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SITE OF ACTION OF TWO NOVEL PYRIMIDINE BIOSYNTHESIS INHIBITORS ACCURATELY PREDICTED BY THE COMPARE PROGRAM

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Abstract—The computer algorithm COMPARE provides information regarding the biological mechanism of action of a compound. In this study, excellent correlations were obtained for 2,2′-[3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)diimino]bis-benzoic acid (redoxal) and 1-(*p*-bromophenyl)-2-methyl-1*H*-naphth[2,3-*d*]imidazole-4,9-dione (BNID) and two well-studied dihydroorotate dehydrogenase (DHOD) inhibitors, dichloroallyl lawsone and brequinar, in terms of antiproliferative activity against tumor cell lines *in vitro*. When redoxal and BNID were incubated with MOLT-4 cells for 72 hr, 50% growth inhibition was achieved at 0.7 and 3.5 μM, respectively. After 24 hr of incubation, pyrimidine triphosphate pools were shown to be decreased by 50% by redoxal (1 μM) and BNID (0.25 μM). Addition of either uridine (50 μM) or cytidine (100 μM) antagonized the cellular cytotoxicity caused by either drug; uridine corrected the UTP and CTP deficit, whereas cytidine corrected only the CTP deficit. Exposure of MOLT-4 cells to a 1 μM concentration of either drug for 18 hr followed by a 1-hr exposure to [^{14}C]bicarbonate showed a 97% decrease of incorporation of [^{14}C] into pyrimidine triphosphates accompanied by a 91- and 82-fold increase in radioactive incorporation into L-dihydroorotate and *N*-carbamyl-L-aspartate, respectively. By direct exposure of DHOD prepared from MOLT-4 cell mitochondria to a range of concentrations of the two drugs, apparent K_i values of 0.33 μM (redoxal) and 0.53 μM (BNID) were determined. These data provide direct evidence for inhibition of DHOD by redoxal and BNID in MOLT-4 lymphoblasts.

Key words: brequinar; BNID; DCL; cytidine; uridine; MOLT-4 lymphoblasts; chemotherapy; antimetabolites

The National Cancer Institute's drug screening program tests several hundred compounds a week against a panel of 60 human tumor cell lines [1]. These cell lines represent leukemia, melanoma and cancers of the lung, colon, kidney, ovary and central nervous system. Routine analysis of the data from the screen includes a chart of the differential response pattern on the different cell lines, called a "meangraph" [2] (see Fig. 1). The meangraph assigns a bar length to represent the difference in concentration of the IC_{50}‖ for each individual cell line versus the average IC_{50} concentration for all 60 cell lines. Thus graphed, the bar lengths yield a distinctive silhouette for each compound.

A comparison, then, can be made between the meangraph of any screened agent against any of several databases containing the results of the compounds previously screened, using the computer algorithm COMPARE [2]. One version of the database has the data of each of 176 agents selected for their historic interest to clinical and experimental cancer chemotherapy. The biochemical mechanism of action is presumed to be known for many of the 176 agents. Agents that act by the same or similar mechanisms of action give higher correlation coefficients than agents with unrelated mechanisms of action [3]. Thus, COMPARE analysis often provides useful insights into a new agent's biochemical mechanism of action.

Using this method, good correlations were provided for two compounds, redoxal and BNID, with two standard agents, DCL and brequinar (Fig. 2), as well as other inhibitors of pyrimidine biosynthesis. Brequinar [4] and DCL [5] have been shown to be potent inhibitors of DHOD, the enzyme that catalyzes the fourth reaction in the *de novo* pyrimidine biosynthetic pathway, i.e.

$$\text{L-dihydroorotic acid} \xrightarrow[\text{DHOD}]{\text{ubiquinone}} \text{L-orotic acid}$$

utilizing ubiquinone as the electron acceptor. Based

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‖ Abbreviations: BNID, 1-(*p*-bromophenyl) -2- methyl-1*H*-naphth[2,3-d]imidazole-4, 9-dione; DCL, dichloroallyl lawsone; DHO, L-dihydroorotic acid; DHOD, dihydroorotic acid dehydrogenase; IC_{50}, concentration that inhibits either cell growth or enzyme activity by 50%; OMP, orotidine monophosphate; and redoxal, 2,2′-[(3,3′-dimethoxy[1, 1′-biphenyl]-4, 4′-diyl)diimino]bis-benzoic acid.

