Articles

Selective Inhibition of Human Molt-4 Leukemia Type II Inosine 5′-Monophosphate Dehydrogenase by the 1,5-Diazabicyclo[3.1.0]hexane-2,4-diones†

Betsy J. Barnes,*,‡ Ann E. Eakin,‡,§ Robert A. Izydore,|| and Iris H. Hall‡

Division of Medicinal Chemistry and Natural Products, School of Pharmacy and Department of Biochemistry and BioPhysics, University of North Carolina, Chapel Hill, North Carolina 27599-7360 and Department of Chemistry, North Carolina Central University, Durham, North Carolina 27707

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ABSTRACT: Inosine 5′-monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme in de novo purine biosynthesis. IMPDH activity results from expression of two isoforms. Type I is constitutively expressed and predominates in normal resting cells, while Type II is selectively up-regulated in neoplastic and replicating cells. Inhibitors of IMPDH activity selectively targeting the Type II isoform have great potential as cancer chemotherapeutic agents. For this study, an expression system was developed which yields 35−50 mg of soluble, purified recombinant Type I and II protein from 1 L of bacteria. In addition, three 1,5-diazabicyclo[3.1.0]hexane-2,4-diones were synthesized and shown to act as specific inhibitors of human recombinant Type II IMPDH. The agents are competitive inhibitors with respect to the endogenous substrate IMP and K_i values range from 5 to 44 μM but were inactive as inhibitors of the Type I isoform at concentrations ranging from 0.5 to 500 μM. IC_{50} values for recombinant Type II inhibition were determined and compared to IC_{50} values obtained from Molt-4 cell extracts of IMPDH. Cytotoxicity assays revealed that the compounds inhibited Molt-4 leukemia growth with ED_{50} values of 3.2−7.6 μM. Computational docking studies predict that the compounds bind to IMPDH in the IMP-binding site, although interactions with residues differ from those previously determined to interact with bound IMP. While all residues predicted to interact directly with the bound compounds are conserved in the Type I and Type II isoforms, sequence divergence within a helix adjacent to the active site may contribute to the observed selectivity for the human Type II isoform. These compounds represent the first class of selective IMPDH Type II inhibitors which may serve as lead compounds for the development of isoform-selective cancer chemotherapy.

The pathways involved in nucleic acid metabolism are tightly regulated as evidenced by the increased rates of nucleotide synthesis during cell proliferation. Specifically, the pathways synthesizing IMP,[1] ATP, and GTP are individually regulated in most cells so as not only to control the total amounts of purine nucleotides produced but to also coordinate relative amounts of ATP and GTP. Inosine 5′-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205), a rate-limiting enzyme in the de novo synthesis of purine nucleotides, is positioned at the branch point in the conversion of IMP to adenine and guanine nucleotides. IMPDH catalyzes the nicotinamide adenine dinucleotide (NAD^+)-dependent hydroxylation of the purine ring of IMP forming xanthosine 5′-monophosphate (XMP) which is converted subsequently to GMP (*1*−*3*). It has been reported numerous times that cancer cell proliferation can be controlled by inhibitors of de novo purine metabolism (e.g., allopurinol,

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* To whom correspondence should be addressed. Present address: Oncology Center, Johns Hopkins University School of Medicine, Room 364, Bunting/Blaustein Cancer Research Building, 1650 E. Orleans St., Baltimore, MD 21231. Phone: (410) 955-0650. Fax: (410) 955-0840. E-mail: barnebe@jhmi.edu.

‡ Division of Medicinal Chemistry and Natural Products, School of Pharmacy.

§ Department of Biochemistry and BioPhysics.

|| Department of Chemistry.

[1] Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; IMP, inosine 5′-monophosphate; XMP, xanthosine 5′-monophosphate; NAD, nicotinamide adenine dinucleotide; GMP, guanosine 5′-monophosphate; ATP, adenosine triphosphate; DTT, dithioerythritol; MPA, mycophenolic acid; SAD, selenazole-4-carboxamide adenine dinucleotide; TAD, thiazole-4-carboxamide adenine dinucleotide; LPIM, low-phosphate induction medium.

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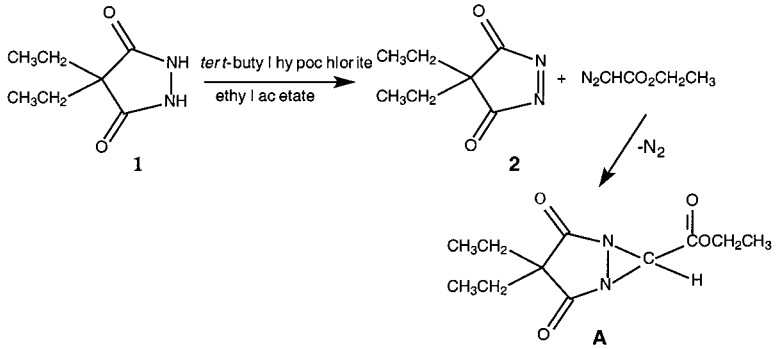
Scheme 1: General Procedure for the Synthesis of 3,5-Pyrazolinedione (**2**) from 4,4-Diethyl-3,5-pyrazolidinedione (**1**) in the Presence of *tert*-butyl Hypochlorite and the Subsequent Reaction with Ethyl Diazoacetate to Obtain **A**



6-mercaptopurine, mycophenolic acid, ribavirin, and tiazofurin) leading to a reduction of nucleotides for RNA and DNA synthesis (*4*). As a result, purine starvation is often responsible for the cellular mechanism of many antineoplastic, antiviral, and immunosuppressive agents (*4*−*6*).

The significance of GTP and dGTP in cancer biochemistry and chemotherapy had been highlighted by the discovery that IMPDH activity is elevated in various murine and human cancers and is particularly high in rapidly growing neoplastic cells such as leukemia cells (*7*). IMPDH activity results from the expression of two isoforms, i.e., Type I and II (*8*−*11*). These two isoforms are regulated differentially in a variety of normal and transformed cell lines. Type I IMPDH is constitutively expressed and is the major isoform found in normal lymphocytes (*12*−*14*). Whereas, the increased level of IMPDH activity found in human leukemias and ovarian tumors appears to be due to the specific induction of the Type II isoform (*12, 14*). Conversely, the expression of the Type II isoform is downregulated with the induction of differentiation in a variety of leukemic and solid tumor cell lines. Normal expression levels are restored with repletion of guanine nucleotide pools (*14*). The discovery of IMPDH isoforms and their differential regulation during neoplastic transformation and cancer cell differentiation provides for the development of isozyme-selective chemotherapeutic agents which target the Type II isoform exclusively.

