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BIOCHEMICAL MODULATION OF IODODEOXYURIDINE BY N^6-[4-(MORPHOLINOSULFONYL)BENZYL]-N^6-METHYL-2,6-DIAMINOBENZ[*cd*]INDOLE GLUCURONATE (AG-331) LEADING TO ENHANCED CYTOTOXICITY

JOSIE PRESSACCO,*† BISERKA MITROVSKI,* DAVID W. HEDLEY,*‡§ RICHARD TSANG‖ and CHARLES ERLICHMAN†¶

*Division of Experimental Therapeutics, The Ontario Cancer Institute, Toronto; Departments of †Pharmacology, ‡Medical Biophysics, and §Medicine, University of Toronto, Toronto; and ‖Department of Radiation Oncology, The Princess Margaret Hospital, Toronto, Canada

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Abstract—Inhibition of thymidylate synthase (TS) may increase incorporation of thymidine analogues into DNA, leading to increased inhibition of colony formation in tumor cells. We have reported previously that TS inhibition by *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6,-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid (ICI D1694 or Tomudex), a folate-based TS inhibitor, increases the cytotoxicity of iododeoxyuridine (IdUrd), a thymidine analogue, in MGH-U1 human bladder and HCT-8 human colon cancer cells. N^6-[4-(Morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diaminobenz[*cd*]-indole glucuronate (AG-331) differs from ICI D1694 in that it is a *de novo* designed lipophilic TS inhibitor, it does not require a specific carrier for cellular uptake, and it does not undergo intracellular polyglutamation. Exposure of MGH-U1 cells to 5 μM AG-331 for 24 hr decreased clonogenic survival by 30%, but almost completely inhibited TS activity. IdUrd is a cytotoxic thymidine analogue, with IC_{50} and IC_{90} values after 24-hr exposures in MGH-U1 cells of 13 and 81 μM, respectively. The combination of IdUrd and AG-331 resulted in an enhanced antitumor effect, as compared with the effect of either agent alone. The cytotoxic IC_{50} of IdUrd decreased from 13 to 1.5 μM, and the IC_{90} decreased from 81 to 5 μM with the addition of 5 μM AG-331. Biochemical studies of the combination revealed that pretreating MGH-U1 cells with 5 μM AG-331 increased IdUrd incorporation into cellular DNA by 3.8-fold. This increased incorporation was associated with a greater proportion of DNA single-strand breaks than observed with either agent alone, and the combination of 5 μM AG-331 plus IdUrd produced up to a 2.5-fold increase in DNA single-strand breaks as compared with IdUrd alone. The effects of AG-331, IdUrd, and the combination of IdUrd and AG-331 on the colony-forming ability of normal human bone marrow CFU-GM cells was determined as a measure of myelosuppression. The combination of IdUrd and AG-331, at the same concentrations as those used in the MGH-U1 cells, produced a wider therapeutic index relative to that of IdUrd alone, and the therapeutic index for the combination was 6.5, as compared with 4.0 for IdUrd plus ICI D1694 in previous studies from this laboratory. These observations suggest that the combination of IdUrd and AG-331 may enhance antitumor effects with minimal myelosuppression *in vivo*.

Key words: thymidylate synthase; inhibitors; iododeoxyuridine; pyrimidine nucleoside; antineoplastic drugs; combination chemotherapy

The thymidine analogue IdUrd** was originally synthesized as a potential antineoplastic agent [1, 2], and its cytotoxicity is dependent upon its cellular uptake and activation to IdUTP by the dTMP salvage pathway, and subsequent incorporation into DNA. Agents such as fluorodeoxyuridine [3–6] and ICI D1694 [7] have been shown to modulate IdUrd incorporation into DNA. Possible mechanisms of modulation of IdUrd incorporation into DNA by these agents can be proposed. Since dTTP competes with IdUTP as a substrate for DNA polymerase, interventions that increase intracellular IdUTP pools and/or decrease dTTP levels could enhance the cytotoxicity of IdUTP [3]. In this regard, the activity of TS, a key enzyme in the *de novo* synthesis of dTMP, is an important target. Inhibition of TS activity by FdUrd or ICI D1694 reduces intracellular dTTP levels [8, 9], thus increasing the cellular ratio of IdUTP to dTTP. A further consequence of TS inhibition may be the concurrent increase in TK and dTMPK activity [10], resulting in an enhanced phosphorylation of IdUrd, although these results are contradictory to those of Refs. 11 and 12. Furthermore, IdUMP is deiodinated extensively *in vivo* by TS [13], and may subsequently be methylated, thus replenishing dTTP pools.

