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Hydroxyurea potentiation of the antineoplastic activity of cyclophosphamide and 4′-(9-acridinylamino)methanesulfon-M-anisidide (AMSA) in the brown Norway rat myelocytic leukemia model*

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Summary. The activities of hydroxyurea (HU), 4′-(9-acridinylamino) methanesulfon-*M*-anisidide (AMSA) and cyclophosphamide (CY) were examined in the brown Norway rat myelocytic leukemia model in experiments designed to determine the synergy, optimal drug sequencing, and therapeutic index of combinations of these agents. A single dose of CY or four consecutive daily doses of AMSA produced increased survival in leukemic rats, with a positive-slope dose-response curve up to the maximum tolerated dose (MTD). HU at ½ MTD produced a minimal antileukemic effect but significantly potentiated the antineoplastic activity of ½ MTD of CY or AMSA with no significant toxic death rate. Drug-sequence experiments demonstrated that maximal synergy was achieved when HU was given immediately after CY but immediately before or during AMSA administration. No significant cure rate was seen with any CY/HU or HU/AMSA sequence. The three drugs given in the sequence of CY followed 3 days later by HU and AMSA simultaneously, however, was curative in the majority of rats with advanced leukemia, whereas other sequences were more toxic or less effective. Each of the drugs in these experiments was given at ½ of its single-agent MTD. HU significantly potentiates the antineoplastic effect of CY and AMSA in a drug-sequence-dependent manner in this model, apparently with an improved therapeutic index.

Introduction

4′-(9-Acridinylamino)methanesulfon-*M*-anisidide (AMSA) is a potent, new antineoplastic drug that produces cytotoxicity by the intercalation of DNA and inhibition of topoisomerase II/DNA interaction [11]. This drug shows significant antineoplastic activity in patients with acute myelocytic leukemia (AML) who have failed present first-line chemotherapy using cytosine arabinoside and an anthracycline [7]. Recent in vitro studies demonstrate significant potentiation of AMSA cytotoxicity when L1210 leukemic cells are preincubated with hydroxyurea (HU) [9], another drug with significant human anti-leukemic activity [5]. HU inhibits ribonucleotide reductase and is cytotoxic by virtue of the inhibition of DNA synthesis and repair [13]. In addition, HU alters the synthesis of nuclear proteins in some cell systems [4].

The brown Norway myelocytic leukemia (BNML) developed by van Bekkum and colleagues is a good model for human AML [3, 6, 14], being similar to the latter in cytologic characteristics, cytochemical reactions, in vivo and in vitro growth, and response to chemotherapeutic agents. This leukemia has been transferred to the hardier and more readily available Lewis X brown Norway (LBN) rat with the preservation of its desirable features [17]. This F1 hybrid model has proved useful for studies relevant to human AML in drug-sequence studies using cyclophosphamide (CY) and cytosine arabinoside [2, 16]. In this model we conducted a series of studies to examine HU potentiation of the antineoplastic activity of AMSA and CY as well as the dose and sequence timing determinants of optimal combination chemotherapy using these agents.

Methods

The rat model. Female LBN rats, obtained from Harlan Sprague-Dawley (Indianapolis, Ind.), were allowed 1 week of acclimatization, then were kept in single hanging cages and given fresh tap water daily and laboratory rat chow ad libitum while bearing leukemia or being given chemotherapy. The BNML was kept in serial passage in LBN rats and in multiple aliquots of an early LBN passage frozen at −90° C. Every eight passages, a new aliquot was thawed to reduce the risk of any spontaneous change in growth pattern or drug sensitivity. Single-cell suspensions of leukemia were made from leukemic bone marrow by gentle mincing in RPMI 1640 and serial aspiration through needles of decreasing size to 25 gauge. Suspensions were then adjusted to yield a cell count of 1×10^6 trypan blue viable cells/ml. Each LBN rat was given 1 ml by tail vein immediately after preparation, and cell count and trypan blue dye exclusion viability was repeated on the residual inoculum. The viable cell count on the residual inoculum was always > 85% of the count on the inoculum prior to initiation of the injections.

Chemotherapy experiments for advanced disease (analogous to the usual clinical setting) were begun when the bone marrow of sample rats contained approximately 80% leukemia cells and the spleen was twice its normal weight (days 13–16 after 10^6 BNML cells i.v.). In all experiments, control rats given no chemotherapy survived 21–24 days after the injection of 10^6 cells.

