Acta Oncologica Vol. 33, No. 8, pp. 953–961, 1994

ANTILEUKEMIC ACTIVITY STUDIES AND CELLULAR PHARMACOLOGY OF THE ANALOGUES OF 2-HYDROXY-1H-ISOINDOLE-1,3-DIONE (HISD) ALONE AND IN COMBINATION WITH CYTOSINE ARABINOSIDE (ara-C) AGAINST HUMAN LEUKEMIA CELLS CEM/0

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The 2-hydroxy-1H-isoindole-1,3-dione (HISD) derivatives are inhibitors of ribonucleotide reductase (RR). Among the seven new compounds of the PL series, three were found to be active in cell lines sensitive (CEM/0) or resistant to ara-C (CEM/ara-C/7A; CEM/dCk[−]). The compounds PL4, PL7 and PL8 exhibited IC_{50} values in micromolar range against the three CEM cell lines. The 3 compounds showed 100- to 1000-fold higher cytotoxicity compared to HU against all three CEM cell lines. Combination of each of the three active PL compounds with ara-C showed high degree of synergism in comparison to the combinations of HU with ara-C against both CEM/0 and CEM/ara-C/7A cell lines. The DNA synthetic capacity of CEM/0 cells treated with 10 μM PL4 for 24 or 48 h was inhibited ≥ 99.8% simultaneously the cellular NTP pools were severely depleted. Treatment of CEM/0 cells with 10 μM PL4 showed steady depletion in all the dNTP pools at 1 or 2 h and complete depletion by 4 h. The enzyme activity did not recover up to 48 h in presence of the drug suggesting irreversible inhibition of RR.

Among the enzymes that show elevated levels of activity in tumor cells, ribonucleotide reductase (RR) (E.C. 1.17.4.1) is one of the most important. It is an allosterically regulated enzyme that converts the nucleoside diphosphates (NDPs) to their corresponding deoxynucleoside diphosphates (dNDPs) through a complex regulatory mechanism involving one or several electron transfer pathways (1–4). Reduction of the ribonucleotides by RR enables the DNA polymerases to utilize the deoxyribonucleotides (dNTPs) during the process of DNA replication. RR activity is well coordinated to the process of cellular proliferation and is markedly increased in the late G1 and the early S-phase when the bulk of DNA synthesis occurs (5, 6). The important role of RR in the de novo synthesis of DNA makes it a target for chemotherapeutic agents.

Several chemotherapeutic agents have been designed and developed targeting several key sites on the two subunits of RR (7–11) and among these, hydroxyurea (HU) is the only compound that is extensively used clinically against leukemias. The majority of known potent RR inhibitors like thiosemicarbazones, 2,3-dihydro-1H-pyrazolo-2,3-imidazole (IMPY), 3,4-dihydroxybenzohydroxamic acid (didox) and guanazole showed toxicity in phase I/II clinical trials (12–15). The most common toxicities associated with these compounds are gastrointestinal toxicity and/or myelosuppression.

HU is known to interact with the M2 subunit of RR, specifically with the tyrosyl free radical (16, 17) and also with the ferric ions which are essential for enzymatic

Received 28 September 1993.
Accepted 3 August 1994.
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Paper presented at 18th International Congress of Chemotherapy, Stockholm, Sweden, June 27–July 2, 1993.

 ISSN 0284-186X

activity (18). The use of HU alone is limited because of its low cytotoxicity (due to a highly polar nature, low molecular weight), a very short half-life, and rapid development of resistance. In developing new HU derivatives the stepping-up principle was adopted (19). The compounds retain the pharmacophore of HU [-C(=O) N(OH)-] but have increased hydrophobicity and/or molecular size to overcome the drawbacks of HU. The new series of HISD compounds are related to HU and possess moderate to strong electron-withdrawing groups at position 6. The 2-hydroxy group is blocked either by methylsulfonyl or isopropylsulfonyl group to impart lipophilicity to the molecules.

It is well documented that HU in combination with ara-C exhibits synergism against CEM/0 cells (20, 21). The synergism is attributed to blockade of de novo dCTP synthesis by RR thus lowering the intracellular dCTP concentration which in turn reduces the level of competition between ara-CTP and dCTP, the natural substrate for the DNA polymerases. Hence, synergistic cell kill of HU and ara-C is the consequence of greater inhibition of DNA polymerases by higher levels of ara-CTP in the presence of lowered levels of dCTP. Three active compounds from the new series (PL4, PL7 and PL8) have been combined separately with ara-C to assess the synergistic cell kill activity against CEM/0 cells and CEM/ara-C/7A.

In order to understand the mechanism of action of the HISD compounds, the effect of PL compounds on DSC, NTP pools and RR inhibition were also examined.

Material and Methods

Hydroxyurea was purchased from Aldrich Chemical Co., Milwaukee, WI. Cytosar-U (Cytosine arabinoside, ara-C) was purchased from Upjohn Co., Kalamazoo, MI. RPMI 1640 growth medium was purchased from Irvine Scientific, Irvine, CA, and heat-inactivated fetal calf serum was obtained from Gemini Bioproducts, Calabasas, CA. The PL compounds were synthesized in our laboratories and their purity were determined by TLC, the sharpness of the melting points and elemental analyses (percentage of carbon and hydrogen). The compounds were identified and characterized by ^{111}NMR and IR spectroscopy (22).

The CCRF-CEM/0 human leukemia cell line was obtained from DCT, Tumor Bank, NCI, NIH, Fredrick, MD and cultured in our laboratory at Childrens Hospital Los Angeles. The CEM/dCk(−) cell line (resistant to ara-C due to lack of deoxycytidine kinase activity) was provided by Dr. Arnold Fridland, St. Jude's Children's Research Hospital, Memphis, TN (23). The CEM/ara-C/7A cell line was developed in our laboratory by consecutive treatment with several high doses of ara-C and is a partially resistant to ara-C. The amount of ara-CTP formed in these resistant cells was about 50% lower than in the wild type CEM/0 cells after a identical treatment.

