Increased Sensitivity of Hydroxyurea-resistant Leukemic Cells to Gemcitabine

Stuart J. Wong, Michael S. Myette, Janine P. Wereley, and Christopher R. Chitambar
Division of Hematology/Oncology, Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

ABSTRACT
Tumor cell resistance to certain chemotherapeutic agents may result in cross-resistance to related antineoplastic agents. To study cross-resistance among inhibitors of ribonucleotide reductase, we developed hydroxyurea-resistant (HU-R) CCRF-CEM cells. These cells were 6-fold more resistant to hydroxyurea than the parent hydroxyurea-sensitive (HU-S) cell line and displayed an increase in the mRNA and protein of the R2 subunit of ribonucleotide reductase. We examined whether HU-R cells were cross-resistant to gemcitabine, a drug that blocks cell proliferation by inhibiting ribonucleotide reductase and incorporating itself into DNA. Contrary to our expectation, HU-R cells had an increased sensitivity to gemcitabine. The \( IC_{50} \) of gemcitabine was 0.06 ± 0.03 \( \mu \text{M} \) for HU-R cells versus 0.16 ± 0.02 \( \mu \text{M} \) for HU-S cells \( (P = 0.005) \). The cellular uptake of \( ^3\text{H} \) gemcitabine and its incorporation into DNA were increased in HU-R cells. Over an 18-h incubation with radio-labeled gemcitabine (0.25 \( \mu \text{M} \)), gemcitabine uptake was 286 ± 37.3 fmol/10^6 cells for HU-R cells and 128 ± 8.8 fmol/10^6 cells for HU-S cells \( (P = 0.03) \). The incorporation of gemcitabine into DNA was 75 ± 6.7 fmol/10^6 cells for HU-R cells versus 22 ± 0.6 fmol/10^6 cells for HU-S cells \( (P < 0.02) \). Our studies suggest that the increased sensitivity of HU-R cells to gemcitabine results from increased drug uptake by these cells. This, in turn, favors the incorporation of gemcitabine into DNA, resulting in enhanced cytotoxicity. The increased sensitivity of malignant cells to gemcitabine after the development of hydroxyurea resistance may be relevant to the design of chemotherapeutic trials with these drugs.

INTRODUCTION
Tumor cell resistance to chemotherapy is one of the major obstacles to the successful treatment of cancer. Hence, knowledge of cross-resistance among different antineoplastic agents is important because it provides direction for the design of clinical trials.

Because of its key role in the synthesis of deoxiribonucleotides, ribonucleotide reductase is an important target for antineoplastic drugs (1, 2). Mammalian ribonucleotide reductase is composed of two subunits termed R1 and R2 (or M1 and M2; Refs. 3–5). The R1 subunit has substrate and effector binding sites, whereas the R2 subunit contains a nonheme iron center and a tyrosyl free radical, both of which are essential for enzyme activity (3–6). Of the various ribonucleotide reductase inhibitors available, hydroxyurea has been in clinical use for over two decades, and its action on malignant cells in vitro and in vivo has been studied extensively (7). It is known that hydroxyurea inhibits ribonucleotide reductase activity by action on the R2 subunit (6, 8) and that resistance to cell growth inhibition by hydroxyurea results from overexpression of the R2 subunit (9–12).

Gemcitabine (2',2'-difluorodeoxycytidine) is a new deoxynucleoside analogue that has shown clinical antitumor activity against a variety of malignancies (13, 14). This drug exerts its cytotoxicity by action on ribonucleotide reductase and incorporation into DNA, the latter of which results in a block in DNA replication (15).

In the present investigation, we have examined whether lymphoid leukemic cells that are resistant to hydroxyurea by virtue of overexpression of the R2 subunit of ribonucleotide reductase are cross-resistant to gemcitabine. Contrary to our expectation, we found that HU-R \(^3\) CCRF-CEM cells take up and incorporate a greater amount of gemcitabine into DNA than HU-S cells and display an increased sensitivity to growth inhibition by gemcitabine.