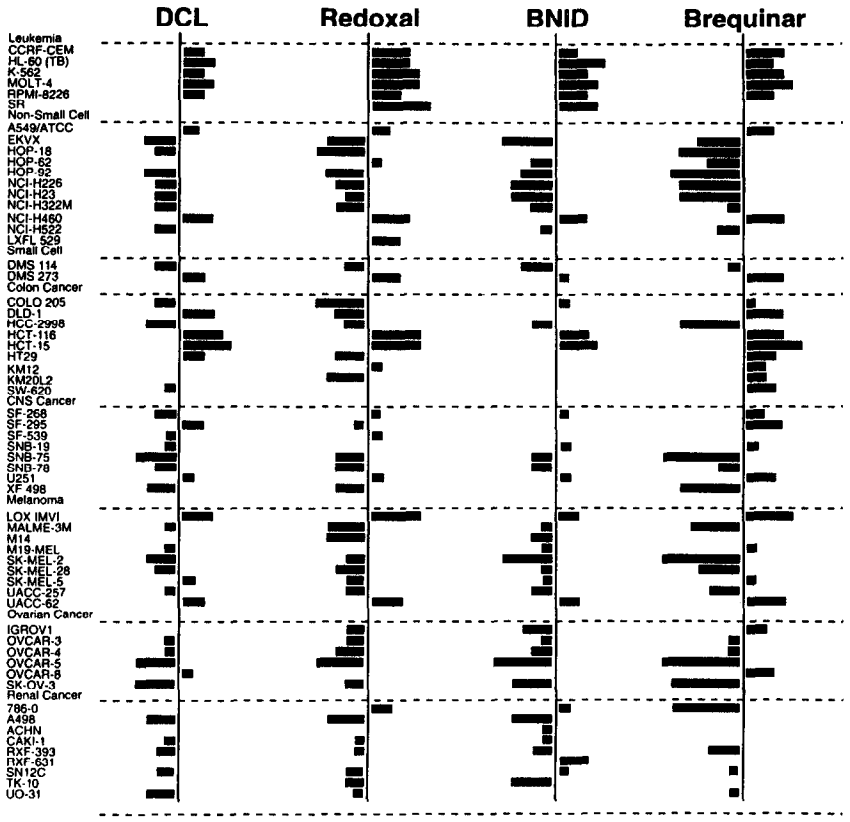

Fig. 1. Fingerprint comparison of the meangraphs for DCL, redoxal, BNID, and brequinar. The central vertical line represents the mean IC_{50} of all cell lines for each drug. Bars to the right indicate a more potent effect of the drug, bars to the left indicate a less potent effect than the average.

on the COMPARE correlations, we initiated biochemical studies to determine whether the mechanism of action and site of activity of redoxal and BNID were, in fact, confined to the same locus attacked by brequinar and DCL. In the present paper, the results of these studies are described.

MATERIALS AND METHODS

Growth inhibitory studies (IC_{50}). MOLT-4 human leukemia cells were added to 96-well microtiter plates at 30,000 cells/well in 100 μL of RPMI 1640 containing 5% fetal bovine serum and 5 mM L-glutamine. This cell density produces a linear growth phase for 72 hr. Cells were preincubated for 4 hr at 37° in 5% CO_2 at 100% relative humidity. Drugs and/or nucleosides in growth medium and gentamicin (50 μg/mL) were then added to the plates. For the growth inhibition studies, brequinar, DCL, redoxal and BNID were added in 10 different concentrations—log and 1/3 log dilutions from a maximum concentration of 1×10^{-4} M, in 100 μL of growth medium. For nucleoside rescue studies, counteragents were added in 50 μL of growth medium at the appropriate concentrations, followed by the drugs in 50 μL of growth medium. Plates containing cells and drugs were incubated for a further 72 hr and then acid-fixed [1,6] with 50 μL of cold 80% (w/v) trichloroacetic acid, refrigerated for 1 hr at 4°, rinsed five times with deionized water, and allowed to air dry. Then plates were incubated for 10 min at room temperature with 100 μL of 0.4% sulforhodamine-B (w/v in 1% acetic acid); this stain binds to the basic amino acids of cellular proteins and thus provides a means of quantifying them [6]. The plates were then rinsed five times with 1% acetic acid and air dried. Bound stain was solubilized with 100 μL of 10 mM unbuffered Tris base (pH 10.5). Optical densities were read on an automated spectrophotometric plate reader (Bio-Tek, model EL 312) at 515 nm. The cells on two plates were fixed with trichloroacetic acid just prior to drug addition to establish the protein content of cells present at the time of treatment. Using this initial cellular protein content, concentration responses were calculated from the protein increase in the control and drug-treated wells. The drug concentration causing a 50% reduction in net protein increase in control cells during drug incubation (IC_{50}) was calculated from the concentration–response curve.