IC_{50} against CEM/0, CEM/ara-C/7A and CEM/dCk(−) human leukemia cell lines. The IC_{50}s of the new compounds were determined against CEM/0, CEM/ara-C/7A and CEM/dCK(−) cells. The 24 well plates (2 ml/well, Costar, Mark II, No. 3424) were used for detemining the IC_{50} values. The compounds were dissolved in dimethylsulfoxide (DMSO) and the final concentration did not exceed 1%. The drug solutions were sterilized through 0.22 μm × 13 mm Millipore filters (Millipore GSWP). The stock solutions (10^{-2} M) were diluted using the RPMI 1640 growth medium. Appropriate controls were run to account for any effect of DMSO. Each well received 0.9 ml of the cell suspension containing 2×10^5 cells. The treatment wells received 0.1 ml of drug solution and the control wells received 0.1 ml of culture medium (11). Each drug concentration (10^{-4} to 10^{-9} M) was plated in triplicate and incubated at 37 C, in an atmosphere of 95% air and 5% CO_2. The cells were incubated with various concentrations of the compounds for 24, 48 or 72 h, and were counted in a Coulter counter after the desired incubation period. The results were evaluated as percent of control after correcting for cell viability by the trypan blue dye-exclusion test. Probit analysis was performed to obtain the IC_{50} values (24). Hydroxyurea was used as positive control in identical studies.

Design of combination treatment of HU or PL compounds with ara-C against CEM/0 and CEM/ara-C/7A cells. The experimental procedure requires two 24 well-rectangular plastic incubation plates. Two of these plates were set-up lengthwise to create a 8 × 6 matrix. Serial concentrations of ara-C were prepared fresh in growth medium and sterilized. The ara-C drug solutions were added in columns on the grid and those of PL4, PL7, PL8 or HU were filled in rows on the grid. Appropriate DMSO controls were also performed. The amount of DMSO never exceeded 1% v/v and there was no detectable cytotoxicity at this concentration. The drug concentrations ranged from 10^{-4} to 10^{-10} M at intervals of one log unit for each drug. The diagonal wells of the grid had ara-C and PL4, PL7, PL8 or HU together in 1:1 molar ratio. Each well received 0.8 ml of cell suspension containing 2×10^5 (CEM/0 or CEM/ara-C/7A) cells. The wells that received a single drug had 0.8 ml of cell suspension, 0.1 ml of drug suspension and 0.1 ml of culture medium. The combination wells received 0.1 ml of both drugs to attain a final volume of 1 ml. The controls received an appropriate volume of the culture medium to have a final volume of 1 ml. The plates were incubated for 48 h, and the well contents were counted in triplicate and corrected for viability. Sequential exposure of PL7 and ara-C was similarly studied against CEM/0 wild type cells. The cells were pretreated with several concentrations of PL7 for 24 or 48 h and then exposed to different concentrations of ara-C for additional 24 h. The cells were not washed before adding ara-C to mimic the in vivo condition (5–7 elimination half-lives of the drugs are

required to wash out all the drugs from the system). The data were analyzed by isobologram and Median effect principle technique.

Construction of isobologram. The isobolograms were constructed according to the method of Berenbaum (25). In the combination experiments a 70% cell kill (f_a fraction affected = 0.7) was selected as the desired effect since the combinations tested were very cytotoxic. Hence the isobolograms constructed were ED_{70} isobols. From the cytotoxicity results the respective fractional concentrations of the two drugs were evaluated. The I (index) values, a measure of synergy, were calculated from the fractional concentration values. The equation describing the mechanics of isobologram is given in the Appendix.

Construction of median effect plots. The theory of median effect plots was derived from the principle of mass action which has been described in detail by Chou & Talalay (26). This method, unlike the classical isobologram method, can assist to determine synergism, additivity and antagonism at any effect level (i.e., for any f_a = fraction affected value). The combination indices (CI) were calculated for the combinations of the two compounds as described in the Appendix. CI plots were prepared by plotting CI on Y-axis and f_a on X-axis for mutually nonexclusive and/or exclusive case of synergism.

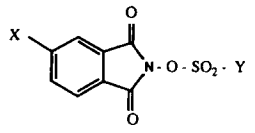
DNA synthetic capacity (DSC) following PL4 treatment for 24 and 48 h. To determine DNA synthetic capacity (DSC), the amount of tritiated thymidine (^{3}H-Thd) incorporated into cellular DNA of 1×10^7 CEM/0 wild type cells exposed to different concentrations of PL4 for 24 or 48 h was counted using a scintillation counter as described earlier. The cells were harvested, washed and incubated with tritiated thymidine for 1 h. Cells were then washed to remove the adhering surface radioactivity and lysed with 0.4 (N) perchloric acid (PCA). The acid insoluble fraction was redispersed in PBS and counted in a scintillation counter after adding 10 ml of scintillation cocktail. Since the drug was dissolved in DMSO, an appropriate control experiment was done with 1% DMSO. Each experiment was done in triplicate. The results were expressed as percentage of control (27).