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Formulation and administration of chemotherapy. HU, obtained as a sterile powder from Squibb Pharmaceuticals, was dissolved in normal saline to achieve the desired dose for a 200 g rat in a volume of 1 ml. The appropriate volume per weight was then injected i.p. q8h for 4 days.

AMSA was obtained from the National Cancer Institute as a concentrated solution in dimethylacetamide, diluted in lactic acid to a concentration of 4 mg/kg, then further diluted in 5% dextrose to a final concentration such that the desired mg/kg dose was contained in a volume of 2 ml for a 200 g rat. The appropriate volume per weight was then injected daily via the tail vein for 4 days.

Commercially produced, clinically formulated CY (Bristol Myers) was obtained from the University of Nebraska Medical Center Pharmacy and injected i.p. in normal saline in a single dose at the volume required to give the desired mg/kg dose in a volume of 1 ml for a 200 g rat.

Experimental design. Because of the expense of animal purchase and care (approximately $ 20/rat), and to satisfy the requirements of the animal resource committee of the University of Nebraska Medical Center, preliminary experiments were typically carried out with five animals per group and repeated with larger numbers only as needed for confirmation of the results. The MTD of each of the drugs used as a single agent was determined in rats with advanced leukemia. Advanced-stage disease was used for all therapeutic experiments except studies on the effect of pretreatment tumor volume on the response to the drug. Each experiment contained controls receiving no treatment and single-agent controls. Toxic deaths (usually due to combined marrow and gastrointestinal toxicity) were identified by timing and necropsy findings of small spleen and grossly infected abdominal or thoracic cavities. The increase in the median life span (ILS) over controls in rats surviving toxicity was determined by observation to determine if antineoplastic synergy was produced. As an initial screen for drug synergy, rats with advanced-stage LBN-ML were treated with either the MTD or ½ MTD of each of the drugs and then simultaneous combinations of the drugs at ½ MTD of each.

Statistical analysis of the difference between two treatments was done using the Wilcoxon rank-sum test. We made no attempt at statistical analysis or modeling of continuous variable studies such as dose-response curves or multiple-time-point sequential therapy studies.

Results

Dose response and MTD of HU and AMSA in LBN-ML

The MTD of HU in the LBN rat is approximately 300 mg/kg when HU is given on a q8h schedule for 4 days to normal rats or rats with advanced disease (day 13 after 10^6 BNML cells i.v.). At the MTD, HU produced a maximum of 4 days' prolongation of survival in rats with advanced LBN-ML compared with the median in control rats given no drug or vehicle only (Fig. 1). If the regrowth of this leukemia after therapy occurs at the same rate as that without therapy [10], this 4-day survival increase corresponds to approximately 1 log kill of leukemic cells with HU at the MTD. At ½ MTD, HU produced no significant prolongation of survival in advanced LBN-ML or rats treated 1 day after inoculation with leukemia.

The dose-response curve for AMSA in LBN-ML (Fig. 2) demonstrated the MTD to be approximately 4 mg/kg per day when given four times daily. In contrast to HU, AMSA produced significant antileukemic effects at as little as ¼ MTD; even at ¼ MTD, AMSA was as effective as the full MTD of HU.

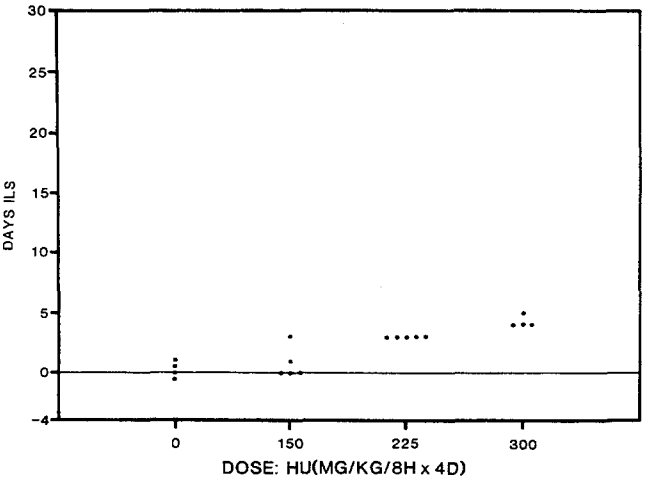

Fig. 1. Effect of HU on LBN-ML, advanced disease. LBN rats were inoculated i.v. with 10^6 BNML and given HU 13 days later at the doses indicated every 8 h for 4 days. ●, death due to leukemia; O, toxic death; *ILS*, increased life span beyond the median in control leukemic rats given no therapy. Survival at 300 mg/kg > 225 mg/kg > 150 mg/kg HU ($P < 0.05$); survival at 150 mg/kg not significantly greater than that in control rats given no therapy