Cells were also investigated for their ability to recover from 8- and 72-hr drug treatment. This involved aspiration of the drug 8 and 72 hr after treatment, addition of fresh medium, and then feeding with 100 μL of new medium over time, as

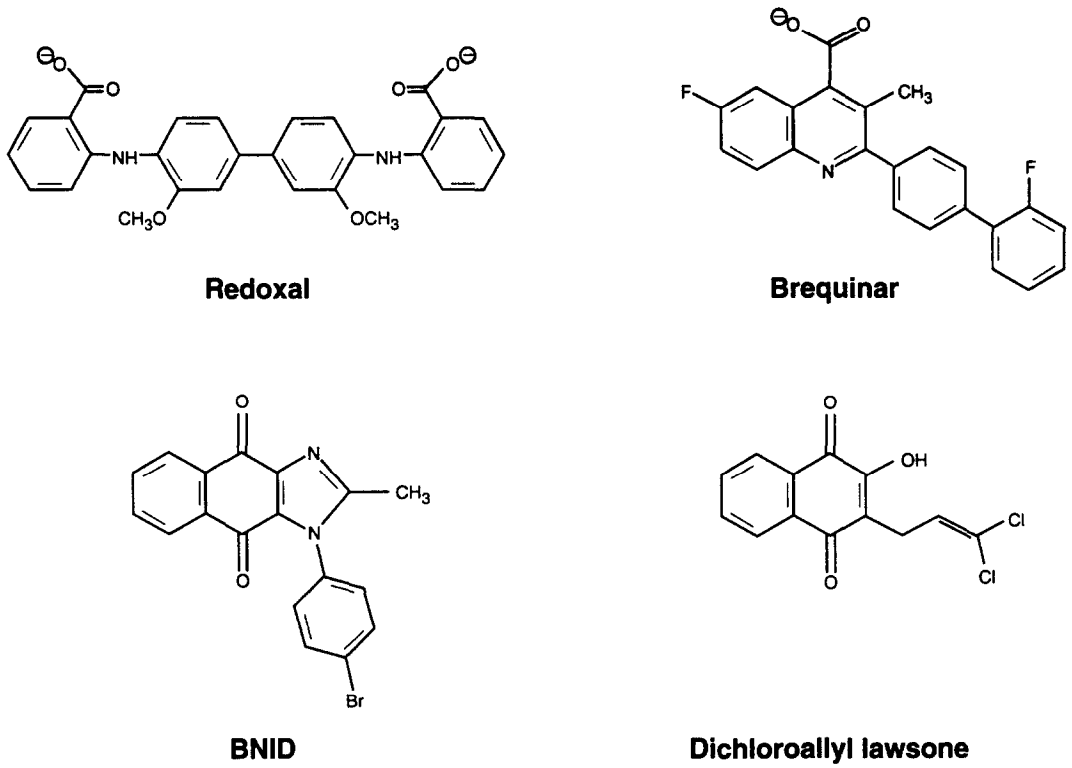

Fig. 2. Structures of *redoxal*, 2,2′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)diimino]bis-benzoic acid; *brequinar*, 6-fluoro-2-(2″-fluoro-1,1″-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid; *BNID*, 1-(*p*-bromophenyl)-2-methyl-1*H*-naphth[2,3-*d*]imidazole-4,9-dione; and *DCL*, 2-hydroxy-3-(3,3-dichloro-allyl)-1,4-naphthoquinone.

appropriate. For a period of 3 weeks thereafter, plates were observed microscopically on a daily basis, fixed, and stained to determine any resumption of cell growth following treatment-induced growth arrest and wash-out.

Measurement of pyrimidine nucleotide pools in MOLT-4 cells. MOLT-4 cells were grown in 50 mL of complete medium for 24 hr in the presence or absence of redoxal (10 μM) or BNID (2 μM). Cytidine (50 μM), uridine (50 μM) or saline was added simultaneously with the drug. Cells were pelleted by centrifugation at 800 *g* for 10 min, and the supernatant was removed. The pellet was then resuspended in 60% MeOH (1 mL/100 million cells), heat-inactivated for 2 min at 95°, centrifuged at 16,000 *g* for 6 min and the supernatant injected onto a 4.6 × 250 mm anion exchange column (Whatman Partisil SAX 10 μm). The column was equilibrated and developed isocratically with 2.5 mM $NH_4H_2PO_4$ (titrated with phosphoric acid to pH 2.8) for the first 15 min followed by a 27-min linear gradient from 2.5 mM $NH_4H_2PO_4$ (pH 2.8) to 0.7 M $NH_4H_2PO_4$ (titrated with phosphoric acid to pH 3.7). This second more concentrated buffer was held isocratic for 2 min followed by 6 min of a reverse linear gradient. The lower concentration buffer was then run isocratically for the final 10 min to re-equilibrate the column for the next sample. UTP and CTP peaks were identified at 36.4 and 37.4 min, respectively, by their absorption at 254 and 280 nm and co-elution with known standards, and quantitated by their absorbance at 254 nm.