Nucleoside triphosphate pools (NTPs) following treatment with PL4 for 24 and 48 h. To determine the effect of PL4 on the cellular nucleoside triphosphate pools (NTPs), CEM/0 cells were pretreated with different concentrations of PL4. Since each test flask contained 1% DMSO, controls with 1% DMSO were performed. About 1×10^7 cells treated with the various concentrations of drugs were harvested and washed. The cells were then incubated with ^{3}H-Cyd for 2 h. Nucleotides were extracted from 1×10^7 human leukemic cells using 0.4 (N) PCA as described elsewhere (27). The neutralized PCA extracts were assayed using gradient HPLC method performed on Waters 840 liquid chromatograph (Waters Associates, Milford, MA) equipped with model 510 gradient pumps, a U6K universal injector, and a 440 UV-VIS detector. The entire unit was controlled by a PC PRO 350 Controlled Equipment Digital Computer (Waters Associates, Milford, MA). The elution buffers were $NH_4H_2PO_4$, 0.005 M, (pH 2.8) and $NH_4H_2PO_4$, 0.75 M, (pH 3.6) at a combined flow rate of 2 ml/min. The method employed a 35 min linear gradient of a binary solvent system, from 0% to 100% of 0.75 M $NH_4H_2PO_4$ (pH 3.6), followed at the final conditions up to 42 min, after which the column was returned to the initial condition for equilibration for 10 min (0.005 M $NH_4H_2PO_4$, pH 2.8). Under these conditions the separation of nucleosides, bases, mono-, di- and triphosphates were achieved (27). The fractions were collected and counted by a scintillation counter. The depletion of NTPs was expressed as percentage of control.

Inhibition of ribonucleotide reductase. The whole cell RR assay was adopted (28) to measure the inhibition of RR by a representative HISD compound (PL4). Exponentially growing CEM/0 human leukemia cells were treated with 10 μM of PL4. Samples (4×10^8 cells) were collected at 1, 2, 4, 14, 24 and 48 h and washed with fresh RPMI 1640 growth media. The cells were resuspended in fresh growth medium containing 50 μl of [^{3}H] cytidine and 100 μl of 20 μM cytidine and incubated at 37°C for 1 h. After incubation, the cells were washed to remove the adhering radioactivity. Cells were then extracted with 0.4 (N) PCA, neutralized and stored at −20°C prior to analysis. To estimate the dNTP pools, the periodate oxidation method of Neu & Heppel was adopted (29). The procedure selectively removes the ribonucleotides sparing only the deoxyribonucleotides. An aliquot ($\approx$ 500 μl) containing 1×10^8 cell extract was subjected to periodate oxidation. The periodate oxidation method is as follows: The tubes were wrapped in aluminum foil to prevent photodegradation. About 0.5 ml of the PCA extract was treated with 0.2 ml of 0.1 M sodium periodate ($NaIO_4$) for 20 min at room temerature. To the reaction mixture, 0.2 ml of 1 M cyclohexamine was added, and the mixture was incubated for 90 min at 45°C. Glycerol, 0.2 ml (0.1 M) was then added to it and incubated at room temperature for 30 min. Finally 0.1 ml of 2 M formic acid (pH = 6.0) was added to the reaction tube and incubated for 30 min at room temperature. The final pH was adjusted to 7.0 prior to analysis. The deoxynucleotide triphosphates (dNTPs) were analyzed on a SAX-10 HPLC column on the same day. Fractions were collected and counted by a scintillation counter. An isocratic elution with 75% buffer A (0.005 M $NH_4H_2PO_4$, pH 2.8) and 25% buffer B (0.75 M $NH_4H_2PO_4$, pH 3.6) was maintained for 20 min at a flow rate of 3.0 ml/min. Then a linear gradient was adopted leading to 31% buffer A and 69% buffer B over 23 min. The total run time was 45 min and the retention times of the dNTPs were evaluated from those of the standards. Both the NTP and dNTP pools were analyzed at each time point and the results were expressed as percentage of control.

Table 1

2-hydroxy-1H-isoindole-1,3-diones and hydroxyurea along with the biological activities against three different CEM cell lines. ND: Not determined



Name	X	Y	Molecular weight	Melting point (°C)	IC_{50} CEM/0 (μm)	IC_{50} CEM/araC/7A (μm)	IC_{50} CEM/dCk(−) (μm)
PL-1	$-CH_3$	$-CH_3$	255.25	155.1–157.3	> 100	ND	ND
PL-2	–Cl	$-CH_3$	275.67	170.8–171.5	> 100	ND	ND
PL-4	–I	$-CH_3$	367.13	179.0–180.5	5.66 ± 0.18	4.95 ± 1.15	17.44 ± 1.87
PL-5	–Br	$-CH_3$	320.13	168.7–170.2	> 100	ND	ND
PL-6	–Br	$-CH(CH_3)_2$	348.18	138.2–138.8	> 100	ND	ND
PL-7	$-CH_3$	$-CH(CH_3)_2$	283.31	134.9–136.3	0.64 ± 0.24	2.0 ± 0.14	16.04 ± 2.36
PL-8	–Cl	$-CH(CH_3)_2$	303.73	128.9–130.0	3.50 ± 0.56	2.19 ± 0.26	81.49 ± 5.48
HU					609 ± 22.0	654 ± 11.4	990.3 ± 10.98

Results

IC_{50} of the HISD compounds or HU against leukemia cell lines in vitro. The IC_{50} values for the seven new HISD compounds and HU were determined against CEM/0, CEM/ara-C/7A and CEM/dCk(−) human leukemia cell lines in vitro. No appreciable cytotoxicity of HISD compounds was observed against CEM/0 cells after 24 h of exposure. Optimum cytotoxicity was achieved after 48 h of continuous treatment with the HISD compounds and the cytotoxicities were comparable after 48 or 72 h of treatment. Hence, the cytotoxicities of HISDs in all three cell lines reported here were determined after 48 h of continuous exposure to the compounds (Table 1). PL1, PL2, PL5 and PL6 had IC_{50} values greater than 100 μM against CEM/0 wild type cells, therefore they were excluded from further screening in the resistant cell lines. PL4, PL7, and PL8 had IC_{50} value of 5.66 ± 0.18 μM, 0.64 ± 0.24 μM and 3.50 ± 0.56 μM against CEM/0 cells. The same three compounds (PL4, PL7, and PL8) had IC_{50}s in the range of 2–5 μM against CEM/ara-C/7A cells and from 16–81 μM against CEM/dCk(−) cells. The IC_{50}s in the latter case was about 3- to 20-fold lower than those observed against both CEM/0 and CEM/ara-C/7A cell lines. Among the compounds tested, only PL7 was about 3-fold more toxic against CEM/0 cells than against CEM/ara-C/7A cells. Hydroxyurea, on the other hand, had IC_{50} of 609 ± 22 μM, 654 ± 11 μM, and 990 ± 11 μM against CEM/0, CEM/ara-C/7A, and CEM/dCk(−) cells respectively (Table 1). Apparently there seems to be a cross-resistance between ara-C and HU in these cell lines. Thus the three newly synthesized compounds were approximately 100- to 1000-fold more active than hydroxyurea against all three CEM cell lines.