Despite the availability of IMPDH inhibitors, which either mimic substrate or cofactor (e.g. nucleotide analogues, ribavirin and tiazofurin) or resemble neither (e.g., MPA), lack of specificity to the Type II isoform remains a difficulty for their clinical use. To date, the two isoforms have proven to be kinetically indistinguishable, and no significant differences can be detected between the Type I and II enzymes with any known inhibitors. To examine whether specific inhibitors of IMPDH Type II could be identified, a new class of agents which resemble the previously studied 1-acyl and 1,2-diacyl-4,4-diethyl-3,5-pyrazolidinediones were designed and synthesized (*15*). A number of nonnucleoside small molecular weight mono- and bicyclic agents have been synthesized and thoroughly investigated in this laboratory as antineoplastic agents (*15*−*18*). The 3,5-pyrazolidinediones were chosen for further examination due to their cytotoxic activity and inhibition of whole cell IMPDH activity in Molt-3 T cell leukemia (*15*). Derivatives from this parent chemical structure that incorporated modifications at both nitrogen atoms yielded enhanced inhibition of IMPDH activity (*15*). As a result, similar chemistry as was proposed for the synthesis of the 6-alkoxycarbonyl-1,3,5-triazabicyclo-[3.1.0]hexane-2,4-diones (*19*−*21*) was explored here in order to optimize studies at these two nitrogen atoms. The substitution patterns shown in the chemical structures of compounds **A**, **B**, and **C** at positions 3 and 6 are illustrative of the ongoing study to explore spatial requirements necessary for the specific inhibition of IMPDH activity. Substitutions on the 3,5-pyrazolidinedione ring were thus investigated by synthesizing agents with novel bicyclic structures. Reported herein is a novel group of compounds, the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones, which demonstrate selective inhibition of the human Type II IMPDH isoform.

MATERIALS AND METHODS

Materials. Plasmids containing the cloned Type I and II IMPDH cDNAs from human Molt-4 leukemia cells (*22*) were provided by Dr. Beverly Mitchell (Departments of Pharmacology and Internal Medicine, UNC, Chapel Hill, NC). pBAce plasmid (*23*) was provided by Dr. Sydney P. Craig III (School of Pharmacy, UNC, Chapel Hill, NC). H712 [genotype: *fhuA2*, *lacY1*, *tsx-70*, *glnV44*(AS), *gal-6*, *λ-*, *trpC45*, *his-68*, *guaB22*, *tyrA2*, *rpsL125*(strR), *malT1*(λ^R), *sylA7*, *mtlA2*, and *thi-1*] cells, a mutant *Escherichia coli* strain deficient in IMPDH, were obtained from the Yale Genetic Stock (Princeton, NJ) and used as a host for expression procedures. All restriction endonucleases and ligases were purchased from New England Bioloabs (Beverly, MA). All other reagents were from Sigma Chemical Co. (St. Louis, MI) and of the highest quality commercially available.

*Synthesis of Compounds **A**, **B**, and **C**. General Procedure for the Synthesis of the 3,5-Pyrazolinediones (Scheme 1).* A modification of the procedure reported by Evnin et al. (*24*) was used to synthesize the starting 3,5-pyrazolinediones. To a slurry of 4,4-diethyl-3,5-pyrazolidinedione (**1**) (3.44 g, 22.0 mmol) in dry ethyl acetate (25 mL) under nitrogen was added *tert*-butyl hypochlorite (2.17 g, 20.0 mmol) over a period of 20 min while maintaining the reaction at room temperature (rt). After the addition was complete, the resulting bright blue suspension was stirred for a further 45 min at room temperature. The reaction mixture was filtered, and the solvent removed under reduced pressure below 40 °C. The last traces of solvent were removed under high-vacuum (0.1 Torr). The product was transferred under nitrogen into a sublimation apparatus and sublimed (0.1 Torr) to give bright

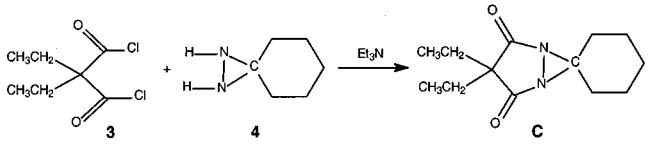
blue crystals of **2**. The crystals were washed off the coldfinger into a single-neck flask with dry dichloromethane. The resulting solution was reacted directly with the diazoalkanes as described below.

*6-Ethoxycarbonyl-3,3-diethyl-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**A**). General Procedure for the Reaction of the 3,5-Pyrazolinediones with Diazoalkanes (Scheme 1).* To the above solution of **2** in dry dichloromethane (20 mL) under nitrogen at 0 °C was added dropwise over 20 min ethyl diazoacetate ($N_2CHCO_2CH_2CH_3$, 20 mmol). The reaction was then stirred for 15 h at room temperature during which the color of the mixture turned from deep blue to yellow. The reaction mixture was filtered, concentrated under reduced pressure, and dried under high vacuum to obtain the crude product as a deep yellow liquid. The liquid was chromatographed on a silica gel (230−400 mesh) column (ethyl acetate/hexane, 60:40). The fractions containing the product were combined and dried under vacuum to obtain a fluffy off white to pale yellow solid. This crude solid was dissolved in dichloromethane:chloroform, and hexane was added dropwise to precipitate impurities. The solvent was concentrated, and the resulting solid dried under vacuum (56 °C, 0.1 Torr) for one week to yield pure **A** as a white solid. (24%): mp 81−95 °C (dec); IR (Nujol) 1765, 1700 cm^{-1}; ^1H NMR (acetone-d_6) broad unresolved peaks at δ 0.78−1.00 (6 H), 1.24−1.36 (3 H), 1.75−1.95 (4H), 4.18−4.37 (3H); MS (rel int) *m*/*z* 240 (90). Anal. calcd for $C_{11}H_{16}N_2O_4$: C, 54..99; H, 6.71; N, 11.66. Found: C, 54.80; H, 6.86; N, 11.45.

*6-Benzoyl-3-ethyl-3-(4-methylphenyl)-1,5-diazabicyclo-[3.1.0]hexane-2,4-dione (**B**).* The procedure for synthesizing compound **B** was identical to that described for compound **A** except for the diazoalkane used; in this case diazoacetophenone was added. (29%): mp 136−148 °C (dec); IR (Nujol) 1758, 1715, 1688 (PhCO) cm $^{-1}$; ^1H NMR ($CDCl_3$) broad unresolved peaks at δ 0.2−1.0 (3 H), 1.8−2.4 (5 H), 4.3 (1 H), 6.7−7.8 (9 H); MS (rel int) 334 (1). Anal. calcd for $C_{20}H_{18}N_2O_3$: C, 71.80; H, 5.40; N, 8.40. Found: C, 71.59; H, 5.63; N, 8.21.