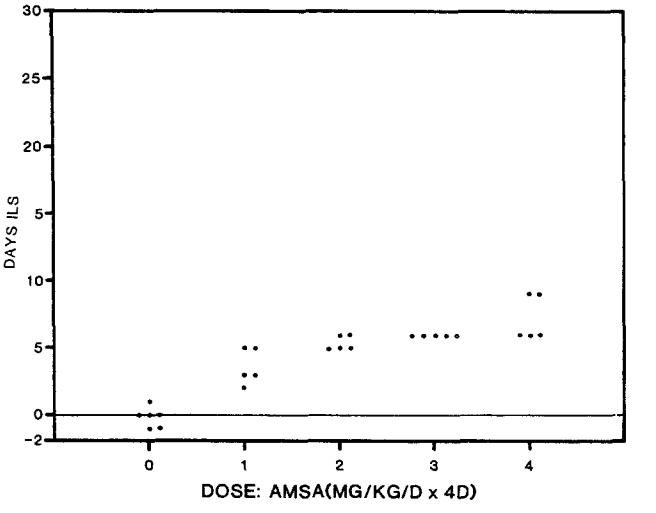

Fig. 2. Effect of AMSA on LBN-ML, advanced disease. LBN rats were inoculated i.v. with 10^6 BNML and given AMSA 13 days later i.v. at the daily doses indicated for 4 days. ●, death due to leukemia; O, toxic death; *ILS*, increased life span beyond the median in control rats given no therapy

HU/AMSA synergy studies

Since HU at ½ MTD produced no significant cell kill in LBN-ML, any increase in survival produced by the addition of this dose of HU to AMSA was considered to represent synergy. A synergistic antileukemic effect without excess toxicity was seen when ½ MTD of HU was given to rats with advanced-stage LBN-ML for the 4-day period during which the animals received ½ MTD of AMSA

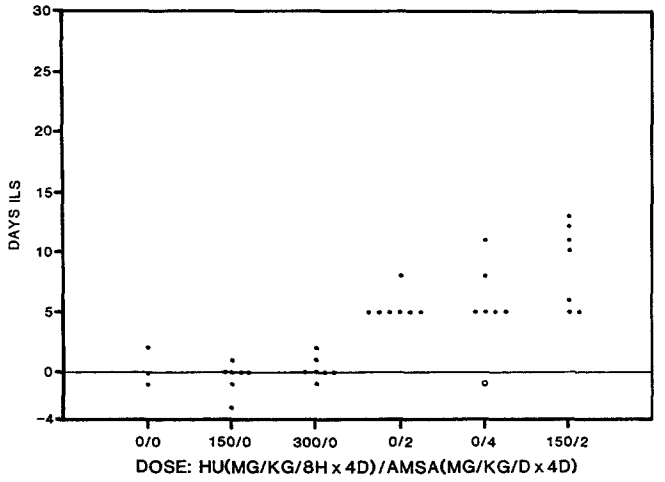

Fig. 3. HU/AMSA combination chemotherapy in LBN-ML, advanced disease. LBN rats were inoculated i.v. with 10^6 BNML and 13 days later given either no therapy, AMSA only at 2 mg/kg per day for 4 days (*½ MTD*) or at 4 mg/kg per day for 4 days (*MTD*), HU only at 150 mg/kg q8h for 4 days (*½ MTD*) or at 300 mg/kg q8h for 4 days (*MTD*), or the simultaneous combination of ½ MTD of HU and ½ MTD of AMSA. ●, death due to leukemia; ○, toxic death; *ILS*, increased life span beyond the median in control rats given no therapy. Survival of 150/2 > 0/2 ($P < 0.05$)