We hypothesize that inhibition of TS will increase

¶ Corresponding author (and present address): Dr. Charles Erlichman, Department of Oncology, Mayo Clinic, 200 First Street, S.W., Rochester, MN 55905. Tel. (507) 284–3902; FAX (507) 284–1803.

** Abbreviations: IdUrd, iododeoxyuridine; IdUTP, iododeoxyuridine triphosphate; dTMP, thymidylate; dTMPK, thymidylate kinase; dTTP, 5′-deoxythymidine triphosphate; TS, thymidylate synthase; TK, thymidine kinase; IdUMP, iododeoxyuridine monophosphate; AG-331, N^6-[4-(morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diaminobenz[*cd*]indole glucuronate; and ICI D1694, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid.

IdUTP incorporation into DNA, increase DNA single-strand breaks, and be associated with enhanced cytotoxicity.

AG-331 is a lipophilic TS inhibitor [14], and differs from the folate-based TS inhibitors in that it does not utilize a folate transporter and it is not polyglutamated [14, 15]. While polyglutamation may have positive implications, such as enhanced cellular retention and increased TS specificity [16], it may also contribute to normal tissue toxicity.

In this study, we have shown that AG-331, via inhibition of TS, can increase the incorporation of IdUrd into DNA, increase DNA damage, and significantly enhance the cytotoxic effect of IdUrd.

MATERIALS AND METHODS

Chemicals. AG-331 was a gift from R. Jackson, Agouron Pharmaceuticals Inc. (La Jolla, CA) and readily dissolves in water. IdUrd, pepsin, fluorescein conjugated sheep anti-mouse IgG, and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). IdUrd was protected from light, and drug dilutions were made in PBS. Mouse anti-IdUrd (IU-4) was purchased from Caltag Laboratories (San Francisco, CA). Media, PBS, antibiotics, and trypsin were purchased from Gibco (Grand Island, NY). Plasticware was purchased from Falcon (Bedford, MA).

Cell culture. MGH-U1, a human bladder cancer cell line, was maintained as a monolayer in Alpha Minimum Essential Medium (MEM) supplemented with 0.1% streptomycin, 0.1% penicillin, and 10% fetal bovine serum (Whittaker, Walkersville, MD, and P.A. Biologicals, Sydney, Australia) at 37° in a 5% CO_2 humidified atmosphere [17, 18] and was subcultured twice weekly until passage 20. Under these conditions, the doubling time of the cells growing exponentially was approximately 24 hr, and plating efficiency was >80%. Exponentially growing asynchronous cultures were used in all experiments.

Cytotoxicity assay. The clonogenic survival of drug-treated cells was performed as described previously [17–19]. Briefly, 1×10^6 cells were seeded in a 75-cm^2 flask in 10% dialyzed fetal bovine serum and nucleoside-free MEM. After 24 hr, the exponentially growing cells were exposed to various drug concentrations and combinations for an additional 24 hr. Cells were then washed three times in calcium- and magnesium-free PBS, trypsinized, syringed to obtain a single cell suspension, counted, and plated in serial dilutions in replicates of 6. Two weeks after plating, colonies were stained with methylene blue solution, and then counted. Survival was expressed as a fraction relative to untreated control.

TS inhibition assay. Exponentially growing MGH-U1 cells were incubated with various concentrations of IdUrd or AG-331 for 24 hr. Following drug incubation, cells were washed with PBS, and TS activity was determined in intact cells as previously demonstrated by Yalowich and Kalman [20]. All values were corrected for background counts (the amount of tritiated water production as a result of the spontaneous breakdown of the radioisotope). Concentration–response curves were obtained by expressing the amount of tritium released from each treated culture as a percentage of the radioactivity measured in untreated controls.