Isobologram analysis of the combination studies. The isobolograms were constructed from the survival data following the combination treatment of PL4, PL7, PL8 or HU and ara-C at 1:1 molar ratio against wild type CEM/0 cells and CEM/ara-C/7A cells. Several combinations showed synergism (I < 1), a few combinations showed additivity (I ≈ 1), and there were also some combinations that exhibited antagonism (I > 1). The I values, which are measures of drug synergism are shown in parenthesis (Fig. 1). When PL4 and ara-C were combined at 1:1 molar ratio, strong synergism was obtained with 10^{-6} to 10^{-9} M of ara-C and 10^{-7} to 10^{-11} M of PL4. The strongest combination (I = 0.01) was observed with 10^{-7} M of ara-C

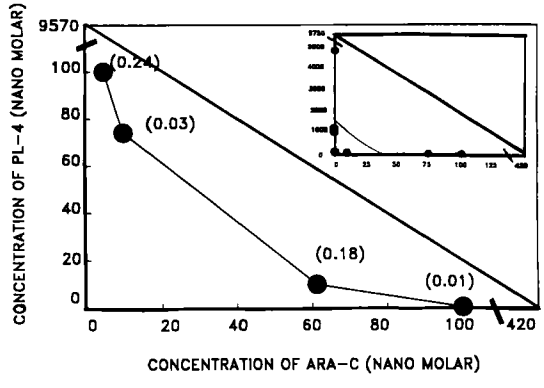


Fig. 1. The ED_{70} isobologram of PL4 and ara-C at 1:1 molar concentration ratio after 48 h of concurrent incubation with the drugs against CEM/0 cells showing strong synergistic activity. The numbers in parentheses represent the index (I) value which is a measure of synergy. The inset is the ED_{70} isobologram showing all the synergistic combinations between PL4 and ara-C in non-amplified axes.

Table 2

Combination treatment of PL series compounds and ara-C against CEM cell lines, showing the D_m value of the combination and degree of synergism compared to ara-C

Compound 1	Compound 2	Treatment	Ratio	D_m	Synergism (Compared to ara-C)	Cell line
PL4	ara-C	Concurrent; 48 h	1:1	0.052 nM	238	CEM/0
PL4	ara-C	Concurrent; 48 h	10:1	3.30 nM	55	CEM/0
PL4	ara-C	Concurrent; 48 h	1:1	0.11 pM	93	CEM/ara-C/7A
PL7	ara-C	Concurrent; 48 h	1:1	17.17 pM	28503	CEM/ara-C/7A
PL8	ara-C	Concurrent; 48 h	1:1	0.96 nM	118	CEM/ara-C/7A
PL7	ara-C	Sequential (24 h PL7 + 24 h ara-C)	1:1	0.005 pM	5×10^7	CEM/0
PL7	ara-C	Sequential (48 h PL7 + 24 h ara-C)	1:1	67.5 pM	6815	CEM/0
HU	ara-C	Concurrent; 48 h	1:1	0.14 μM	3	CEM/0
HU	ara-C	Concurrent; 48 h	1:1	2.93 μM	10	CEM/ara-C/7A
–	ara-C	Continuous; 24 h (Control)	–	350 nM	–	CEM/0
–	ara-C	Continuous; 48 h (Control)		28 nM	–	CEM/0
–	ara-C	Continuous; 48 h (Control)		291 nM	–	CEM/ara-C/7A

and 3.1×10^{-10} M of PL4. The combination of 10^{-5} M ara-C and 1.05×10^{-9} M PL4 had an additive effect. Antagonism was observed with a decrease in ara-C concentration and a subsequent increase in concentration of PL compounds. This pattern was observed in other studies as well. The strongest antagonism was observed with a combination of 1.57×10^{-10} M ara-C and 10^{-4} or 10^{-5} M PL4. This could be due to the sub-therapeutic concentration of ara-C (4 $\log_{10}$ concentration lower than the IC_{50} of ara-C). The isobolograms of PL4, PL7, or PL8 with ara-C against CEM/ara-C/7A cells showed an even higher degree of synergism. The HISD compounds increased the sensitivity of the partially resistant cells to ara-C by more than 1 000-fold. The survival results obtained after sequential exposure of CEM/0 cells to PL7 followed by ara-C exhibited a high level of synergism (Table 2). Similar drug synergism studies with HU and ara-C against CEM/0 and CEM/ara-C/7A cell lines exhibited a somewhat lower degree of synergism, 3- to 10-fold. In addition, significantly higher concentrations of HU and ara-C were necessary to achieve comparable drug synergism that was observed with lower concentrations of ara-C and PL compounds.