*3,3-Diethyl-6,6-pentamethylene-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**C**). General Procedure for the Reaction of Malonyl Chlorides (**3**) with 3,3-Pentamethylenediaziridine (**4**) (Scheme 2).* To a solution of **4** (69.5 mmol) [mp 107−108 °C (lit. mp 104−107 °C)] (*25*) and triethylamine (175 mmol) in anhydrous ether (75 mL) under N_2 at 0 °C was added **3** (70 mmol) dropwise over 20 min. The reaction was stirred at room temperature until the precipitation of triethylamine hydrochloride (Et_3NHCl) was complete (24−48 h). The reaction mixture was filtered and the ether layer washed with water (35 mL) and 10% HCl (2 × 35 mL) then dried ($MgSO_4$). The resulting solution was then concentrated under reduced pressure, and dried under vacuum to yield a white solid. The solid was recrystallized (abs. EtOH) and the resulting solid washed with benzene to give pure **C** as a white solid. (21%): mp 81.5−82 °C; IR (Nujol) 1737 cm^{-1}; ^1H NMR (DMSO-d_6) peaks at δ 0.936 and 0.943 (overlapping t, 6 H), 1.45−1.55 (br m, 2 H), 1.59 (q, 2 H), 1.6−1.7 (br m, 8 H), 1.97 (q, 2 H); ^{13}C NMR (DMSO-d_6) δ 7.2, 8.1, 20.4, 23.5, 23.7, 23.9, 28.9, 29.8, 35.7, 60.8, 81.1, 190.0. Anal. calcd for $C_{13}H_{20}N_2O_2$: C, 66.07; H, 8.53; N, 11.85. Found: C, 65.92; H, 8.68; N, 11.68.

Scheme 2: General Synthetic Procedure for the Reaction of Malonyl Chlorides (**3**) with 3,3-Pentamethylenediaziridine (**4**)



Expression and Purification of Human Type I and II IMPDH. Human Type I and II IMPDH cDNA, originally isolated by RT-PCR from Molt-4 T cell RNA (*22*), were subcloned into pBAce (*23*) creating pBHI and pBHII and expressed in *E. coli* H712 (*26*). The transformants, pBHI/H712 and pBHII/H712, were maintained on solid NZCYM media supplemented with 15 μg/mL adenine, guanine, and hypoxanthine; 50 μg/mL histidine; 10 μg/mL tryptophan, tyrosine, and thiamine; 100 μg/mL ampicillin; and 100 μg/mL streptomycin. pBHI/H712 and pBHII/H712 cells were cultured with shaking at 37 °C in 10 mL of supplemented NZCYM to an OD_{600} of 0.3−0.6. Expression was induced by innoculating 1 L of low phosphate induction media (LPIM) with 100 μL of the log growth culture and grown for ∼24 as described by Craig et al. (23). The media was supplemented as described above. Maximum induction of recombinant protein was achieved when the cultures reached an optical density of 1.0 at a wavelength of 600 nm, in a 1 cm path-length cell. Cells were harvested and washed with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM PMSF, and stored frozen at −80 °C.

Both enzymes were purified by the same procedure at 0−4 °C. Frozen cells (approximately 7 g) were suspended in 3 vol (wet weight/volume) of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl, and 0.1 mM PMSF. Cells were lysed in this buffer with lysozyme (0.5 mg/mL, added fresh) by a process of freeze−thawing three times using a 37 °C water bath and dry ice/ethanol. Cells were then centrifuged 20 min at 14 000 rpm, 4 °C. The supernatant was batch loaded onto phosphocellulose that had been equilibrated with 10 mM potassium phosphate, 0.1 M KCl, and 0.1 mM EDTA, pH 7.3. The column was washed with 10 mM potassium phosphate, 0.1 M KCl, 1 mM DTT, 0.1 mM EDTA, pH 7.3, and protein eluted with a 0.1 to 1.0 M KCl gradient in the same buffer. Purity of the human Type I and II IMPDH proteins were estimated to be >99% by Coomassie-stained SDS/PAGE. Sample fractions from the anion exchange column yielded 0.8−1.5 mg/mL active protein without further concentrating. Initial attempts were made to concentrate enzymes further, however, concentrations greater than 2−3 mg/mL led to Type I and II protein precipitation.

Enzymatic Activity Assay. The specific activities for recombinant Type I and Type II IMPDH were determined by spectrophotometric assay using a Beckman DU-640 UV−vis spectrophotometer equipped with Peltier electronic temperature control (*27*). IMPDH activity was measured by monitoring the absorbance increase at 340 nm due to formation of NADH (ϵ_{340} = 6200 $M^{-1}cm^{-1}$) at 37 °C. Specific activity calculations were made using Beer's Law and expressed in units per milligram (μmol/min/mg). In addition to the substrates IMP and NAD, the reaction mixture consisted of 50 mM Tris HCl, pH 8.0, 100 mM KCl, 3 mM EDTA, 1 mM DTT, and 0.2−0.4 μM purified enzyme.

Reactions were performed in 1 cm path-length cuvettes and initiated by the addition of purified enzyme. Steady-state apparent kinetic parameters were evaluated by direct fit of initial velocity data versus substrate concentration to the Michealis-Menten equation using a weighted nonlinear regression method in the program "Enzyme Kinetics" by Exeter. Protein concentration was determined using the Bio-Rad Bradford Protein Assay and bovine IgG as a standard.

IC_{50} Values. IC_{50} values for compounds **A**, **B**, and **C** were determined using purified recombinant Type I and Type II enzyme. Inhibitors were tested with a range of concentrations from 0.5 to 500 μM (dependent on compound solubility) prepared in 0.05% Tween 80/water by Dounce homogenization. Analysis of inhibitor activity was performed with a reaction mixture consisting of 60 μM IMP, 240 μM NAD, 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 3 mM EDTA, 1 mM DTT, 0.2−0.4 μM Type I IMPDH enzyme, and inhibitors. With the Type II enzyme, 50 μM IMP and 170 μM NAD were used. Initial velocities were determined from continuous time courses by extrapolation of the linear portions of the curves to zero time so that the rates reflected conversion of less than 10% of the limiting substrate. IC_{50} values were then estimated from a semilog plot of inhibitor concentration vs % inhibition of enzyme activity for Type I or Type II IMPDH.

Inhibition Studies. Inhibition experiments were performed similarly as K_m and V_{max} determination but with variable concentration of inhibitor and one of the substrates, while the second substrate concentration was either saturating or below saturation. The type of inhibition (e.g., competitive, uncompetitive, or noncompetitive) with respect to IMP was determined from double reciprocal plots of $1/v$ vs 1/[IMP] at saturating and subsaturating concentrations of the nonvariable substrate [NAD] and increasing concentrations of inhibitor. In addition, studies were conducted to test interactions of the inhibitors with the NAD binding site by using a fixed concentration (saturating and subsaturating) of IMP as the nonvariable substrate and NAD at varying concentrations in the presence of increasing concentrations of the inhibitors. For the determination of K_is, the apparent K_m and V_{max} at each inhibitor concentration were determined by nonlinear regression assuming Michaelis−Menten kinetics. These values (K_m/V_{max}) were replotted against inhibitor concentration, and the K_i taken as the negative of the *x*-intercept of the least-squares fit line. Calculations were carried out using "Enzyme Kinetics" from Exeter Software. MPA and ribavirin were used as positive controls for all inhibition studies. Reactions for K_i determinations of MPA were performed at 14 nM enzyme.