MATERIALS AND METHODS
Gemcitabine and \( ^3\text{H} \) gemcitabine (specific activity, 20 Ci/mmol) were obtained from Lilly Research Laboratories (Indianapolis, IN). Hydroxyurea was purchased from Calbiochem, whereas MTT was obtained from Sigma Chemical Co. (St. Louis, MO). \( ^{32}\text{P} \) dCTP was obtained from New England Nuclear Life Sciences (Boston, MA). The cDNA for the R2 subunit of human ribonucleotide reductase cloned into vector pCRII (Invitrogen, Carlsbad CA) was kindly provided by Dr. Yun Yen (City of Hope, Duarte, CA) and has been described previously (16). The R2 cDNA insert was excised from the plasmid using

Received 10/7/98; revised 10/29/98; accepted 11/8/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by USPHS Grant RO1 CA68028 and funds from the Sampson family. This work was presented in abstract form at the 89th Annual Meeting of the American Association for Cancer Research held March 28-April 1, 1998 in New Orleans, Louisiana.

2 To whom requests for reprints should be addressed, at Division of Hematology/Oncology, Medical College of Wisconsin, 9200 West Wisconsin Avenue, Milwaukee, WI 53226. Phone: (414) 805-4604; Fax: (414) 805-4606; E-mail: chitambar@mcw.edu.

3 The abbreviations used are: HU-R, hydroxyurea-resistant; HU-S, hydroxyurea-sensitive; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCA, perchloric acid.
Gemcitabine and Hydroxyurea Resistance

Kodak, Co., Rochester, NY) with intensifying screens at diographed by exposing it to Kodak XAR-5 film (Eastman recommended by the manufacturer. The membrane was autora-

tions. R2 mRNA was detected by hybridization of the mem-

NH) by capillary blotting. Equal loading of RNA on the gel was

tained in culture medium containing 90 µM hydroxyurea.
The effects of hydroxyurea and gemcitabine on the gemification of HU-S and HU-R CCRF-CEM cells were measured by MTT assay as described by Mosmann (18), with modifications as reported previously by us (19). In this assay, cells were plated at a density of 2 × 10^5 cells/ml in 96-well microwell plates in the presence of increasing concentrations of hydroxyurea or gemcitabine (shown in the figures) and analyzed for growth after 72 h of incubation.

**RNA Isolation and Northern Blotting.** Total cellular RNA was isolated from cells using RNAzol (Tel-Test, Inc., Friendswood, TX) as recommended by the manufacturer, and the integrity of the RNA was verified by agarose gel electro- phoresis before use. RNA (20 µg) was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and transferred from the gel to a Nytran membrane (Schleicher & Schuell, Keene, NH) by capillary blotting. Equal loading of RNA on the gel was confirmed visually by ethidium bromide staining of the RNA bands. R2 mRNA was detected by hybridization of the membrane to 32P-labeled cDNA probe (1.2 × 10^6 cpm/ml) using QuickHyb Hybridization Solution (Stratagene, La Jolla, CA) as recommended by the manufacturer. The membrane was autoradiographed by exposing it to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying screens at 70°C for 24–48 h.

**Western Blotting.** R1 and R2 protein levels in HU-S and HU-R cells were detected by Western blotting using an enhanced chemiluminescence Western blotting detection system (Amersham, Arlington Heights, IL). Cells in exponential growth phase were harvested and lysed in 200 µl of radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris, and 150 mM NaCl) containing 100 µg/ml phenylmethylsulfonyl fluoride, 75 µg/ml aprotinin, and 1 mM sodium orthovanadate. The protein content of the clarified lysate was measured by the BCA protein assay (Pierce, Rockford ILL). SDS-PAGE of the samples was performed as described by Laemmli (20), and proteins were transferred from the gel onto a nitrocellulose membrane as described by Towbin et al. (21), using a Transblot system (Bio-Rad, Richmond, CA). Membranes were incubated with specific primary antibodies against R1 or R2, followed by secondary antibodies conjugated to horseradish peroxidase. For detection of protein bands, membranes were immersed in enhanced chemiluminescence detec-