Median effect plots. The median effect plots of the PL compounds and ara-C alone or in combinations were constructed with $\log(f_a/f_u)$ on Y-axis and log molar concentration on the X-axis. The diagonal of the 8×6 grid had a molar ratio of 1:1 of both compounds and was used for the analysis. The D_m values ($\approx IC_{50}$) were 6.12 μM for PL4 and 0.012 μM for ara-C against CEM/0 cells, and matched well with their respective IC_{50} values determined from other experiments. The median effect plot of 1:1 molar concentration of PL4 and ara-C had D_m ($\approx IC_{50}$) value of 0.052 nM, which was lower than either compound alone (Fig. 2). This combination was several-fold more potent than PL4 alone and was 238-fold more potent than ara-C alone. The correlation coefficients (r) in all the cases were above 0.95. The CI (combination index) was calculated from the data for the case when the two compounds are mutually non-exclusive because it is hypothesized that HISD compounds of the PL series are potential inhibitors of ribonucleotide reductase and ara-C is known to inhibit DNA polymerases and therefore their mechanisms of action do not overlap. A plot of CI against f_a

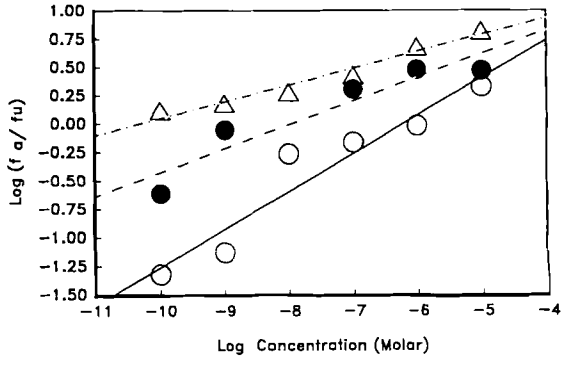


Fig. 2. The median effect plot of PL4 or ara-C alone and 1:1 molar combination of PL4 and ara-C after 48 h of continuous incubation with the drugs against CEM/0 cells. The open circles (○) represent PL4 alone; filled circles (●) represent ara-C alone; open triangles (△) represent PL4 and ara-C in 1:1 molar ratio. Approximately $2\frac{1}{2}$ $\log_{10}$ of drug synergism is obtained with PL4 and ara-C when compared to ara-C alone. An even higher degree of synergism (4 $\log_{10}$) is seen when compared to PL4 alone.

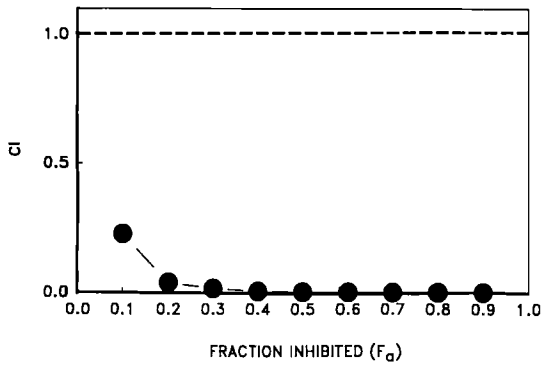


Fig. 3. The combination index (CI) plot derived from the median effect plot of PL4 and ara-C at 1:1 molar ratio against CEM/0 cells when PL4 and ara-C are considered to be mutually non-exclusive drugs. The dashed line of one represents the line of additivity. The CI data of the combination regimen between PL4 and ara-C are close to zero strongly suggesting a high degree of drug synergism.

shows that all the points are below 1 (the line of additivity), which indicates that the combinations are highly synergistic (Fig. 3). The combination test results of PL4, PL7, or PL8 with ara-C at a 1:1 molar ratio against CEM/ara-C/7A cells showed that the D_m values of the PL compounds also matched fairly well to their IC_{50} values against the same cell lines. The IC_{50} of ara-C against CEM/ara-C/7A cell line after 48 h of continuous incubation was 291 nM which was about 10-fold lower than that observed against CEM/0 cell lines (28 nM). The D_m values, along with the degrees of synergy compared to that of ara-C, are shown in Table 2. The combination of PL compounds with ara-C increased ara-C activity by more than a thousand-fold. The results from the sequential exposure experiments analyzed similarly are also shown in Table 2. The pretreatment of the cells with PL7 for 24 or 48 h increased the efficacy of the 24 h ara-C posttreatment, although a 24 h pretreatment with PL7 followed by 24 h ara-C posttreatment is significantly better than the 48 h pretreatment with PL7 followed by 24 h exposure to ara-C. The concurrent treatment of CEM/0 and CEM/ara-C/7A cells with HU and ara-C exhibited lower synergism as compared to the combinations of PL compounds with ara-C. The combinations against CEM/0 or CEM/ara-C/7A cell lines were 3- or 10-fold synergistic respectively when compared to ara-C alone.

DNA synthetic capacity. A substantial reduction in DNA synthetic capacity (DSC) was observed following 24 or 48 h of treatment with PL4 in wild type CEM/0 cells. At 10 μM of PL4, (about the IC_{50} value for this compound) the DSC was 0.2% and 0.1% of control after 24 or 48 h incubation respectively. At a concentration of 1 μM the DSC was 25% and 14.5% of control at 24 or 48 h respectively. At PL4 concentrations of 0.1 μM and 0.01 μM, no

Table 3

DNA synthetic capacity (DSC) of CEM/0 cells following treatment with PL4 for 24 or 48 h

Treatment	DSC after 24 h	DSC after 48 h
Control	100	100
DMSO control (1% v/v)	101.68 ± 5.88	107.25 ± 5.63
0.01 μM PL4	104.05 ± 9.97	73.77 ± 11.73
0.01 μM PL4	95.52 ± 8.96	83.31 ± 14.23
1.0 μM PL4	24.83 ± 7.83	14.48 ± 3.86
10.0 μM PL4	0.19 ± 0.05	0.07 ± 0.03

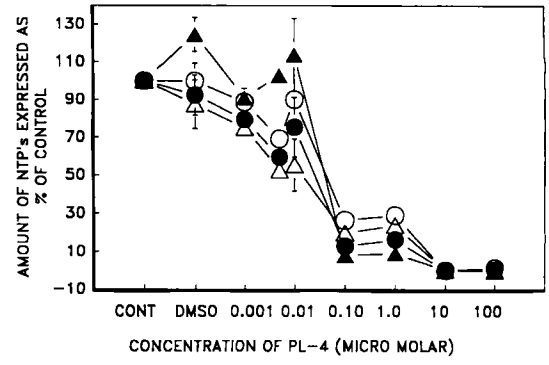


Fig. 4. The reduction of NTP pools following continuous treatment of CEM/0 cells with five $\log_{10}$ different concentrations of PL4 for 48 h. The data are expressed in percentage of control. The open circles (○) represent the CTP, filled circle (●) represent UTP, open triangle (△) represent ATP and filled triangle (▲) represent GTP pools. The data represent the mean ± standard deviations (n = 3) corresponding to each concentration point and for each of the NTP pools.

detectable change in DSC was observed after 24 h, however after 48 h of treatment with 0.1 and 0.01 μM of PL4, the DSC was 83% and 74% of the control respectively (Table 3).