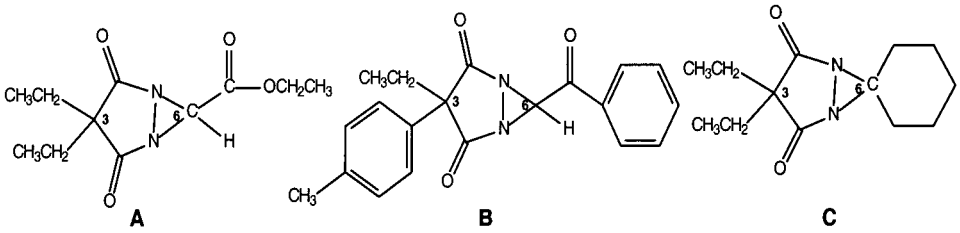
In Vitro Cytotoxicity Assay. The effects of compounds **A**−**C** and control agents, MPA and ribavirin, on the inhibition of Molt-4 T cell leukemia growth were examined. Human Molt-4 acute lymphoblastic T cell leukemia was grown in RPMI-1640 with 10% fetal bovine serum and kanamycin/gentamician. Ribavirin, MPA, **A**, **B**, and **C** were prepared as 1 mg/mL solutions in 0.05% Tween 80/H_2O by homogenization with 10 strokes by a Dounce homogenizer and filtered through a 45 μm acrodisc. Antiproliferative screens were conducted according to NIH protocol with tumor cells, growth medium, antibiotics, and agents to be tested with a range of concentrations from 10^{-4} to 10^{-10} M (*28*). Incubations were conducted by the method of Geran et al. (*28*) with 5×10^4 cells in a final volume of 1 mL for 72 h at 37 °C in a 5% CO_2 atmosphere. The number of cells per milliliter was determined using trypan blue exclusion and a hemocytometer/microscope. ED_{50} values (i.e., the concentration at which 50% inhibition of cell growth occurs) were determined by making an estimation from a semilog plot of compound concentration versus % inhibition of cell growth.

Cellular IMPDH Activity Assay. IMPDH activity was measured by the method of Becker and Lohr (*29*). Molt-4 T cells (10^6) were incubated with reaction mixture containing 0.1 mCi [8-^{14}C]inosine-5′-monophosphate (56 Ci/mmol), 0.5 mmol NAD, 50 mmol KCl, 0.5 mmol EDTA, and 25 mmol of potassium phosphate buffer, pH 7.4. Compounds (25, 50, or 100 μM) were also added to the reaction, for a final volume of 1 mL. The tubes were incubated for 1 h at 37 °C. Addition of 1 vol of ice cold 95% ethanol terminated the reaction and the samples were centrifuged at 1200*g* for 10 min to remove precipitated protein. Aliquots of the final supernatant (100 μL) were spotted on silica gel TLC plates and were eluted with 0.5 M $(NH_4)_2SO_4$. Radiolabeled xanthine monophosphate (XMP) was then scraped from each plate according to R_f values as determined for cold standards, and counted for radioactivity. Results were expressed as dpm of XMP formed per 1 h/10^6 cells. IC_{50} values were determined from a semilog plot of compound concentration versus % inhibition of IMPDH activity.

Guanosine Recovery of Antiproliferative Effects on Molt-4 T Cell Leukemia Growth. Cytotoxicity screens were conducted as described above. The effects of exogenous guanosine were determined by co-incubation of the agents at their ED_{50} values with guanosine, at 5−50 μM. The number of cells per milliliter was determined using trypan blue exclusion and a hemocytometer/microscope.

Molecular Modeling FlexiDock Studies. Energy minimization routines were performed on compounds with Tripos Associates SYBYL 6.5 software to gain insight into the degree of strain energies and allowable conformations. The structures were built and optimized in vacuo to a gradient of 0.005 kcal/mol using Tripos force field and Gasteiger-Marsili charges. Rotatable bonds were assigned and a conformational search performed allowing bonds to rotate with a chosen stepwise increment of the dihedral angle (30°). The internal energy corresponding to each valid conformation was evaluated by a molecular mechanics method where the fitting attempt forces the molecular features chosen as reference to an optimized fit at the cost of some conformational energy. The molecules relax to the closest minima, which may not always coincide with the global minimum energy. The minimized structures obtained for **A**, **B**, and **C** were then used in docking studies to establish how and where the compounds might be interacting in the IMP or NAD-binding site of Chinese Hamster IMPDH (*30*). The Chinese Hamster IMPDH crystal structure was determined with MPA bound in the NAD-cofactor binding site and XMP covalently bound to cysteine 331 in the IMP binding site. Both MPA and the covalently bound intermediate were removed from the crystal structure and the chemical properties of cysteine 331 restored for computational docking studies with the compounds. SYBYL was used for docking studies with the FlexiDock module. Important H-bonding sites with donor/acceptor atoms in the entire active site, including the IMP and NAD-

Scheme 3: 6-Ethoxycarbonyl-3,3-diethyl-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**A**), 6-benzoyl-3-ethyl-3-(4-methylphenyl)-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**B**), and 3,3-diethyl-6,6-pentamethylene-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**C**)



binding regions, were assigned based on the Chinese Hamster IMPDH crystal structure (*30*), and for the compounds, assignments were based on chemical properties. Docking procedures were repeated several times with different initial orientations of side chains and inhibitor in order to evaluate possible interactions with residues in both substrate and cofactor binding regions. The final output from the program were calculated interaction energies for each inhibitor, based on chemical interactions with residues in the entire active site.

RESULTS

Synthesis of Inhibitors. Three novel 1,5-diazabicyclo[3.1.0]-hexane-2,4-diones with substitutions at position 3 and 6 were successfully synthesized and purified (Scheme 3). IR, NMR, MS, and elemental analysis confirmed the composition and purity of the agents.

Bacterial Expression and Purification of Human Type I and II IMPDH. By using the pBAce expression vector constructs containing the cloned human IMPDH Type I and Type II from Molt-4 T cell leukemia, recombinant enzymes successfully were overexpressed in soluble, active form in *E. coli* H712. Anion-exchange chromatography using a phosphocellulose column afforded recombinant enzyme purified to near homogeneity by eluting with approximately 300 mM KCl from the gradient, as shown on a Coomassie-stained 10% SDS polyacrylamide gel (Figure 1). Consistently, 35−50 mg of purified, soluble recombinant Type I or Type II protein from 1 L of bacteria culture could be recovered without the use of standard solubilizing agents such as urea.

Type I and II Enzyme Activity. The final specific activities of 0.8−1.1 units/mg for the Type I enzyme and 0.5−0.9 untis/mg for the Type II enzyme were similar to specific activities of IMPDH enzyme purified by Hager et al. from the pET/BL21(DE3) system (*31*). Michaelis constants for recombinant Type I (IMP, 11−15 μM; NAD, 48 μM) and Type II (IMP, 8−11 μM; NAD, 35 μM) IMPDH confirmed the similarity of IMP and NAD K_m values for each enzyme.