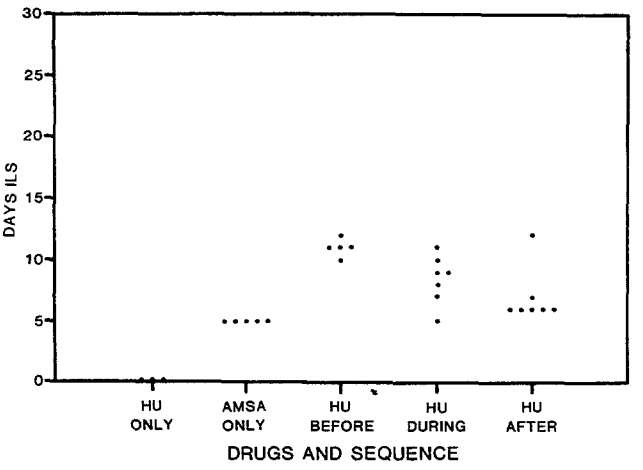

Fig. 5. Effect of the sequence of administration on HU/AMSA synergy against LBN-ML. LBN rats with advanced disease (day 13 after i.v. inoculation of 10^6 BNML) were treated with HU alone at 150 mg/kg q8h i.p. for 4 days (*½ MTD*), AMSA alone at 2 mg/kg i.v. daily for 4 days, or the same dose of both drugs, with HU given for the 4 days before AMSA, the 4 days during AMSA, or the 4 days after AMSA administration. ●, death due to leukemia; ○, toxic death; *ILS*, increased life span beyond the median in control rats given no therapy. Survival of HU before > HU after AMSA ($P < 0.05$)

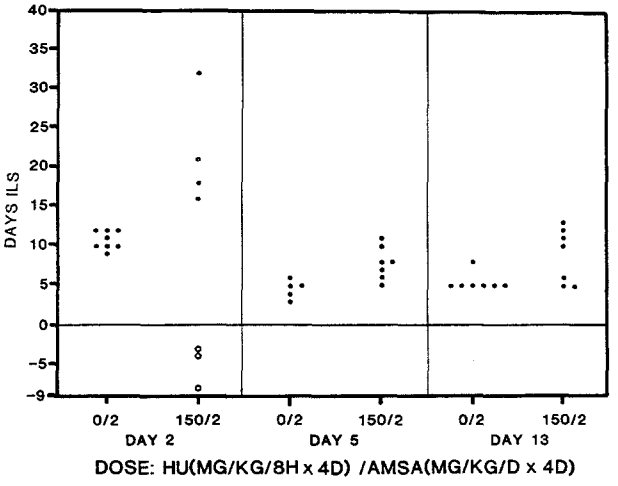

Fig. 4. HU/AMSA synergy against LBN-ML as a function of tumor volume. On days 2, 5, or 13 after i.v. inoculation with 10^6 BNML cells, LBN rats were given 2 mg/kg AMSA i.v. daily for 4 days (*½ MTD*) either as a single agent or in combination with 150 mg/kg HU i.v. daily for 4 days (*½ MTD*). ●, death due to leukemia; ○, toxic death; *ILS*, increased life span beyond the median in control rats given no therapy

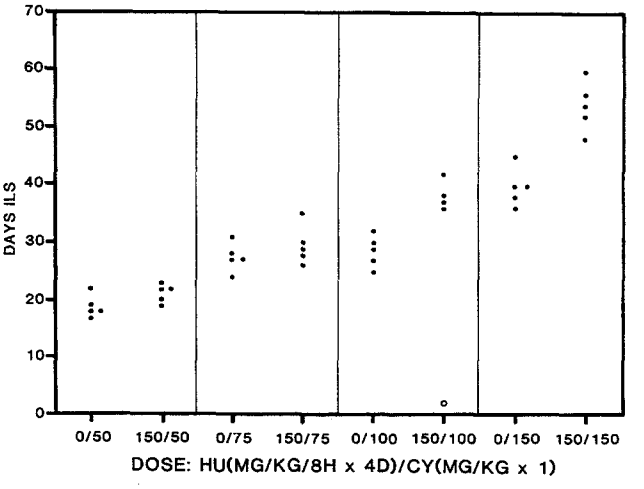

Fig. 6. HU/CY combination chemotherapy. LBN rats were inoculated with 10^6 BNML cells and given CY 13 days later at the doses indicated as a single i.p. injection either alone or followed immediately by HU at a dose of 150 mg/kg q8h for 4 days. ●, death due to leukemia; ○, toxic death; *ILS*, increased life span beyond the median in control rats given no therapy. Survival of 150/100 > 0/100, and survival of 150/100 > 0/150 ($P < 0.05$)

(Fig. 3). In fact, ½ MTD of HU plus ½ MTD of AMSA produced a greater antileukemic effect than the full MTD of AMSA. HU/AMSA synergy was seen with low tumor volume (equivalent to the adjuvant or minimal residual disease setting) as well as during intermediate and advanced disease states (Fig. 4).

Finally, the effect of drug sequence on HU/AMSA synergy was studied. AMSA preceded by HU was significantly more effective ($P < 0.05$, Wilcoxon rank-sum test) than AMSA followed by HU. HU and AMSA given simultaneously had an intermediate effect (Fig. 5).