Nucleoside triphosphate pools following treatment with PL4. Significant reductions in both the purine and the pyrimidine pools were observed following the treatment of CEM/0 cells with PL4. Reductions of all the NTP pools were observed after treatment with 0.1 μM PL4 for 48 h, but at lower concentrations no significant reductions in the NTP pools were seen (Fig. 4). No statistically significant changes in the NTP pools were observed with cells treated with 1% DMSO for a length of 24 or 48 h. At higher concentrations of PL4 (10 and 100 μM), all of the NTP pools were severely depleted after 24 or 48 h. Reductions in GTP and UTP were approximately 7-fold, while depletion of the ATP pool was about 5-fold, followed by a 2-fold depletion of the CTP pools. After treatment with 0.1 μM of PL4 for 24 h, the reduction in the NTP pools varied from 25% to 50% of control (Table 4), but at lower concentrations the depletion of the NTP pools was not significant.

Table 4

Nucleoside triphosphate levels expressed as percent of control following treatment with PL4 for 24 and 48 h against CEM/0 cells

Treatment	CTP	UTP	ATP	GTP
Control	100	100	100	100
24 h				
1% DMSO				
control	94.72 ± 8.94	87.65 ± 7.24	93.53 ± 5.78	88.26 ± 11.29
10.0 μM PL4	20.10 ± 3.22	11.23 ± 1.63	19.56 ± 2.57	12.62 ± 0.91
1.08 μM PL4	67.62 ± 1.08	51.57 ± 1.79	75.02 ± 2.55	58.14 ± 2.18
0.1 μM PL4	92.31 ± 7.61	73.66 ± 3.88	90.43 ± 7.12	79.63 ± 7.02
0.01 μM PL4	89.84 ± 2.50	73.60 ± 3.09	92.30 ± 1.26	81.03 ± 2.37
0.001 μM PL4	91.37 ± 8.97	67.83 ± 2.80	84.18 ± 3.81	72.36 ± 0.78
48 h				
1% DMSO				
Control	99.77 ± 9.54	92.44 ± 10.66	87.55 ± 12.93	124.58 ± 9.07
100 μM PL4	1.45	0.62	0.05	0.86
10.0 μM PL4	0.22 ± 0.07	0.18 ± 0.01	0.27 ± 0.15	0.26 ± 0.12
1.0 μM PL4	29.15 ± 2.16	16.43 ± 1.57	23.91 ± 1.15	9.28 ± 2.41
0.1 μM PL4	26.57 ± 2.25	12.97 ± 1.08	19.80 ± 0.61	8.17 ± 0.56
0.01 μM PL4	90.13 ± 5.60	75.45 ± 15.89	95.43 ± 13.68	113.91 ± 19.30
0.005 μM PL4	69.22 ± 0.87	59.22 ± 2.40	52.65 ± 1.02	102.87 ± 4.17
0.001 μM PL4	88.71	79.42	75.03	91.08

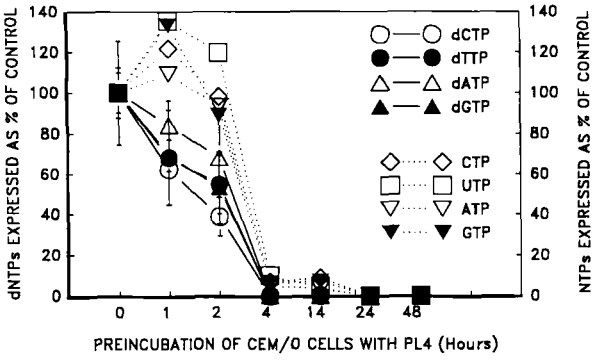


Fig. 5. The reduction of dNTP and NTP pools following treatment with 10 μM PL4. The data represent the mean ± standard deviations (n = 5) corresponding to each time point and for each of the dNTP and NTP pools.

Inhibition of ribonucleotide reductase. The RR activity in CEM/0 cells was reduced by 40% or 70% following 1 or 2 h incubation with 10 μM of PL4, and there was no apparent reduction in the NTP pools at 1 or 2 h. However, no dNTP pools were detectable after PL4 exposure for 4, 14, 24 or 48 h and after 4 or 14 h, the NTP pools were about 4 to 10% of the control, but there were no detectable NTPs after 24 or 48 h exposure to PL4 (Fig. 5). The RR activity was completely inhibited after 4 h of 10 μM PL4 treatment, and did not recover after 14, 24 or 48 h in the presence of the drug. The results suggest a possible irreversible enzyme inhibition.

Discussion

The compounds (PL4, PL7 and PL8) were 100-to 1 000-fold better than HU alone against the three CEM cell lines. The compounds did not show significant cytotoxicity following 24 h treatment suggesting that at least one cell-cycle is required to exhibit their cytotoxic activity, and that their activity is also cell-cycle dependent. Optimum cytotoxicity was observed after 48 h treatment and the IC_{50} values following 48 or 72 h exposure were highly comparable. The activity of HISD compounds against ara-C resistant cell lines suggest that these compounds do not require activation by deoxycytidine kinase and thus, have an entirely different mehcanism of action from that of ara-C. However, it is not known whether these compounds need activation by other enzymes in the cell.