To determine the concentration of the drug that would result in an approximate 50% reduction of UTP and CTP pools, MOLT-4 cells were grown for 24 hr in the presence of redoxal (0.5, 2.0, 5.0, and 10.0 μM) or BNID (0.2, 0.5, 1.0, and 2.0 μM) and then extracted as described above. The nucleotide levels were determined by ion-exchange HPLC as described previously.

Effects of redoxal and BNID on the flux of [^{14}C]-*bicarbonate into cellular nucleotides.* MOLT-4 cells were grown for 18 hr in sodium bicarbonate-free, RPMI 1640 medium in the presence or absence of redoxal (10 μM) and BNID (10 μM). Cells were then incubated for 1 hr in medium containing 4 μCi/mL of [^{14}C]sodium bicarbonate [7], and extracted as described above; the nucleotide pools in the supernatants were analyzed by ion-exchange HPLC with online radiometric detectors.

Separation of L-*DHO,* L-*orotic acid and* N-*carbamyl-*L-*aspartic acid by HPLC.* DHO, L-orotic acid and *N*-carbamyl-L-aspartic acid were also separated by ion-exchange chromatography (Partisil SAX, 10 μm column). To achieve complete separation in a shorter time period, the elution gradient described earlier was modified as follows. The column was equilibrated isocratically with 3.9 mM

$NH_4H_2PO_4$ (titrated with phosphoric acid to pH 3.0) for the first 15 min followed by a 2-min linear gradient from 3.9 mM $NH_4H_2PO_4$ (pH 3.0) to 0.7 M $NH_4H_2PO_4$ (titrated with phosphoric acid to pH 3.7). This second, more concentrated buffer was held isocratic for 2 min, followed by 6 min of a reverse linear gradient, and then re-equilibration of the column with the lower concentration buffer for 5 min. Retention times were found using known standards.

Mitochondrial preparation. MOLT-4 cells growing in log phase were centrifuged (800 *g*, 10 min), and the cell pellet was suspended in buffer containing 0.02 M Tris–HCl and 2 mM dithiothreitol, pH 8.4 (10^6 cells/0.5 mL). The suspension was homogenized gently using Tissue Tearer (Biospec Products Inc., Racine, WI) for about 7 sec at the lowest setting. This procedure disrupted cell membranes while leaving the mitochondria intact. Cellular debris was removed by a brief centrifugation (16,000 *g*, 3 sec), and the mitochondrial fraction was pelleted from this supernatant at 16,000 *g* using a 15-min centrifugation at 4°. The mitochondrial pellet was resuspended in a buffer containing 0.01 M Tris, 2 mM dithiothreitol in sterile normal saline (pH 8.4). After a gentle agitation, the suspension was centrifuged (16,000 *g*, 15 min) to remove L-dihydroorotase contamination (> 85% efficiency). The resultant pellet was resuspended in the above buffer at a concentration of 200×10^6 cells/mL, after which the sample was agitated gently to achieve uniformity. As the enzyme DHOD is membrane bound [9], the final suspension of intact mitochondria maintains both the enzyme and its ubiquinone cofactor, which is also membrane bound. The enzyme was used immediately after preparation. The protein concentration was measured using a standard nomogram, extrapolating the protein concentration from the ultraviolet absorbance at 260 and 280 nm.

Enzyme assay for DHOD. The enzyme activity was determined by incubating a 10-μL aliquot of the mitochondrial preparation (as described above) in a total reaction volume of 30 μL (0.5 mg/mL protein) with [^{14}C]L-dihydroorotic acid (Moravek Biochemicals, Brea, CA) (2.75 mCi, 50 μM). Inhibition studies were conducted by measuring the initial reaction velocities in the presence or absence of either redoxal (0.01 to 1 μM) or BNID (0.01 to 1 μM). The enzyme preparation was preincubated with the respective inhibitor approximately 5 min before the addition of substrate. The reaction was allowed to proceed for 10 min at 37°, before termination by heating at 95° for 2 min. The concentration of [^{14}C]L-orotic acid formed was determined by the ion-exchange chromatography described earlier, using an online radioactivity detector.

RESULTS

COMPARE program. Using the COMPARE program [2], with a database of the principal synthetic compounds tested in the NCI screen up until this time (> 25,000 compounds), redoxal, used as the seed compound, gave a good correlation coefficient with both DCL (0.80) and brequinar (0.72) and even better with BNID (0.84). Other pyrimidine biosynthetic pathway inhibitors, PALA [*N*-(phosphoacetyl)-L-aspartic acid] and pyrazofurin, also gave close correlation coefficients with BNID (0.56, 0.60) and redoxal (0.66, 0.56), respectively. When the DCL meangraph (Fig. 1) was matched against the database, BNID and redoxal appeared as the third and fifth most closely correlated patterns, respectively.