Flow cytometry analysis of IdUrd incorporation into DNA. Flow cytometry was used to quantitate IdUrd incorporation into DNA following TS inhibition by AG-331 [21]. Exponentially growing cells were exposed to various concentrations of IdUrd for the last 2 hr of a 24-hr exposure to 5 μM AG-331. The penultimate 2-hr incubation time for IdUrd treatment was chosen as there was active drug incorporation taking place during this time. Nuclei were extracted from cells with a solution of 0.4 mg/mL pepsin in 0.1 M HCl for 30 min at 37°. The sample was filtered subsequently through a 35 μm nylon mesh and centrifigued at 1000 g. The pellet was then resuspended in 2 M HCl solution for 10 min at room temperature, centrifuged, and resuspended in PBS. This sample was first exposed to monoclonal antibody IU-4, which specifically binds to bromodeoxyuridine (BrdUrd) and IdUrd, then to fluorescein conjugated sheep anti-mouse IgG, and lastly to propidium iodide. Each of these exposures were 1 hr in duration at room temperature. Fluorescence for IU-4 binding and propidium iodide staining was detected for 20,000 cells with green and red fluorescence, respectively.

DNA damage. The alkaline comet assay was used to detect DNA single-strand breaks, with some modifications to the methods described previously [22]. Cells were embedded in 1% agarose that gels at a low temperature, lysed in a 1 M NaCl/0.03 M NaOH solution for 20 min, and subjected to electrophoresis (1 V/cm) for 10 min. After staining with 2.5 μg/mL propidium iodide for 30 min, a characteristic comet appeared with a tail consisting of DNA fragments. The samples were examined using a 25X objective with an Olympus epifluorescence microscope fitted with an intensified CCD camera, interfaced to a SAMBA 4000 image analysis system (IPI, Chantilly, VA). Tail moment was defined [23] as the product of the relative amount of DNA in the comet tail (the total fluorescence of the image located in the comet tail) multiplied by a measurement of the tail length (the distance between the means of distributions for the head and tail of the comet). The tail moment represents the DNA single-strand breaks caused by IdUrd and AG-331, either alone or in combination.

Normal human bone marrow CFU-GM toxicity studies. Cells were separated and grown as described previously [24]. Briefly, 1×10^6 CFU-GM cells were exposed to the same drug treatments as in the cytotoxicity assay. After 24 hr of drug treatment, 5×10^4 and 1×10^5 cells were cultured in Iscove's Modified Dulbecco's MEM, 2-mercaptoethanol (5×10^{-5} M), 10% fetal bovine serum, 5% phytohemagglutinin (PHA-LCM), and 0.9% methylcellulose. These cultures were incubated for 14 days at 37° in a humidified atmosphere supplemented with 5% CO_2. Colonies were detected and counted using an inverted microscope (Carl Zeiss Ltd., Don Mills, Canada).

RESULTS

Cytotoxicity studies in MGH-U1 cells. As previously

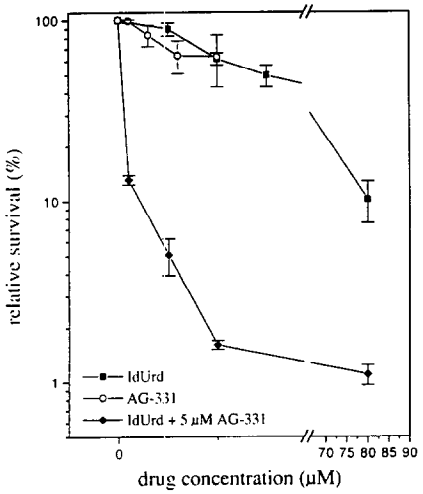

Fig. 1. Clonogenic survival of MGH-U1 cells treated with AG-331, IdUrd, or the combination of AG-331 plus IdUrd for 24 hr. Each point represents the mean ± SD of at least three experiments.