The combinations of ara-C and PL compounds against CEM/0 or CEM/ara-C/7A cells exhibited a substantial degree of mutually nonexclusive synergism at several concentrations. The synergism can be explained by depletion of the intracellular dCTP by PL compounds thereby reducing the competition between dCTP and ara-CTP for the same site in the DNA polymerases. A reduction of dCTP enhances ara-CTP incorporation into DNA thereby increasing the toxicity towards ara-C (30, 31). Antagonism was predominant at a very low dose of ara-C in conjunction with a moderately high dose of PL4. It is known that at concentrations of 0.1, 1 or 10 μM, PL compounds significantly deplete the NTP pools after 48 h. Therefore, at higher concentrations of PL compounds the ATP pool

is severely depleted and since ATP is utilized by deoxycytidine kinase to phosphorylate ara-C, the ara-C activation could be incomplete thus showing antagonism. Moreover, antagonism may be aggravated by low concentration of ara-C or due to improper scheduling of the two compounds (32). The synergism with lower concentrations of PL compounds and moderate concentrations of ara-C against both CEM/0 and CEM/ara-C/7A cells can be similarly explained. At a very high dose of ara-C (10 or 1 μM) additivity was observed because it probably induced leukemic cell death without additional contribution from PL4. The treatment of CEM/ara-C/7A cell with PL compounds increased ara-C toxicity several folds probably by lowering dCTP pool thereby activating dCk thus stimulating ara-CTP production.

The sequential administration of PL7 followed by ara-C in CEM/0 cells was better than concurrent administration of the two compounds. The 24-h pretreatment with PL7 was much better than 48-h pretreatment. The 48-h PL7 treatment reduced the ATP pool more than 24-h treatment. So when the cells were pretreated with PL7 for 24 h, there might be sufficient ATP concentration remaining in the cells to phosphorylate ara-C to ara-CTP which was reflected in a higher degree of synergism in case of 24-h pretreatment compared to 48-h pretreatment.

The treatments of CEM/0 or CEM/ara-C/7A cell lines with HU and ara-C showed that the combinations were synergistic. However, the degree of synergism was significantly lower than that observed with combinations of each of the PL compounds with ara-C against same cell lines. Both isobologram and median-effect methods have their limitations (33), but in this case comparable results were obtained. Although the median-effect plot is suitable to analyze a wide variety of molar combinations, caution must be applied at every step before considering the results.

The RR inhibition studies, DNA synthetic capacity (DSC) studies, along with the analysis of NTP pools that were undertaken using PL4, shed some light on the possible mechanism of action of this series of compounds. The 40% or 70% inhibition of RR activity in CEM/0 cells following treatment with 10 μM PL4 for 1 or 2 h and complete inhibition of enzyme activity by 4 h and continuing up to 48 h in the presence of the drug suggests that the PL compounds are potent and irreversible inhibitors of RR. At a 10 μM concentration of PL4, the DSC was almost completely inhibited in CEM/0 cells after 24 or 48 h. When the NTP pools were analyzed at the same concentration, all the pools were found to be severely depleted. The purine nucleotide pools were depleted preferentially over the pyrimidine nucleotide pools probably due to a greater degree of structural similarities between the PL compounds and the purine nucleotides than pyrimidine nucleotides.

Hence, the combinations of each of the PL compounds with ara-C may be beneficial as they required lower concentrations of either compound and provided improved efficacy. This can also reduce the toxic manifestations of ara-C, especially its dose-limiting toxicities, like myelosuppression, mucosities, seizures, and myalgia. Finally, combination regimens, utilizing one of the investigated HISDs, and other nucleoside analog drugs, such as ara-C, may provide an improved therapeutic effect, which may benefit patients with leukemia or lymphoma.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Neil Bogart Memorial Laboratories by the T. J. Martell Foundation for Cancer, Leukemia and AIDS Research.

REFERENCES

1. Holmgren A. Hydrogen donor system for E. Coli Ribonucleoside diphosphate reductase dependent upon glutathione. Proc Natl Acad Sci USA 1976; 73: 2275–9.
2. Thelander L. Reductase of ribonucleotides. Ann Rev Biochem 1979; 46: 133–58.
3. Ashley GW, Stubbe J. Current ideas on the chemical mechanism of ribonucleotide reductase. Pharmac Ther 1985; 30: 301–29.
4. Stubbe J. Ribonucleotide reductase: Amazing and confusing. J Biol Chem 1990; 265: 5329–32.
5. Cory JG, Whitford Jr, TW. Ribonucleotide reductase and DNA synthesis in Ehrlich ascites tumor cells cancer. Cancer Res 1972; 32: 1301–6.
6. Hammarstan E, Reichard P, Saluste E. Pyrimidine nucleosides as precursors of pyrimidines in polynucleotides. J Biol Chem 1950; 183: 105–9.
7. Young CW. Inhibition of DNA synthesis by hydroxyurea: SAR. Cancer Res 1967; 27: 535–40.
8. Hahn MA, Adamson RH. Pharmacology of 3,5-diamino-1,2,4-triazole (guanzole). JNCI 1972; 48: 783–90.
9. Moore EC, Sartorelli AC. Inhibition of ribonucleotide reductase by α-(N)-heterocyclic carboxyaldehydethiosemicarbazones. Pharmacol Ther 1984; 24: 439–47.
10. Elford HL, Van't Riet B. Inhibition of nucleoside diphosphate reductase by hydroxybenzohydroxamic acid derivatives. Pharmacol Ther 1985; 29: 239–54.
11. Koneru PB, Lien EJ, Avramis VI. 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). Pharmacol Res 1993; 10: 515–20.
12. Haas CD, Joseph U, Belt RJ, Hoogstraten B. Phase I evaluation of IMPY in a twice weekly schedule. Cancer Treat Rep 1981; 65: 439–41.
13. Caoli EM, Talley RW, Smith F, Salem P, Vaitkevicius VK. Guanazole (NSC 1895). A phase I clinical study. Cancer Chemother Rep 1975; 59: 1117–21.
14. Krakoff IH, Etcubanes E, Tan C, Mayer K, Bethune V, Burchenal JH. Clinical trial of 5-hydroxypicolinaldehyde thiosemicarbazone (5-HP; NSC 107392), with special reference to its iron chelating properties. Cancer Chemother Rep 1974; 58: 207–12.
15. Veale D, Carmichael J, Cantwell BM, et al. A phase I and pharmacokinetic study of didox: A ribonucleotide reductase inhibitor. Br J Cancer 1988; 58: 70–2.