*IC_{50} Values and Inhibition Constants for Compounds **A**, **B**, and **C** with Recombinant Type I and II IMPDH.* The 1,5-diazabicyclo[3.1.0]hexane-2,4-diones, **A**, **B**, and **C**, gave IC_{50} values of 32, 22, and 63 μM, respectively, with purified recombinant Type II IMPDH (Table 1). MPA and ribavirin afforded IC_{50} values of 0.082 and 79 μM, respectively. IC_{50} values for compounds **A**, **B**, and **C** could not be obtained with purified, recombinant Type I IMPDH. Agents were inactive as inhibitors of this isoform at concentrations ranging from 0.5 to 500 μM. Instead, these agents increased the activity of the purified recombinant Type I isoform by ~10−15% (data not shown). IC_{50} values for MPA and ribavirin could be obtained with the Type I isoform, 0.101 μM for MPA and 121 μM for ribavirin, showing that the recombinant enzymes are active and displayed inhibition patterns similar to enzymes purified from mammalian cells.

Table 1: Summary of Kinetic and Inhibition Constants for **A**, **B**, and **C** and Effects on Recombinant Type I and II IMPDH[a]

inhibitors	Type I IC_{50}[b] (μM)	Type II IC_{50} (μM)	Type II K_I (μM)	type of inhibition
A	n.o.	32	11.7	competitive
B	n.o.	22	5.1	competitive
C	n.o.	63	44.2	competitive
MPA	0.1	.082	.037	uncompetitive
ribavirin	121	79	37	competitive

[a] The type of inhibition was determined from Lineweaver−Burk plots of steady-state reactions with IMP as the varied substrate, except for studies with MPA where NAD was the varied substrate. Specific reaction conditions are described in the Materials and Methods section. K_i values were determined by a weighted nonlinear regression method from replots of K_m/V_{max} vs inhibitor concentration. Results are expressed as the mean of two or three experiments and the deviations in values are within 2.5%. [b] n.o. = no inhibition was observed at the highest concentration permitted by compound solubility (500 μM for **A** and **B**, 200 μM for **C**).

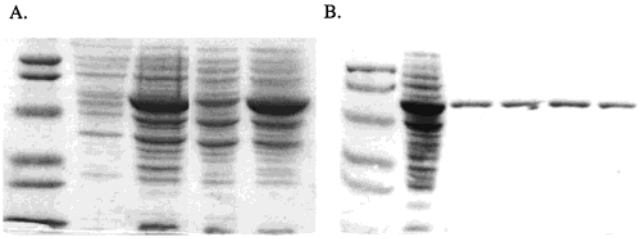


FIGURE 1: Soluble expression of IMPDH Type I and II isoforms in H712 *E. coli*. In panel A, a 10% SDS gel shows overexpression of Type I and Type II IMPDH. Lane 2 depicts levels of insoluble Type I protein found in the pellet and lane 3 shows the amount of soluble Type I protein after lysing without the presence of urea or any solubilizing agent. Lane 4 illustrates the level of Type II protein found in the pellet in insoluble form. Lane 5 shows the amount of soluble Type II protein after lysing cells. (B) 10% SDS gel illustrating a purification profile of Type II IMPDH using an anion-exchange column, Phosphocellose. In lane 2, the amount of induced, soluble Type II protein found in the bacterial lysate is shown. Lanes 3−6 show samples from fractions collected off the phosphocellulose column. These fractions were purified to near homogeneity, as shown from one column run, and were loaded directly onto the gel after collecting from the column. In both gels, lane 1 is the molecular weight marker.

The enzymatic reaction rates were measured in the presence of compounds **A**, **B**, and **C** at various concentrations of either IMP or NAD, above and below their respective K_m values, to determine interactions at both binding sites. In addition, this enabled the comparison of the binding of inhibitor before and after the association of the two substrates. The kinetics of inhibition by compounds **A**, **B**, and **C** were competitive with respect to IMP, giving intersecting

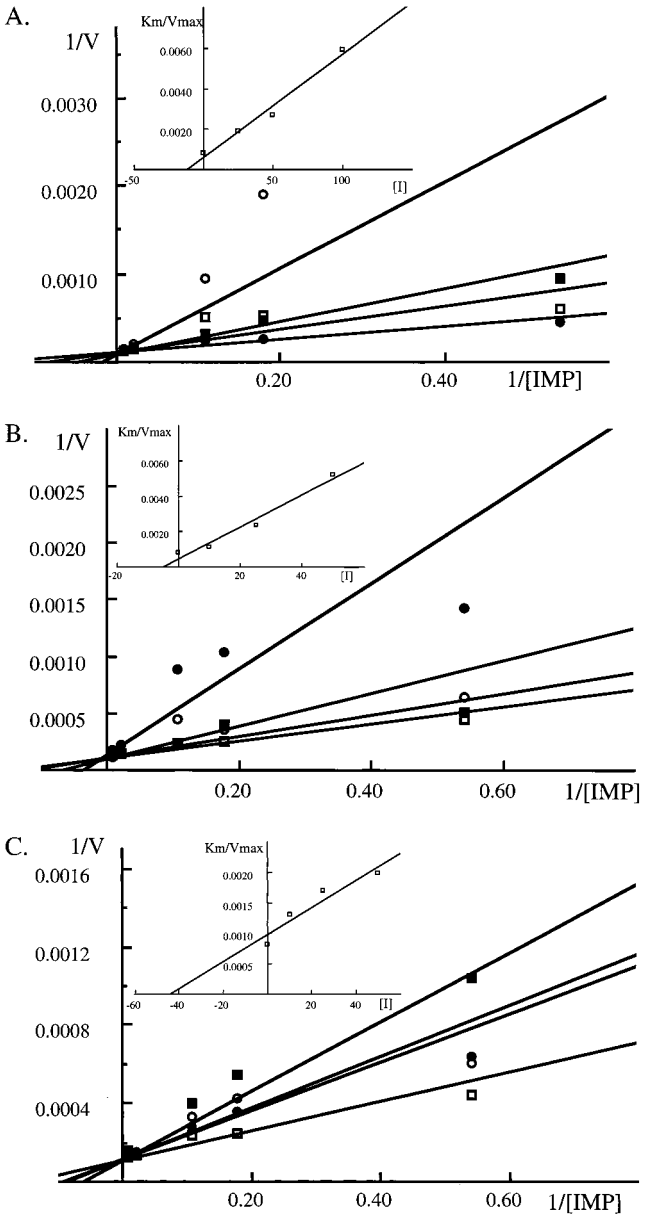


FIGURE 2: Kinetics of inhibition for compounds **A**, **B**, and **C**. Plots A, B, and C are Lineweaver−Burk plots, $1/V$ (s^{-1}) as a function of 1/[IMP] (μM^{-1}), for the respective compounds examined, (**A**−**C**) Measurements were made as competitive inhibitors with respect to IMP in the IMPDH reaction unless noted, while NAD was held constant at 170 μM. The inset of plot A is a replot of $K_{m,app}/V_{max}$ ($\mu M\ s^{-1}$) versus different concentrations of **A** (μM): closed circles, 0 μM; closed squares, 25 μM; open squares, 50 μM; and open circles, 100 μM. For inset replots of graphs B and C, concentrations of **B** and **C** tested: open squares, 0 μM; open circles, 10 μM; closed squares, 25 μM; and closed circles, 50 μM.