HU/CY synergy studies

HU at ½ MTD was given to LBN-ML rats with advanced disease for the 4 days immediately after a single dose of CY ranging between ¼ and ¾ MTD. The median survival of leukemic rats was slightly greater when HU was added to CY at all doses of the latter, but significant synergy ($P < 0.05$, Wilcoxon rank-sum test) was seen only with ½ or ¾ MTD of CY (Fig. 6). The fact that animals tolerated ¾ MTD of CY plus ½ MTD of HU suggested that the antineoplastic synergy of HU with CY might be achieved with

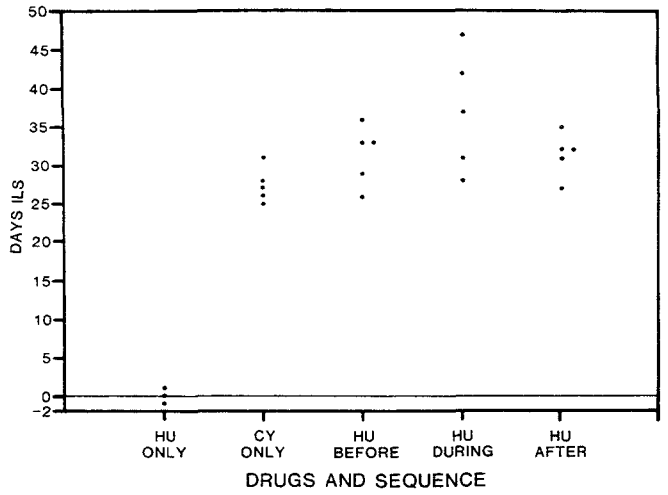

Fig. 7. Effect of the sequence of administration on HU/CY synergy against LBN-ML. LBN rats were inoculated with 10^6 BNML cells and given 100 mg/kg CY 13 days later as a single i.p. injection (*½ MTD*) either alone, preceded by HU at 150 mg/kg q8h for 4 days (*½ MTD*) (*before*) and followed immediately by the same dose of HU (*during*), or followed by the same dose of HU beginning 4 days later (*after*). ●, death due to leukemia; ○, toxic death; *ILS*, increased life span beyond the median in control animals given no therapy

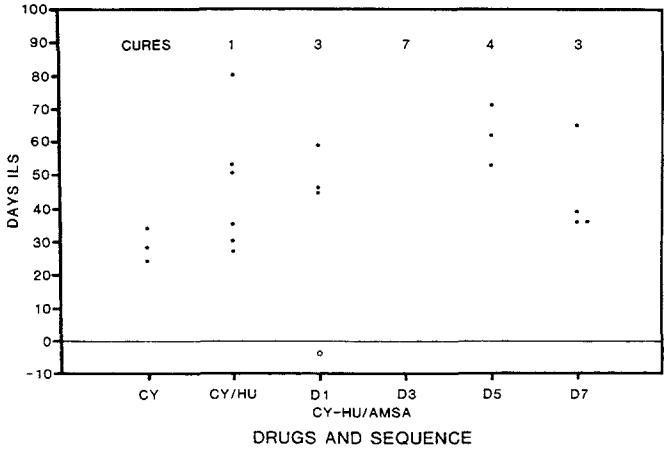

Fig. 8. Effect of the sequence of administration on CY/HU/AMSA synergy against LBN-ML. LBN rats with advanced disease (day 13 after i.v. inoculation of 10^6 BNML) were given 100 mg/kg CY as a single i.p. dose (*½ MTD*), followed by 150 mg/kg HU q8h i.p. for 4 days (*½ MTD*) and 2 mg/kg AMSA i.v. daily for the same 4 days (*½ MTD*) beginning on the day indicated after CY. Survival of rats treated with CY followed by HU without AMSA on day 3 is also shown. ●, death due to leukemia; ○, toxic death; *ILS*, increased life span beyond the median in control rats given no therapy

an improved therapeutic index (improved toxic:therapeutic ratio).

When the sequence of administration of HU and CY was examined, the results were less dramatic than for HU/AMSA sequential therapy and a different trend emerged (Fig. 7). HU/CY synergy was most pronounced when HU was given immediately after CY ("during" the period of alkylating agent activity).