Growth inhibition. All four compounds were cytotoxic. The IC_{50} values in MOLT-4 cells incubated for 72 hr were: 0.1 μM for brequinar, 4.0 μM for DCL, 0.7 μM for redoxal, and 3.5 μM for BNID (Fig. 3). Figure 3 also demonstrates that 50 μM uridine abrogated the toxicity of all four compounds, whereas 100 μM cytidine was less effective at modulating drug toxicity. Uridine protection was concentration dependent, with slight amelioration observed at 1 μM, and maximum reversal reached at 12.5 ± 2.5 μM. In the presence of 100 μM OMP, there was greater than a 10-fold decrease in the potency (measured by IC_{50}) of all the drugs, whereas 100 μM cytidine, CMP and thymidine resulted only in sufficient protection to decrease drug potency by a factor of two. Other metabolites (at 100 μM) that had no effect in altering drug toxicity were: orotidine, orotic acid, *N*-carbamyl-L-aspartic acid, DHO, cytosine, and uracil.

To evaluate the ability of cells to recover from drug toxicity, the cells were exposed for 8 and 72 hr, then washed and re-fed with fresh medium. Following an 8-hr incubation with the IC_{50} concentration given earlier, for all four agents, MOLT-4 cells resumed normal proliferation in 3–4 days. After a 72-hr incubation with redoxal, cells again recovered in 4 days. However, it required 6 days for full recovery from 72 hr of incubation with DCL, 7 days following 72 hr of incubation with brequinar, and 8–9 days before full recovery was seen with 72-hr BNID treatment. This indicates some differential in the ability of MOLT-4 cells to recover from prolonged exposure to equitoxic concentrations of these agents.

Redoxal and BNID as pyrimidine inhibitors. When logarithmically growing MOLT-4 cells were incubated for 24 hr with 10 μM redoxal or 2 μM BNID, the pool sizes of UTP and CTP present in methanol extracts of these cells fell dramatically (Table 1). To further quantify the depletion of pyrimidine ribonucleoside-5′-triphosphates produced by these agents, MOLT-4 cells were incubated at 37° for 24 hr with graduated concentrations of the two drugs, and that concentration achieving a 50% depletion of UTP and CTP pools was estimated by graphical analysis (Fig. 4). From these diagrams it can be concluded that the median antipyrimidine effect was achieved with redoxal at 1.0 μM, and with BNID at 0.25 μM. Noteworthy is the finding that redoxal and BNID both produced a considerable elevation of ATP pools (Table 1). The basis for this effect was not studied further.

As both redoxal and BNID proved capable of profoundly depleting both the UTP and CTP pools, we next examined the ability of preformed uridine, cytidine and a mixture of the two to (a) antagonize the cytotoxicity of these agents, and (b) achieve

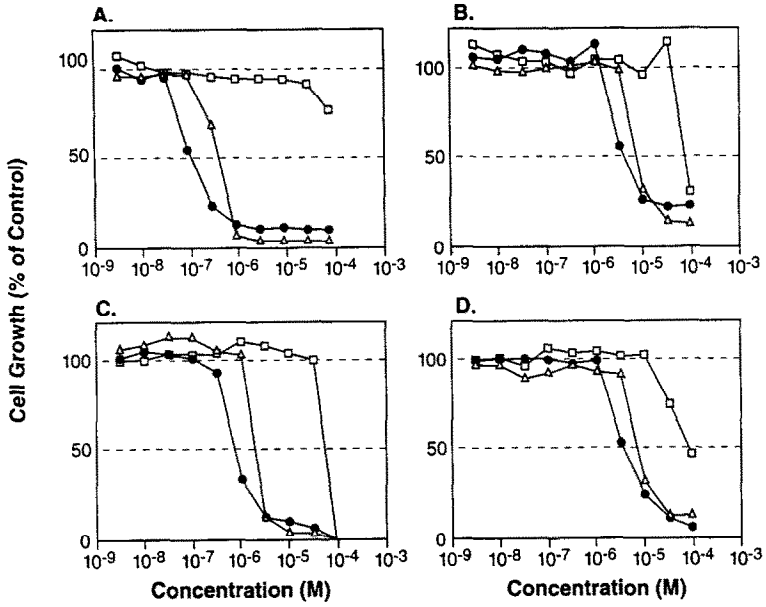

Fig. 3. Comparative cytotoxicities of brequinar, DCL, redoxal and BNID. MOLT-4 cells (3×10^4 cells in 100 μL) were incubated for 72 hr in the presence of (●) (A) brequinar, (B) DCL, (C) redoxal, and (D) BNID at the concentrations indicated. Proliferation was also measured in the presence of the drugs indicated above plus two rescue agents added simultaneously: (□) 50 μM uridine, and (△) 100 μM cytidine. Redoxal at 10^{-4} M displayed cell kill of 16% (negative growth), which failed to recover in the presence of either pyrimidine. This graph is representative of multiple experiments.