lines on Lineweaver−Burk plots which are characteristic of this mechanism (Figure 2). The compounds did not display inhibition when NAD was used as the variable substrate, suggesting that the compounds do not interact with the enzyme at the cofactor binding site. Thus, K_i values for **A**, **B**, **C**, and ribavirin were determined using IMP as the variable substrate, whereas the variable substrate for studies on MPA was NAD. Compounds **A** and **B** had lower K_i values of 12 and 5 μM, respectively, than ribavirin (37 μM) for Type II IMPDH. However, these values were significantly higher than those obtained for MPA, with a K_i of 0.037 μM (Table 1). Both ribavirin and MPA were more active than compound **C** with a K_i value of 44 μM. The observed kinetics indicated that all three inhibitors were competitive with the IMP substrate. Inhibition constants for the agents could not be obtained with the Type I enzyme because they were not active in inhibiting this isoform. However, K_i values for MPA and ribavirin could be obtained with the Type I isoform, 0.042 μM for MPA and 47 μM for ribavirin, yielding slightly higher results than what has been reported previously (*31*).

Table 2: In Vitro Human Molt-4 Whole Cell Cytotoxicity and Inhibition of IMPDH Activity by **A**, **B**, and **C**

inhibitors	cytotoxicity ED_{50} (μM)[a]	IMPDH IC_{50} (μM)[b]
A	3.3	52
B	7.6	54
C	3.2	55
MPA	8.9	nd[c]
ribavirin	42.1	81

[a] Molt-4 T cells were preincubated in the RPMI-1640 medium in the presence or absence of varying concentrations of inhibitors, ranging from 10^{-4} to 10^{-10} M, for a period of 3 days. After preincubation, cells were counted by staining with trypan blue/hemocytometer to determine cell viability. [b] Whole cell extracts were used to determine IMPDH inhibition by compounds **A**−**C** as described in the Materials and Methods section. Ribavirin was incubated in a cellular homogenate of Molt-4 to determine IMPDH inhibition. [c] nd = value was not determined. Results are expressed as the mean of 4 experimental values obtained with standard deviations within 3%.

*In Vitro Cytotoxicity and Cellular IMPDH Inhibition by Compounds **A**, **B**, and **C** in Human Molt-4 T Cell Leukemia.* To further investigate the selective inhibition of human IMPDH Type II activity and to correlate its inhibition with inhibition of cancer cell proliferation, effects of the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones on cell growth and cellular IMPDH activity were assessed. In addition, results from these studies were then compared to results obtained with two nonspecific Type II inhibitors, MPA and ribavirin. As illustrated in Table 2, the three 1,5-diazabicyclo[3.1.0]hexane-2,4-diones examined were more effective than either ribavirin or MPA in reducing Molt-4 T cell growth. Compounds **A**−**C** gave cytotoxic ED_{50} values ranging from 3.2 to 7.6 μM, while ribavirin exhibited an ED_{50} of 42.1 μM and MPA 8.9 μM.

The effects of the agents on cellular IMPDH activity were determined two ways: (1) by using whole cell Molt-4 extracts and (2) by using cellular homogenates of Molt-4 cells. These two methods were employed to establish how well inhibitors could enter the cell in order to inhibit IMPDH activity, thus leading to the observed inhibition of Molt-4 cell growth. IC_{50} values for the nucleoside drug, ribavirin, using whole cell extracts to determine IMPDH inhibition, gave substantially higher values than those observed using a cellular homogenate. Even so, by establishing an IC_{50} value for ribavirin (81 μM) using homogenized cells, the inhibitory activity of agents **A**, **B**, and **C** determined by using whole cells was significantly better (52−55 μM). These three agents were also examined for their inhibition of IMPDH activity using cellular Molt-4 homogenates, as was used for studies on ribavirin, to determine the difference in inhibition due to transport through the cell membrane. IC_{50} values for compounds **A**−**C** were significantly reduced by 35−55% by this method. Results from these studies indicate that the inhibitors accumulated inside the cells, when the cell membrane was

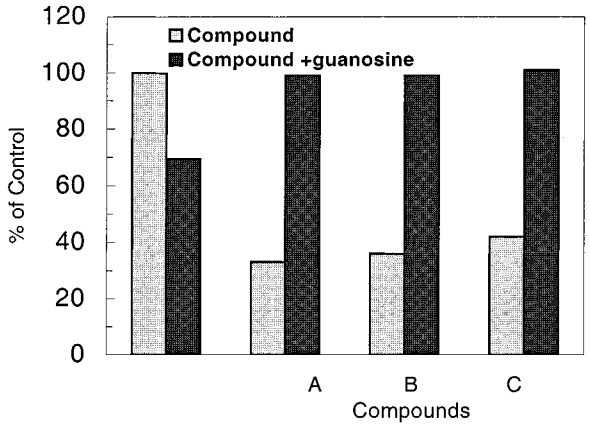


FIGURE 3: Effects of **A**, **B**, and **C** on Molt-4 T cell growth by coincubation with exogenous guanosine. Molt-4 cells in log growth were incubated with 3.3 μM (A), 7.6 μM (B), 3.2 μM (C), or no inhibitor. Parallel incubations were carried out with guanosine added at 5 μM and incubated for 3 days. Aliquots of samples were taken and counted for cell viability. Results are expressed as the mean of four experimental values obtained and standard deviations were within 4.4%.

not disrupted by homogenization, yet, significantly lower IC_{50} values could be obtained when the cell membrane had been destroyed by homogenization.

*Guanosine Recovery of Molt-4 T Cell Leukemia Growth by Compounds **A**, **B**, and **C**.* To examine the specificity of the observed inhibition on IMPDH activity, guanosine was added to the cell culture medium when the inhibitors were tested. If enhanced levels of exogenous guanosine reduced the observed inhibition by the agents, it would suggest that the inhibitors are interacting specifically with IMPDH and the excess guanosine is allowing the cells to bypass the need for IMPDH activity. Cell culture growth over 72 h was measured in the presence of cytotoxic concentrations of the inhibitors plus increasing levels of guanosine. Incubation of **A**, **B**, and **C** at their respective ED_{50} values led to $\geq$50% reduction in cell growth. Coincubation of the agents with low concentrations of guanosine (5 μM) afforded a complete reversal of cell growth inhibition observed when agents were incubated alone (Figure 3). Incubation of guanosine alone with Molt-4 cells led to decreased levels of cell growth, as compared to the normal growth without inhibitor or guanosine added.