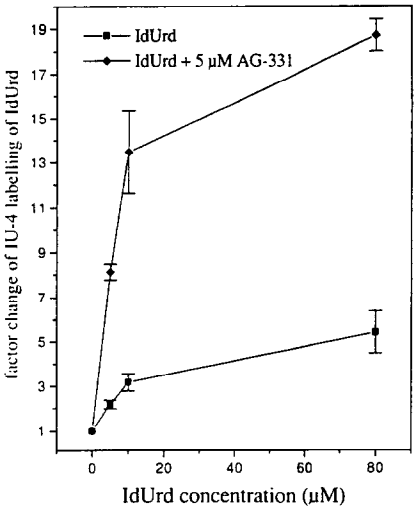

Fig. 2. Incorporation of IdUrd alone or in the presence of 5 μM AG-331 into the cellular DNA of MGH-U1 cells. Cells were exposed to IdUrd for the last 2 hr of a 24-hr exposure to 5 μM AG-331. Each point represents the mean ± SD of three experiments.

reported [7], IdUrd was cytotoxic to MGH-U1 cells. The cytotoxic IC_{50} (drug concentration producing 50% clonogenic survival) and IC_{90} (drug concentration producing 10% clonogenic survival) for IdUrd were 13 and 81 μM, respectively (Fig. 1). After a 24-hr exposure to AG-331, clonogenic survival was decreased to 59 ± 20% at a concentration of 20 μM consistent with previous studies [25]. In our studies, a 24-hr exposure of MGH-U1 cells to 5 μM AG-331 decreased clonogenic survival by a maximum of 30% (Fig. 1). Combination studies with 5 μM AG-331 and various concentrations of IdUrd for 24-hr exposures exhibited enhanced IdUrd cytotoxicity (Fig. 1). The IC_{50} of IdUrd decreased from 13 to 1.5 μM, and the IC_{90} from 81 to 5 μM with the addition of 5 μM AG-331.

Inhibition of TS activity. The inhibition of TS by AG-331 was observed in MGH-U1 cells, consistent with previous studies [25]. The IC_{50} and IC_{90} values for TS inhibition in MGH-U1 cells exposed to AG-331 for 24 hr were 0.7 and 3.0 μM, respectively. IdUrd at concentrations up to 160 μM showed no evidence of TS inhibition in this cell line. We had reported previously [7] a lack of TS inhibition by IdUrd in both MGH-U1 and a human colon cancer cell line, HCT-8.

IdUrd incorporation into DNA. The incorporation of IdUrd was examined in the DNA fraction of MGH-U1 cells. The incorporation of IdUrd increased in a concentration-dependent manner (Fig. 2). Cells that were pretreated for 22 hr with 5 μM AG-331 showed an enhanced incorporation of IdUrd as compared with those that were not pretreated, at the same IdUrd concentrations (Fig. 2).

DNA damage. DNA single-strand breaks following 24-hr drug exposure were measured in individual cells using a sensitive microelectrophoresis method, which has shown previously that the number of

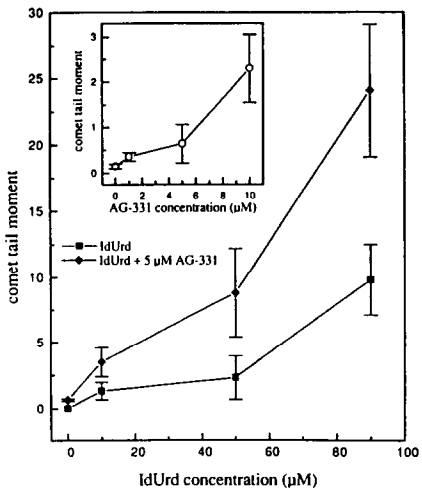

Fig. 3. DNA single-strand breaks caused by IdUrd, AG-331, and the combination of 5 μM AG-331 plus IdUrd, as determined by comet tail moment. Cells were exposed to AG-331 and IdUrd for 24 hr. Each point represents the mean tail moment of thirty images from three separate experiments. Error bars represent the SD.