**RESULTS**

**HU-R Cells Overproduce R2 mRNA and Protein.** The growth of HU-S and HU-R CCRF-CEM cells in the presence of hydroxyurea is shown in Fig. 1. HU-R cells were approximately 6–7-fold more resistant to growth inhibition by hydroxyurea than HU-S cells. To confirm that our HU-R cells had an increase in the expression of the R2 subunit of ribonucleotide reductase similar to that reported by others (9–12), steady-state R2 mRNA levels were measured by Northern blotting. These studies showed that R2 mRNA was indeed increased in HU-R cells.
(Fig. 2). Consistent with prior reports, the cDNA probe for R2 detected two transcripts of 3.4 and 1.6 kb (16). Western blot analysis revealed that HU-R cells had a corresponding increase in their content of R2 subunit protein but no increase in R1 subunit protein (Fig. 3).

Effect of Gemcitabine on Cell Growth. HU-S and HU-R cells were incubated with gemcitabine, and the effects on cell growth were measured by MTT assay. As shown in Fig. 4, HU-R cells were approximately 2.5-fold more sensitive to gemcitabine than HU-S cells. The IC50 for gemcitabine was $0.16 \pm 0.02$ and $0.06 \pm 0.03 \mu M$ for HU-S and HU-R cells, respectively ($P = 0.005$). The differences between all of the data points shown in Fig. 4 for HU-S and HU-R cells are highly significant ($P < 0.0005$).

Gemcitabine Uptake and Incorporation into DNA. To gain an insight into the mechanism responsible for the increased sensitivity of HU-R cells to gemcitabine, the cellular uptake of $[^3H]$gemcitabine and its incorporation into DNA were measured after an 18-h incubation of cells with radiolabeled drug. As shown in Fig. 5A, the uptake of $[^3H]$gemcitabine was significantly greater in HU-R cells than in HU-S cells ($286 \pm 37.3$ fmol/10⁶ cells for HU-R cells versus $128 \pm 8.8$ fmol/10⁶ cells for HU-S cells (mean ± SE); $P = 0.03$). In addition, as shown in Fig. 5B, the incorporation of $[^3H]$gemcitabine into DNA was approximately 3.4-fold greater in HU-R cells than in HU-S cells ($75 \pm 6.7$ fmol/10⁶ cells for HU-R cells versus $22 \pm 0.6$ fmol/10⁶ cells for HU-S cells (mean ± SE); $P < 0.02$).

DISCUSSION
In the present investigation, we sought to determine whether cross-resistance exists between hydroxyurea and gemcitabine. We developed HU-R CCRF-CEM cells, and, consistent with other reports (9–12), these cells displayed an increase in the expression of mRNA and protein of the R2 subunit. However, our studies show that rather than being cross-resistant to gemcitabine, HU-R cells displayed a significant increase in sensitivity to gemcitabine. Further examination of the mechanism involved revealed that both the cellular uptake of gemcitabine and its incorporation into DNA were significantly increased in HU-R cells. It is known that in addition to inhibiting ribonucleotide reductase, gemcitabine exerts its cytotoxicity by incorporating into DNA and terminating DNA chain elongation (15). Our results strongly suggest that the increased sensitivity of HU-R cells to gemcitabine is likely to be due to the increased cellular uptake of gemcitabine, resulting in an expanded intra-
Gemcitabine and Hydroxyurea Resistance

A. Gemcitabine Uptake by Cells

B. Gemcitabine Incorporated into DNA

Fig. 5 A, cellular uptake of [3H]gemcitabine. HU-S and HU-R cells were incubated with 0.25 μM [3H]gemcitabine for 18 h, and the total cellular uptake of radiolabeled gemcitabine was measured as described in “Materials and Methods.” B, [3H]gemcitabine incorporation into DNA. Incubation conditions were similar to those described in A. Radiolabeled gemcitabine incorporation into DNA was determined as described in “Materials and Methods.” Values shown represent the means ± SE (n = 3).

cellular gemcitabine pool. The latter, in turn, would favor gemcitabine incorporation into DNA and lead to enhanced cytotoxicity.