FlexiDock Studies Show Differential Binding Compared to IMP. Compounds **A**, **B**, and **C** were examined using SYBYL modeling software to determine low-energy conformations before examining their potential binding to IMPDH. The energy values agreed closely with those calculated for a similar group of compounds, the 1,3,5-triazabicyclo[3.1.0]hexane-2,4-diones. Compound **A** had a calculated relative total energy value of 216.2 kcal/mol, **B** had the lowest energy value at 212.7 kcal/mol, and compound **C** had the highest energy value at 217.6 kcal/mol. The minimized conformations of the compounds were successfully docked into the IMP active site of Chinese Hamster IMPDH using the FlexiDock module. By this method of computational modeling, all three IMPDH inhibitors were predicted to bind with similar low energies to the nucleotide binding site and were predicted to interact poorly (or not at all) with the NAD cofactor binding site. Results from the Flexidock routine did not allow for a correlation to be made between K_i values obtained and calculated interaction energies; however, these docking studies allowed for the visualization of the predicted binding modes for the inhibitors with IMPDH. Figure 4 illustrates a comparison between the binding of IMP and of compound **B** to the IMPDH active site. The docking studies revealed that compounds **A**, **B**, and **C** do not have the same spatial requirements as IMP; therefore, amino acid residues important for binding of the inhibitors may be different from those that bind IMP, especially in the 5′-phosphate binding region. Compounds **A** and **B** were determined to bind nearly identically with

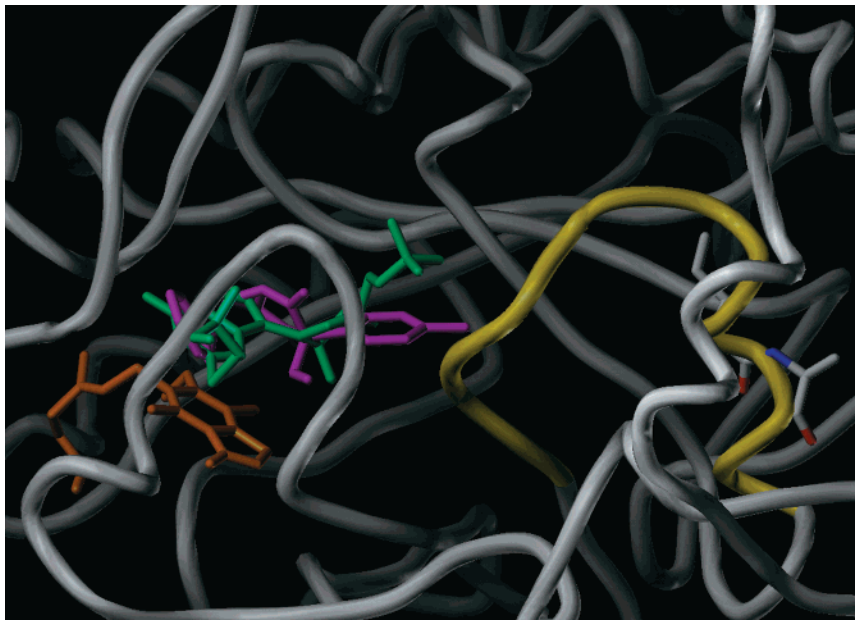


FIGURE 4: Comparison of the binding modes for IMP and compound **B** docked in the IMPDH active site. The crystal structure of Chinese hamster IMPDH (*30*) with bound MPA and IMP was superimposed with the protein model of IMPDH and the computationally docked compound **B**. MPA is highlighted in orange, IMP in green, and compound **B** is colored purple. The alpha helix adjacent to the active site that contains sequence differences between the two isoforms of IMPDH is shown in yellow, and the sites of amino acid substitutions at residues 373 and 374 in this helix are displayed in standard atom colors.

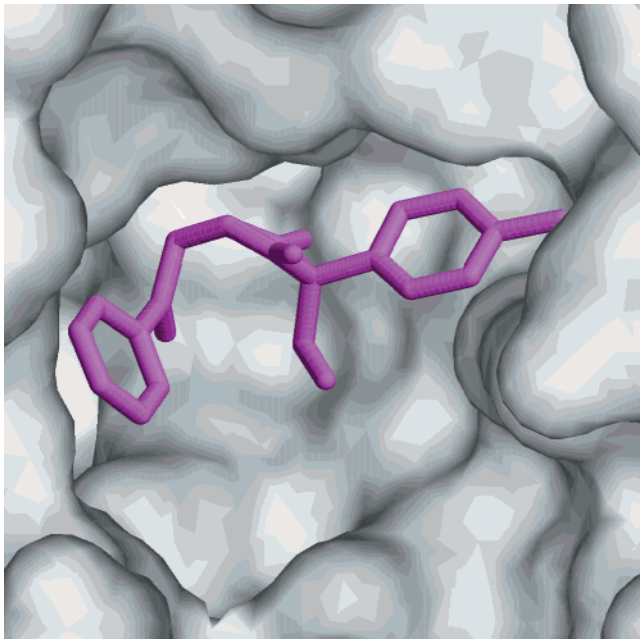


FIGURE 5: Pocket adjacent to the IMP binding site accommodates substituents at the 6-position of the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones. A model of the enzyme was generated using SChisM (*37*) by forming surface contours 6 Å from Cys 331 where IMP is covalently bound. This surface representation highlights a pocket adjacent to the IMP binding site in which the methylphenyl substitution of compound **B** apparently binds.

their substitutions at position 6 interacting with the same residues, yet, **C** was rotated 180 °C with the diethyl substitution at position 3 interacting with these residues. Figure 5 clearly shows that the methylphenyl substitution on compound **B** does not overlap with binding of the phosphate region of IMP. Instead, the methylphenyl substituent at position 6 of compound B appears to interact with a small pocket in the enzyme outside of the IMP binding region (Figure 5). As a result, residues that were not previously determined to be important for binding of IMP based on crystal structure data may be important for specific binding of the diazabicyclic compounds.

DISCUSSION

A successful bacterial expression and purification procedure was established for both isoforms of IMPDH using the pBAce vector system and *E. coli* H712. Soluble, human Type I and Type II IMPDHs were obtained at levels significantly greater than values previously reported in the literature. Greater than 70% of the IMPDH protein, Type I and Type II, was found in the soluble fraction of the bacterial lysate and remained soluble throughout the purification procedures. Kinetic constants for IMP and NAD, along with final specific activities for human recombinant IMPDH Type I and II indicate the similarity of the *E. coli*-expressed enzymes to enzymes from mammalian cells (*32*).