DNA single-strand breaks is related to the extent of DNA migration or tail moment [23]. DNA single-strand breaks were observed with both IdUrd and AG-331, although the latter only showed minimal effects (Fig. 3); 5 μM AG-331 produced a mean tail moment of less than 1.0. The addition of 5 μM AG-331 resulted in enhanced single-strand breaks with the same concentrations of IdUrd (Fig. 3). At 90 μM

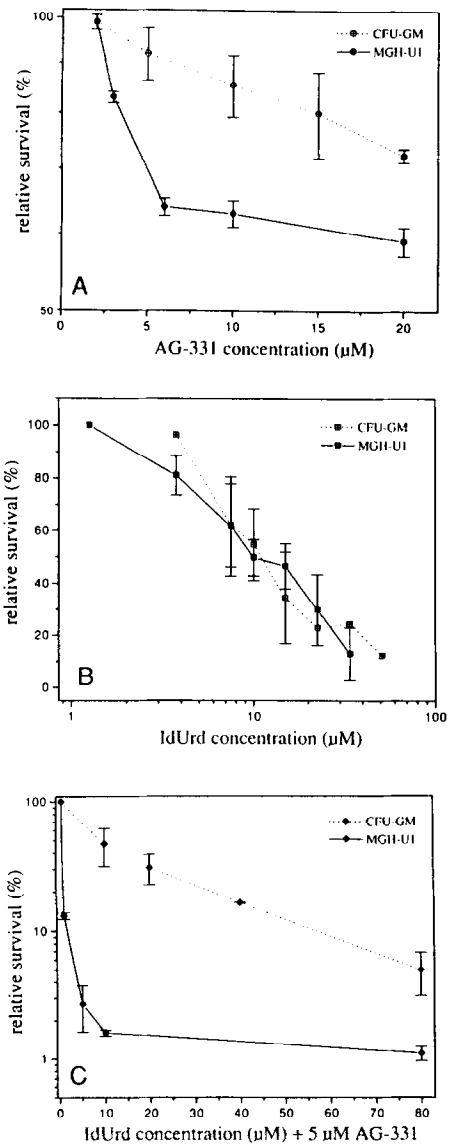

Fig. 4. Cytotoxicity of (A) AG-331, (B) IdUrd and (C) IdUrd + 5 μM AG-331 in MGH-U1 and CFU-GM cells. Graph (B) is a reproduction of previously published results [7] from our laboratory. The drug exposure was for 24 hr for both MGH-U1 and CFU-GM cells. Each point represents the mean ± SD of at least three experiments.

IdUrd, the tail moment increased from 8 to 24 when 5 μM AG-331 was added.

Normal human bone marrow CFU-GM cytotoxicity. The clonogenic survival of CFU-GM cells was investigated with AG-331 and the combination of AG-331 plus IdUrd, as a surrogate for myelosuppression *in vivo*. The cytotoxicity of AG-331 alone or in combination with IdUrd is depicted in Fig. 4. AG-331 at 5 μM had limited toxic effects on CFU-GM cells, with a 92 ± 6% survival after a 24-hr exposure (Fig. 4A). The therapeutic index (TI) was determined as: TI = IC_{50}(CFU-GM)/IC_{50}(MGH-U1). We had previously reported the therapeutic index of IdUrd alone to be 1.17 (Fig. 4B) and that for the combination of IdUrd plus ICI D1694 to be 4.0 in MGH-U1 cells [7]. The combination of AG-331 with IdUrd resulted in a more favorable therapeutic index of 6.5 (Fig. 4C).

DISCUSSION

Previous attempts have been made to exploit differences in pyrimidine salvage between normal and tumor tissue by combining 5-fluorouracil (5-FU) with thymidine analogues [26–28]. Since 5-FU can be incorporated into RNA and DNA, in addition to inhibiting TS [29], interpretation of its interaction with IdUrd is more difficult than is the case with a specific TS inhibitor such as AG-331 or ICI D1694. We previously reported [7] that the combination of IdUrd with the specific and potent TS inhibitor ICI D1694 resulted in an enhanced inhibition of colony formation, which was associated with increased IdUrd incorporation into cellular DNA and increased DNA damage. Since TK activity in tumor cells is elevated as compared with that of normal cells [30], we can exploit this salvage pathway for dTMP formation with the simultaneous inhibition of the *de novo* synthesis pathway of dTMP formation, namely TS activity by ICI D1694. Thus, we had previously suggested that inhibition of TS activity and subsequent exploitation of TK activity by IdUrd would result in selective inhibition of colony formation in tumor cells. If our hypothesis holds true, then any TS inhibitor that is combined with IdUrd should produce similar results. AG-331 differs from ICI D1694 with respect to structure, transport into cells, and inhibition of colony formation. Even though both agents are specific TS inhibitors, AG-331 was a relatively weak cytotoxic agent (Fig. 1) as compared with ICI D1694 [7]. Hence, we chose to study IdUrd in combination with AG-331 so as to investigate the role of TS with minimal interference of cytotoxicity.