Little information exists regarding the effect of hydroxyurea resistance on the cellular uptake of gemcitabine. Recently, however, Mackey et al. (23) have shown that the influx of gemcitabine into different cells, including CCRF-CEM cells, requires specific cell membrane nucleoside transporters, and that the cytotoxicity of gemcitabine varies among cell lines with different nucleoside transporters. Although HU-S and HU-R cells were derived from the same parent cell line, it is possible that the development of hydroxyurea resistance somehow alters the expression of nucleoside transporters responsible for gemcitabine uptake. Increased expression and/or function of a gemcitabine transporter in these cells could render them more sensitive to this drug. Additional studies are planned to elucidate this potential mechanism of action.

The cross-resistance pattern of a variety of ribonucleotide reductase inhibitors has previously been examined by others. However, in most of these studies, hydroxyurea resistance has been associated with parallel resistance to other drugs. Carter and Cory (24) have shown that R2-overproducing L1210 murine leukemic cells display cross-resistance to 2,3-dihydroxy-1H-pyrazolo[2,3-α]imidazole, an inhibitor of the R2 subunit, but remain sensitive to 4-methyl-5-aminol-fornylisoquinoline thiosemicarbazone and 1-isoquionolylmethylene-N-hydroxy-N′-aminoguanidine tosylate, agents that also inhibit R2. Along the same lines, Huang et al. (25) recently showed that HU-R cells with elevated R2 expression exhibit decreased sensitivity to methotrexate and N-[(phosphonacetyl)-l-aspartate, agents that act on targets other than ribonucleotide reductase. Interestingly, Yen et al. (16) found that HU-R KB cells with increased ribonucleotide reductase activity displayed increased sensitivity to 6-thioguanine. The underlying mechanism of this supersensitivity was felt to be increased conversion of 6-thioguanine to the deoxynucleotide, leading to enhanced incorporation into DNA and subsequent cell growth arrest (16).

The results of our studies may be relevant for the use of gemcitabine and hydroxyurea in the clinical setting. Hydroxyurea has activity in hematological malignancies and, to a lesser extent, in certain solid cancers. Gemcitabine is being intensively investigated in Phase II trials in a variety of malignancies and has shown activity against pancreatic cancer, non-small cell lung cancer, head and neck cancer, and urothelial and gynecological malignancies (13, 14). Both hydroxyurea and gemcitabine also appear to be potent radiosensitizing agents (7, 26).

Our studies suggest that a therapeutic strategy in which tumor cells are exposed to hydroxyurea and gemcitabine in an alternating fashion may be more efficacious than treatment with either drug alone. With such treatment, clones of cells that develop resistance to hydroxyurea after exposure to hydroxyurea may be effectively eradicated with subsequent exposure to gemcitabine. Studies are planned to evaluate the effect of gemcitabine in an animal model implanted with HU-R cells and to confirm our in vitro observations. The result of such studies will be highly relevant to the design of clinical trials and the use of gemcitabine in tumors that may be resistant to hydroxyurea.

REFERENCES

Increased Sensitivity of Hydroxyurea-resistant Leukemic Cells to Gemcitabine

Stuart J. Wong, Michael S. Myette, Janine P. Wereley, et al.


**Updated version**
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/2/439

**Cited articles**
This article cites 21 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/2/439.full.html#ref-list-1

**Citing articles**
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/5/2/439.full.html#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.