Three 1,5-diazabicyclo[3.1.0]hexane-2,4-diones (**A**, **B**, and **C**) were initially examined with the Type II enzyme. In the process of determining inhibition constants for those agents, a successful purification routine was identified for Type I, and thus, studies continued with this isoform. The 1,5-diazabicyclo[3.1.0]hexane-2,4-diones were analyzed with a number of batches of purified recombinant Type I enzyme and standard agents, MPA and ribavirin, were used as positive controls to show that the enzyme could be inhibited. Both of the known standards inhibited Molt-4 Type I enzyme activity at values approximating what had been reported in the literature. The nucleoside drug ribavirin was 100 times less active than the active drug, ribavirin 5′-monophosphate (*31*), when assaying with recombinant enzyme, yet was a consistent control for comparison to the activity of the diazabicyclic compounds. The present studies indicate that the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones are the first class of agents that selectively inhibit Type II IMPDH activity in a competitive manner without inhibiting the Type I isoform.

Results from cellular IMPDH studies suggest that the inhibitory activity of the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones, determined after a 60 min incubation with homogenized or nonhomogenized cellular extracts (as described in Materials and Methods), could be enhanced by breaking down the cell wall. However, substantial differences in ED_{50} values obtained for cytotoxic activity compared to IC_{50} values for enzyme inhibition determined with whole cell or homogenized extracts were still observed. Data described here indicate that the agents are probably being metabolized to a more potent form over the 3-day incubation period for determining cell growth and viability in this cellular system as compared to the 60 min whole cell incubation period for determining IMPDH activity. Furthermore, differences in IC_{50} values obtained using Molt-4 whole cells versus homogenized cells also could be explained by the inefficient transport of radiolabeled IMP into the cells. We observed that the conversion of IMP to XMP by cellular IMPDH could be increased by approximately 30% when a cellular homogenate was used. However, reductions in IC_{50} values greater than 30% were observed with the cellular homogenates, suggesting that in addition to drug metabolism, cell transport also is an issue for enhancing the inhibition of IMPDH activity and cytotoxicity by these compounds.

Studies with exogenous guanosine demonstrated that the effects of inhibitors **A**, **B**, and **C** on Molt-4 cell growth could be reversed by added guanosine. These results suggest that the cytotoxic activity in Molt-4 leukemic cells were due to the specific inhibition of IMPDH activity since the metabolic block induced by the agents could be bypassed or circumvented by addition of guanosine to the medium. A high degree of specificity for IMPDH presumably would reduce the likelihood of nonspecific, nonreversible inhibition of cell proliferation. The use of guanosine to prove specificity for IMPDH inhibition can often be complicated by the toxicity of guanosine for certain cells with lymphoid origin (*33*). The cytotoxicity of exogenous guanosine has been reported in the literature previously (*33*, *34*) and is thought to be attributed to an altered regulation of the enzyme purine nucleoside phosphorylase (*33*, *35*, *36*) which results in the accumulation of toxic levels of some guanine nucleotides. These findings have been corroborated in the Molt-4 T cell line by the induced cytotoxicity resulting from coincubation of exogenous guanosine with Molt-4. Coincubation with a specific inhibitor of IMPDH activity, such as MPA or the diazabicyclic compounds, could overcome the cytotoxic effects of guanosine alone on cell growth by enhancing levels back to those seen with untreated Molt-4 cells due to the initial decrease in guanine nucleotides. These results support

the notion that guanine nucleotide biosynthesis is under tight regulation.

IC_{50} values obtained for compounds **A**-**C** using the recombinant Type II enzyme demonstrated the semblance in chemical structure of these agents. Specifically, compounds **A** and **B** resemble each other at position 6 having polar side chains, whereas substitution at position 6 on **C** introduces nonpolar atoms. The slight differences in chemical reactivity at this end of the molecule could explain the differences in IC_{50} values and K_i values observed. Even so, backbones of the three 1,5-diazabicyclo[3.1.0]hexane-2,4-diones are very similar with substitution differing only at positions 3 and 6 yielding very rigid compounds with little flexibility as seen in the energy minimization routines performed by SYBYL. Using space-filling models, spatial requirements were nearly identical, suggesting the compounds bind similarly to each other in the IMP active site.

Although it was determined that IMP and compounds **A**−**C** have different binding requirements, i.e., interactions with different residues in the active site, amino acids that are predicted to interact directly with the bound 1,5-diazabicyclo[3.1.0]hexane-2,4-diones are conserved between the two isoforms. Therefore, selectivity of these agents for the Type II isoform cannot be explained directly by the docking studies. However, further inspection of interactions more remote from the predicted binding sites, coupled with sequence comparisons between human Type II over Type I IMPDH, suggests a possible explanation for the observed selectivity. By performing monomer mutations with SYBYL, the crystal structure of Chinese hamster IMPDH was converted to human Type I IMPDH. This simple manipulation allowed for the visualization of sequence differences between the two isoforms. On the basis of the amino acid sequences of human Type I and II IMPDH, a number of amino acid substitutions were found nearby the binding site yet not within direct contact of IMP nor the compounds. Although most of these substitutions are conservative, even modest changes could perturb the structure enough to alter the geometry of the active site in Type I versus Type II IMPDH. An explanation that remains in the forefront of many possibilities why the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones are specific to human Type II IMPDH involves amino acids 373 and 374 which reside in an α helix adjacent to the predicted binding position of the methylphenyl substitution of compound **B** (Figure 4). These residues are substituted from valines in Type I to isoleucine and alanine in Type II. It is possible that these substitutions could modify the position of the helix and thus alter the pocket that was shown by molecular modeling to be available for binding compound **B** near the IMP active site of Type II IMPDH (Figure 5). Such a possible structural difference between the two isoforms could be an explanation for the observed selectivity of the compounds for human Type II IMPDH.

Results from recent inhibitory studies with 4,4-diethyl-3,5-pyrazolidinedione (Scheme 1, compound **1**), a parent compound for the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones, show that it is an effective inhibitor of IMPDH activity, yet it is not selective for the Type II isoform. This agent inhibited both isoforms similarly but the mode of inhibition with respect to IMP could not be definitively ascertained from Lineweaver−Burk plots (data not shown). However, a K_i value was approximated at 80 μM for Type II that was significantly greater than the K_i values for **A**−**C**. Taken together, these results suggest that the substituent at position 6 plays a major role in determining isoform selectivity and potency of the inhibitor.

Without a crystal structure of the diazabicyclic compounds bound to human Type II IMPDH, one can only hypothesize how the agents might occupy and distort secondary structures in the enzyme based on computer-generated models. However, the findings reported herein suggest that it is possible to design agents which bind at the IMP active site and are selective to the Type II isoform. Further studies focusing on mutational analysis and crystallography would be necessary to determine the exact binding mode of these inhibitors. The IC_{50} or K_i values determined for the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones are not necessarily impressive as immediate clinical drug candidates. However, such compounds provide excellent leads with which to embark on redesign efforts using iterative crystallography and molecular modeling to improve initial inhibitor activity. The three lead compounds, **A**, **B**, and **C**, examined in this study are potent enough to justify further testing and to evaluate the hypothesis that specific inhibitors of IMPDH Type II activity could be used for isozyme-selective chemotherapy in a clinical situation.

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