Table 1. UTP and CTP rescue from the antipyrimidine effects of redoxal and BNID

Compound(s) added to cells	Pool sizes (pmol/10^6/cells)		
	CTP	UTP	ATP
None	470 ± 45	2075 ± 155	3470 ± 230
Redoxal	25 ± 5	75 ± 5	4735 ± 265
Redoxal and cytidine	995 ± 5	65 ± 5	2285 ± 25
Redoxal and uridine	340 ± 10	1515 ± 5	3240 ± 10
BNID	57 ± 3	157 ± 4	4746 ± 67
BNID and cytidine	562 ± 8	200 ± 6	2222 ± 48
BNID and uridine	218 ± 16	808 ± 28	2406 ± 63
Cytidine	590 ± 5	2085 ± 66	3781 ± 137
Uridine	382 ± 9	1819 ± 79	3367 ± 127

MOLT-4 cells were incubated for 24 hr in the presence of toxic levels of redoxal (10 μM) and BNID (2 μM) with and without cytidine (50 μM) and uridine (50 μM) as indicated. Pool sizes were determined by HPLC as described in Materials and Methods. Values are the means ± SD of triplicate experiments.

restoration of the pyrimidine riboside-5′-triphosphate pools. Incubation of MOLT-4 cells with 10 μM redoxal or 2 μM BNID in the presence or absence of 50 μM uridine or cytidine led to the following results with both drugs: exogenous uridine tended to restore both UTP and CTP pools towards the levels measured in untreated cells; and cytidine tended to achieve a normalization principally of the CTP pools with only a minor impact on the UTP levels in these lymphoblasts (Table 1).

Redoxal and BNID as pyrimidine de novo *pathway inhibitors.* Once the depletion of UTP and CTP pools had been established, it became necessary to determine the locus of action of the agents generating this effect. Of the two cellular pathways to pyrimidine ribonucleoside-5′-triphosphates (*de novo* and salvage), the *de novo* pathway seemed to be the most likely target, since exogenous uridine and cytidine were capable of replenishing the CTP and UTP deficiency, indicating integrity of the salvage route. To better understand the mechanism of action, the metabolism of radiolabeled sodium

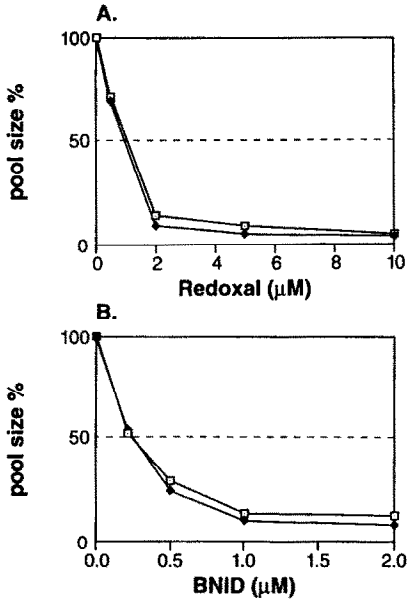

Fig. 4. Percent inhibition of pyrimidine triphosphate pool sizes. MOLT-4 cells were incubated for 24 hr in the presence of (A) redoxal and (B) BNID at the concentrations indicated. The percent decreases of CTP (◆) and UTP (□) pools are shown. In no case did the individual values (3) differ from the mean by more than 5%. The endogenous concentrations of CTP and UTP are: 469 and 1447 pmol/10^6 cells, respectively.