16. Choy BK, McClarty GA, Chan AK, Thelander L, Wright JA. Molecular mechanisms of drug resistance involving ribonucleotide reductase: Hydroxyurea resistance in a series of clonally related mouse cell lines selected in the presence of increasing drug concentrations. Cancer Res 1988; 48: 2029–35.
17. Cory JG, Lasater L, Sato A. Effect of iron-chelating agents on inhibitors of ribonucleotide reductase. Biochem Pharmacol 1981; 30: 979–84.
18. Satyamoorthy R, Chitnis M, Basrur V. Sensitization of P388 murine leukemia cells to hydroxyurea cytotoxicity by hydrophobic iron-chelating agents. Anticancer Res 1986; 6: 329–34.
19. Lien EJ, Gao H, Wang FZ. Stepping down approach (SDA) and stepping up approach (SUA) in new drug design. Med Chem Res 1991; 1: 173–84.
20. Kubota M, Takimoto T, Tanizawa A, Akiyama Y, Mikawa H. Differential modulation of 1-β-D-arabinofuranosylcytosine metabolism by hydroxyurea in human leukemia cell lines. Biochem Pharmacol 1988; 37: 1745–9.
21. Shackney SE, Ford SS, Occhipinti SJ, Ritch PS, Riceardi R, Erickson Jr BW. Schedule optimization of hydroxyurea and 1-β-D-arabinofuranosylcytosine in sarcoma 180 in vitro. Cancer Res 1982; 42: 4339–47.
22. Nandy P, Lien EJ, Avramis VI. Synthesis and testing of 2-hydroxy-1 H-isoindole-1,3-dione (HISD) derivatives against CEM/0 human leukemia cells in vitro alone and in combination with ara-C. Proc Am Assoc Cancer Res 1993; 34: 378.
23. Antonsson BE, Avramis VI, Nyce J. Effect of 5-azacytidine and congeners on DNA methylation and expression of deoxycytidine kinase in the human lymphoid cell lines CCRF/CEM/0 and CCRF/CEM/dCk(−). Cancer Res 1987; 47: 3672–8.
24. Tallarida RJ, Murray R. Manual of pharmacologic calculations with computer programs. New York: Springer-Verlag, 1981.
25. Berenbaum MC. A method for testing of synergy with any number of agents. J Infect Dis 1978; 137: 122–30.
26. Chou T-C, Talalay P. Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984; 22: 27–55.
27. Avramis VI, Biener R, Krailo M. Biochemical pharmacology of high-dose cytosine arabinoside (HD-ara-C) in childhood acute leukemia. Cancer Res 1987; 47: 6786–92.
28. Heinemann V, Plunkett W. Modulation of deoxynucleotide metabolism by the deoxycytidylate deaminase inhibitor 3,4,5,6-tetrahydrodeoxy uridine. Biochem Pharmacol 1989; 38: 4115–21.
29. Neu HC, Heppel LA. Nucleotide sequence analysis of polyribonucleotides by means of periodate oxidation followed by cleavage with an amine. J Biol Chem 1964; 239: 2927 34.
30. Tanaka M, Kimura K, Yoshida S. Mechanism of synergistic cell killing by hydroxyurea and cytosine arabinoside. Jpn J Cancer Res 1985; 76: 729–35.
31. Schilsky RL, Williams SF, Ultmann JE, Watson S. Sequential hydroxyurea-cytarabine chemotherapy for refractory non-Hodgkin's lymphoma. J Clin Oncol 1987; 5: 419–25.
32. Damon LE, Cadman EC. Advances in rational combination chemotherapy. Cancer Invest 1986; 4: 421–44.
33. Nocentini G, Barzi A, Franchetti P. Implications and problems in analyzing cytotoxic activity of hydroxyurea in combination with a potential inhibitor of ribonucleotide reductase. Cancer Chemother Pharmacol 1990; 26: 345–51.

Appendix

Equations of median effect principle method and isobologram method

Isobologram method

$$CI = (A_c/A_e) + (B_c/B_e)$$

where A_e and B_e are the doses of compound A and B alone that are required to inhibit a system by x%.
A_c and B_c are concentrations of A and B in combination that also inhibit x% of the system.

Median effect equation

$$f_a/f_u = (D/D_m)^m$$

where, D = Dose, f_a = Fraction of the system affected by dose D, f_u = Fraction of the system unaffected by dose D, D_m = Dose required to produce the median effect (analogous to IC_{50}), m = Hill-type coefficient signifying sigmoidicity of the dose-effect curve.

$$f_a = f_u = 1$$

$$D = D_m[f_a/(1\text{-}f_a)]^{1/m}$$

$$\log(f_a/f_u) = m\log(D) = m\log(D_m)$$

Combination Index (CI) for mutually exclusive drugs

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$$

Combination Index (CI) for mutually nonexclusive drugs

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_1 \times (D)_2/(D_x)_1 \times (D_x)_2$$

For mutually exclusive or nonexclusive drugs when,

CI < 1, Synergism is indicated.
CI = 1, Additivity is indicated.
CI > 1, Antagonism is indicated.