CY/HU/AMSA sequential therapy

Based on the above results, the combination and sequence of CY followed by HU and AMSA was studied. Leukemia cell kill was remarkably drug-sequence-dependent when the three drugs were combined. In the best sequence (CY followed 3 days later by HU and AMSA given simultaneously), all animals were cured (100-day survival) of leukemia (Fig. 8).

Despite the fact that greater cell kill was achieved in HU/AMSA studies when HU preceded AMSA, the sequence of CY followed by HU on day 1, followed by AMSA on day 3 or 5 (data not shown), was less effective than CY followed by HU and AMSA together on day 3. Only one toxic death was seen with any of these sequences, despite the fact each of these three drugs was given at ½ of its single-agent MTD.

Discussion

In this rat model, these empiric studies have demonstrated that HU potentiates the antineoplastic activity of CY and AMSA. This synergy is drug-sequence- and timing-dependent. However, in none of the sequences and combinations examined did the combination of ½ MTD of HU plus ½ MTD of CY or AMSA produce a greater toxic death rate than the full MTD of CY or AMSA alone. In fact, neither the optimal sequence of ¾ MTD of CY combined with ½ MTD of HU nor the optimal sequence of ½ MTD each of CY, HU, and AMSA produced toxic deaths. Thus, the observed potentiation of the antileukemic effects of CY and AMSA by HU was achieved with an improved therapeutic index. In the best sequential combination chemotherapy identified in these studies (½ MTD of CY followed 3 days later by simultaneous HU and AMSA at ½ MTD each), advanced LBN-ML was cured with a single cycle of drug treatment.

In other experiments (data not shown), we examined combination therapy with HU and 1-*B*-D-arabinofuranosyl cytosine (ARA-C). HU increased the antileukemic effect of ARA-C in this model, but HU/ARA-C combinations and sequences were just as toxic and not as effective as simply increasing the ARA-C dose. Since HU and ARA-C are similar in their mechanisms of cytotoxicity and patterns of clinical side effects, this is perhaps not surprising. In contrast, CY and AMSA are not antimetabolites and HU, CY, and AMSA have different mechanisms of cytotoxicity. Thus, our results are consistent with the notion that an improved therapeutic index of combinations and sequences of drugs is more likely to occur when drugs with different mechanisms of action are combined.

Of interest is that the drugs studied in this rat model have activity similar to their effects in human AML. Thus, HU has some antileukemic effect in human AML but as a single agent does not produce significant prolongation of survival [5]. In contrast, as a single agent AMSA produces a significant percentage of complete remissions in advanced human AML, and in previously nontreated disease it may be as effective in combination with ARA-C as the anthracyclines [1]. Likewise, CY has significant activity in human AML, especially at high doses [12]. Consequently, we have begun a clinical trial of CY followed by simultaneous HU and AMSA in patients with AML who are no longer responsive to and anthracycline combination ther-

apy. Preliminary results of this clinical trial are encouraging [15].

The effect of the timing of administration on HU potentiation of CY and AMSA antineoplastic activity in vivo is consistent with in vitro observations of others and suggests testable hypotheses to guide further investigation and translation of these results into the clinical setting. Lindahl et al. [8] have demonstrated that recovery from sublethal cytotoxicity induced by alkylating agents may be mediated by DNA repair enzymes that require deoxyribonucleotides. Thus, the depletion of deoxyribonucleotides by HU following CY administration might be expected to increase the cytotoxicity of CY.

Of particular interest, however, is the dramatic sequence-dependence of HU/AMSA synergy in vivo. The failure of HU to potentiate the antineoplastic effect of AMSA in this model when HU was given after AMSA is consistent with the conclusion of Pommier et al. [10] and Minford et al. [9], who found that AMSA-induced DNA damage is repaired without new DNA synthesis. The mechanism by which HU pretreatment potentiates the cytotoxicity of AMSA is not known, but Minford et al. [9] have recently reviewed the possibilities. In their in vitro work, HU pretreatment increased AMSA-induced DNA-protein cross-links and cytotoxicity by more than could be achieved by simply increasing the AMSA dose. Our data demonstrate the same phenomenon in vivo. A ½ MTD of HU plus ½ MTD of AMSA produced at least as much ILS as the full MTD of AMSA alone. Our dose-response curve data for AMSA suggests that a transport maximum is achieved or that all available DNA/topoisomerase II-binding sites are saturated at a dose less than the MTD of AMSA. The effect of HU on AMSA uptake, intercalation, and topoisomerase binding can probably be measured, but whatever the explanation for this sequence-dependent synergy between HU and AMSA, its in vivo confirmation has significant implications for clinical trial design.

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