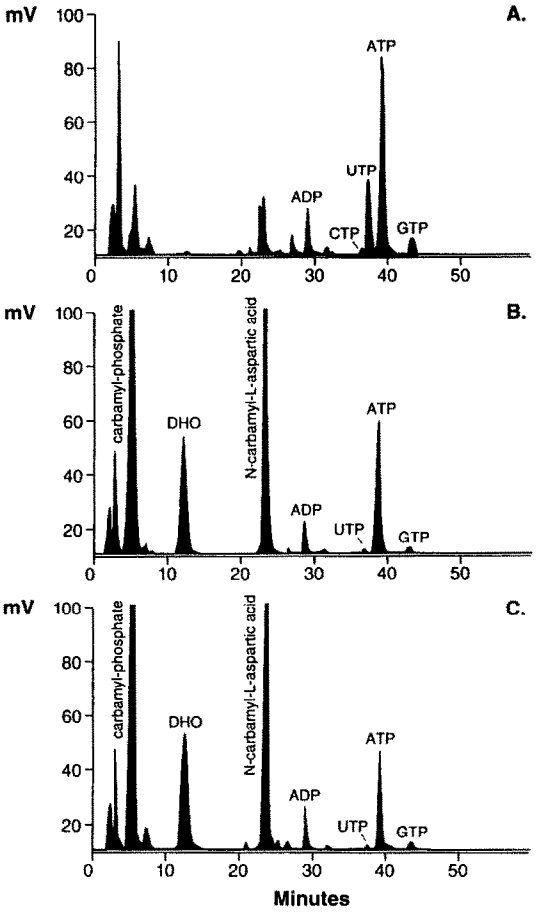

Fig. 5. Sodium bicarbonate flux. HPLC profile of MOLT-4 cell extract obtained after an 18-hr incubation in sodium bicarbonate-free RPMI 1640 medium with (A) saline, (B) redoxal (1 μM), and (C) BNID (1 μM). Then cells were pulsed for 1 hr with 4 μCi/mL of [^{14}C]sodium bicarbonate, and methanolic extracts were examined by HPLC. This is a representative tracing of triplicate experiments.

bicarbonate, the initial compound of the *de novo* pathway, was followed in the presence and absence of the drugs. MOLT-4 cells were incubated for 18 hr with redoxal or BNID (1 μM) in sodium bicarbonate-free RPMI 1640 medium in a sealed flask to produce a CO_2-poor environment, and then pulsed for 1 hr with [^{14}C]sodium bicarbonate. Methanolic extracts of cells treated with either redoxal or BNID showed a lack of [^{14}C] incorporation into cytidine mono-, di-, and triphosphates with a 98.2% (redoxal) to 96.9% (BNID) decrease of radioactive incorporation into uridine triphosphate pools (Fig. 5), further evidence for the *de novo* pathway theory. These cells also showed a considerable increase in radioactive incorporation into DHO (91-fold) and *N*-carbamyl-L-aspartic acid (82-fold) (Fig. 5), the third and second products of the biosynthetic pathway, and just prior to DHOD, pointing to DHOD as the target enzyme.

Redoxal and BNID as DHOD inhibitors. When DHOD had been shown to be a potential target enzyme, the next step was to treat the preparation of partially purified (but still particulate) mitochondrial DHOD directly with the two compounds and establish the potency of inhibition. DHOD was prepared from MOLT-4 cells as described above, and the concentration of [^{14}C]orotic acid, formed from L-[^{14}C]dihydroorotic acid as determined by HPLC, was used to indicate the activity of the enzyme. After establishing that the initial enzyme rate was linear with respect to time and concentration of substrate at 10 min with 50 μM DHO, the reaction was run in the presence of the inhibitors. It should be noted that the concentration of the coenzyme ubiquinone was not varied but remained constant at the level present in MOLT-4 cell mitochondria [8].

By varying the concentration of the inhibitor, the apparent K_i could be calculated by a Dixon plot [9] (Fig. 6). From these diagrams, we can assign an approximate K_i for redoxal of 0.33 μM and for BNID, 0.53 μM.

DISCUSSION

At the National Cancer Institute, initial screening for novel anti-neoplastic agents is conducted in a panel of 60 cultured cell lines, representing the principal human tumors capable of growth *in vitro*. To evaluate the voluminous data generated by such a screen and to pinpoint potentially valuable leads, methods such as COMPARE have been adopted, to provide useful insights into a new agent's biochemical mechanism of action.

In the present experiments, two compounds, the dicarboxylic acid redoxal [10] and the imid-azolonapthoquinone BNID (Fig. 2), were shown to correlate well with the antipyrimidine drugs DCL

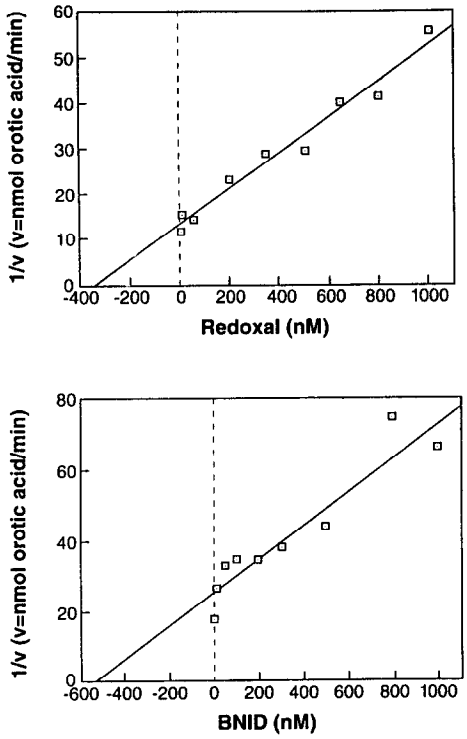

Fig. 6. Dixon plot of the two antipyrimidines. The plot of 1/rate of orotate formation vs test analog concentration [9] is shown, giving apparent K_i values of redoxal (0.33 μM) and BNID (0.53 μM).

