proper scheduling of its combinations with other drugs are needed for a better understanding.

Isobologram and median-effect plots are the two most commonly used methods of analyzing synergism data. The isobologram and median-effect plots gave very similar results in terms of the IC_{50} values of the combination effect. This shows that, despite its limitations, the isobologram method is comparable to the median-effect method under the present set of experimental conditions. On the other hand, the median-effect method is most practical when combinations of drugs other than 1:1 molar ratios need to be analyzed. However, the limitations of median-effect method, as pointed out by Nocentini *et al.* (24), need to be considered as well.

In summary, eight new hydroxyaminoguanidines were

synthesized and their IC_{50} values were determined against CCRF-CEM/0 cells *in vitro*. In addition, the combination of a 1:1 molar ratio of a hydroxyaminoguanidine and cytarabine (ara-C) was shown to be significantly more potent than either drug alone and was synergistic.

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