The goals of this study were to determine whether combining IdUrd with AG-331 would result in enhanced cytotoxicity, to determine the biochemical effects of these agents on DNA, and to measure the effects on normal human bone marrow CFU-GM.

Studies of AG-331 [25, 31] have indicated that it inhibits TS activity. The IC_{50} and IC_{90} values for TS inhibition were 0.7 and 3.0 μM, respectively, for 24-hr exposures in MGH-U1 cells. IdUrd did not inhibit TS activity at concentrations ranging from 5.0 to 160 μM in MGH-U1 cells, consistent with previous studies [7]. We predict that through AG-331's inhibition of TS activity, dTTP pools will be depleted. We have encountered difficulty in measuring the effect of TS inhibitors on dTTP pools, as the increase in dUTP, as a consequence of TS inhibition, coelutes or interacts with the assays for dTTP, creating much difficulty in determining dTTP levels. Further experimentation of the effects of TS inhibitors on dTTP are in progress in our laboratory.

At a concentration that maximally inhibits TS activity while causing only 30% inhibition of clonogenic survival, AG-331 profoundly increased IdUrd cytotoxicity in MGH-U1 cells (Fig. 1). Several

biochemical factors that increase IdUrd cytotoxicity may be operative. TS inhibition by AG-331 could decrease dTTP pools, decrease the de-iodination of IdUMP, and increase both TK and dTMPK activity, which would favor an increased IdUrd incorporation into DNA, possibly leading to DNA damage and cell death. However, TS inhibition could also increase dUMP pools [32], thus increasing the levels of dUTP, which could compete with IdUTP for DNA polymerase. Since we have depleted the cell of dTTP, repair of dUTP misincorporation into DNA is not possible and this would thus lead to DNA damage, which, in turn, might decrease IdUrd incorporation into DNA. Biochemical evaluation of IdUrd incorporation into DNA was warranted.

An alternative flow cytometric method [7], which detects IdUrd labeling of DNA by a mouse monoclonal antibody to BrdUrd and IdUrd, was used to detect IdUrd incorporation into DNA. MGH-U1 cells treated with 5 μM AG-331 showed increased IdUrd incorporation into DNA (Fig. 2), and this was associated with increased DNA single-strand breaks (Fig. 3). The increase in IdUrd incorporation into DNA and the increase in single-strand breaks paralleled the decrease in clonogenic survival. Taken together, these results suggest that TS inhibition increases IdUrd incorporation into DNA, leading to an enhanced inhibition of colony formation.

A successful combination is one that gives a greater increase in damage to tumor cells as compared with normal cells. The therapeutic index is a measure of this differential effect. AG-331 and the combination of AG-331 plus IdUrd have been evaluated in normal human bone marrow CFU-GM cells as a surrogate for myelosuppression *in vivo* [33]. The combination of AG-331 and IdUrd exhibited a larger therapeutic index compared with the therapeutic index of IdUrd alone. The difference in cytotoxicity observed by this combination in normal versus cancer cells could be due to differences in the proportion of cells cycling, differences in doubling time between the tumor and the CFU-GM cells in culture, or differences in drug metabolism between the normal and tumor cells. Elevated levels of TK activity in tumor cells, relative to normal cells, have been reported by Weber [30]. This could result in greater phosphorylation of IdUrd by tumor cells, leading to greater incorporation into DNA and increased tumor cell death, as compared with normal cells.

We had postulated that the inhibition of TS by AG-331 would decrease dTTP pools, which would compete with IdUTP as a substrate for DNA polymerase. Furthermore, the inhibition of TS would decrease the dehalogenation of IdUMP by TS [13, 34], and increase TK and dTMPK activity [10]. Each of these consequences of TS inhibition would favor more incorporation of IdUrd into DNA. The results of these studies support this original premise. Hence, we conclude that biochemical and therapeutic exploitation of differences in TdR salvage in tumor cells may be possible when *de novo* thymidylate synthesis is inhibited.

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