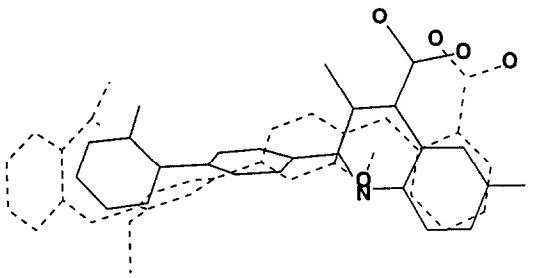

Fig. 7. Superimposed diagrams of the structures of brequinar (——) and redoxal (– – –). Computer-assisted three-dimensional modelling was used to generate this diagram. See discussion for details of the elements used to create this model.

and brequinar. Both of these agents are known for their ability to interrupt the *de novo* pyrimidine pathway at the level of the mitochondrial enzyme DHOD [4,5].

Therefore, several additional lines of inquiry were undertaken with the goal of establishing whether redoxal and BNID share a common site of action with brequinar and DCL. Other mechanisms of action may exist for DCL, redoxal and BNID, as exhibited by inhibition not susceptible to protection by high concentrations of uridine (Fig. 3 B–D); however, they have not been explored in this study.

Initially, MOLT-4 lymphoblasts were incubated for various times in parallel with graded concentrations of the two subject molecules (BNID, redoxal) and the two standards (brequinar, DCL). The cells were extracted and their pyrimidine pools analyzed chromatographically. All four drugs, at concentrations that reduced proliferation, reduced the cellular concentrations of both UTP and CTP considerably. Data for BNID and redoxal are shown in Table 1. Higher concentrations of all four compounds engendered further increases in growth inhibition and parallel depression of pyrimidine nucleoside 5′-triphosphate pools.

It was known that preformed cytidine and uridine both were effective counteragents against the cytotoxicity of brequinar [11] and DCL [5]. Similarly, in the present study, it was found that the salvage of uridine (and to a somewhat lesser extent of cytidine, which is convertible to uridine only in trace amounts [12]) proved capable, under the appropriate experimental conditions, of preventing the retardation of growth produced by both redoxal and BNID. These studies gave every indication that the two new agents were antipyrimidine antimetabolites.

It should be noted at this point that orotic acid and orotidine, while not described by either Chen *et al.* [4] or Bennett *et al.* [5] as rescue agents of brequinar or DCL, were two orotate derivatives which did not serve as rescue agents at concentrations of 100 μM. The basis for this phenomenon remains to be explored.

Further evidence of the antipyrimidine nature of the two new agents included the chromatographic monitoring of the influence of redoxal and BNID on the flux of [^{14}C]bicarbonate through the *de novo* pyrimidine synthetic pathway. In these studies, both agents caused accumulations of both L-[^{14}C]-dihydroorotic acid as well as of *N*-carbamoyl-L-aspartate, the antecedent metabolite in the pathway. Such accumulations are compatible with the imposition of a block subsequent to the biosynthesis of DHO. These results then prompted further direct studies of the impact of the COMPARE-discovered drugs on DHOD, the enzyme responsible for the intramitochondrial conversion of DHO to orotic acid. Using intact mitochondrial preparations (with autochthonous coenzyme Q_{10}), it was demonstrated that both redoxal and BNID were, in fact, highly potent inhibitors of this enzyme (Fig. 6).

The K_i values of redoxal (0.33 μM) and BNID (0.53 μM), under the experimental conditions used, may be compared with those of brequinar 5–8 nM [4], and DCL, 27 nM [5]. Because only a comparatively crude preparation of DHOD was available for the present studies, the formal type of inhibition exerted by redoxal and BNID could not be determined. Such studies will follow our purification of the enzyme from its co-factor.

In summary, the COMPARE program has accurately selected two potent new inhibitors of DHOD. This selection would not have been possible through analysis of structure alone. However, it is relevant that, once the mode of action of these two

subject drugs was pinpointed at DHOD, it was noted that the carboxylic acid and aromatic rings of redoxal are, in fact, suggestive of a possible structural relationship between this molecule and brequinar (Fig. 7). Chen *et al.* [9] have described three requirements for DHOD inhibitory activity in their series of brequinar analogues: (1) a bulky hydrophobic substituent at the C(2) position; (2) a carboxylic acid at C(4); and (3) the benzene portion of the quinoline ring with no bulky substituents at the 7 or 8 position. Figure 7 shows a computer-generated structural fit of redoxal to brequinar that takes these requirements into account.

It is also relevant to note that while brequinar and DCL exhibited excellent antineoplastic activity against experimental tumors in mice and rats [13], comparable activity was not seen in the clinic, possibly because of unmanageable toxicities [14]. It is thus to be hoped that one or both of the novel antipyrimidines described herein will merit consideration for development towards clinical trials should toxicological studies so warrant.

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