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Implications of selective type II IMP dehydrogenase (IMPDH) inhibition by the 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-diones on tumor cell death

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Abstract

It was shown previously that three 1,5-diazabicyclo[3.1.0]hexane-2,4-diones selectively inhibited human Type II IMP dehydrogenase (IMPDH) from $Tmolt_4$ cell leukemia [Barnes *et al.*, Biochemistry 2000;39:13641–50]. The agents acted as competitive inhibitors of this isoform, yet when tested against human Type I at concentrations ranging from 0.5 to 500 μM, Type I was not inhibited. This study focuses on the antineoplastic activity and cellular effects of one of these agents and two new derivatives containing ethoxycarbonyl substitution at position C6. Agents were studied for antiproliferative activity in human $Tmolt_4$ leukemia (EC_{50} 3.3 to 9.2 μM) and alterations in the levels of enzymes involved with cellular metabolism, including DNA and RNA syntheses due to IMPDH inhibition. Results reported here demonstrate that 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-diones are effective inhibitors of DNA synthesis (30–66% inhibition) due to reductions in dGTP pool levels. Collectively, the three agents proved to be selective inhibitors of human IMPDH Type II activity (K_i 11–33 μM), leading to cytotoxicity in a number of suspended and solid tumor lines, notably MCF-7 (EC_{50} 0.7 to 6.0 μM). In addition, negative cytotoxic actions of these agents on WI-38 cell growth, a normal rapidly growing human line, suggest that specific targeting of Type II IMPDH would help to eliminate cell killing in lines where Type I predominates. Furthermore, effects of agents on DNA synthesis and cell death could be reversed by the addition of exogenous guanosine to the medium. Results from *in vitro* studies suggest that the 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-diones may be used as effective isozyme-selective chemotherapeutic agents.

Keywords: Type II IMP dehydrogenase; 1,5-Diazabicyclo[3.1.0]hexane-2,4-diones; Antineoplastic chemotherapy

1. Introduction

IMPDH (EC 1.1.1.205) has proven to be a target for anticancer, antiviral, antiparasitic, and immunosuppressive chemotherapy [1], and is a key enzyme in the *de novo* biosynthesis of guanine nucleotides where it catalyzes the NAD-dependent conversion of IMP to XMP [2,3]. Specific inhibitors of IMPDH, such as MPA, have been shown to produce their biochemical effects by blocking *de novo* guanine nucleotide synthesis, leading to a decrease in guanine nucleotide levels, which, in turn, blocks RNA and DNA syntheses [4–6]. To date, tiazofurin is the most well known IMPDH inhibitor having antileukemic/antineoplastic activity [7,8]. The majority of IMPDH inhibitors, such as MPA and ribavirin, are used as immunosuppressive or antiviral agents [9–11]. The effects of MPA, tiazofurin, and ribavirin, along with all other known inhibitors of IMPDH activity, are due to the inhibition of both Type I and Type II IMPDH isoforms [12]. The activity of IMPDH results from the expression of these two isoforms [12–14]. Type I is constitutively expressed and is the major form found in normal lymphocytes, whereas Type II activity is increased in human leukemias and ovarian tumors [15–18]. In addi-

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Abbreviations: IMPDH, IMP dehydrogenase; MPA, mycophenolic acid; 6-MP, 6-mercaptopurine; and VP-16, etoposide.

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tion, the effects produced by specific inhibitors of IMPDH activity can be reversed by treatment with guanosine [19, 20].

Despite considerable effort within the pharmaceutical industry, there has been no significant progress in devising analogues of MPA with improved activity or with designing new and novel agents that may act as competitive or uncompetitive inhibitors of IMPDH activity for use in antineoplastic chemotherapy. The major focus of the pharmaceutical industry has been in the discovery of new IMPDH inhibitors as potent immunosuppressive agents, such as VX-497 by Vertex Pharmaceuticals, for the treatment of psoriasis and hepatitis C [21]. Recently, however, kinetics data demonstrated that three 3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-diones selectively inhibited the human $Tmolt_4$ Type II IMPDH isoform over the Type I [22]. Before the discovery of these differential effects on the IMPDH isoforms by the 1,5-diazabicyclo[3.1.0]hexane-2,4-diones, there had been no reports of IMPDH inhibitors that showed significant selectivity to either Type I or Type II IMPDH for isozyme-selective chemotherapy. The results herein describe further the biological effects of 6-ethoxycarbonyl-3,3-diethyl-1,5-diazabicyclo[3.1.0]hexane-2,4-dione on tumor cell growth, along with a detailed examination of two new agents containing the identical ethoxycarbonyl substitution.

2. Materials and methods

2.1. Materials

The gene for Types I and II human IMPDH was cloned and expressed in *Escherichia coli* as previously described [22]. All radioisotopes were purchased from New England Nuclear unless otherwise indicated. Radioactivity was determined in Fisher Scintiverse scintillation fluid with corrections for quenching. Substrates and cofactors were obtained from the Sigma Chemical Co.

2.2. Synthesis of compounds **2** and **3**

The general procedure for the synthesis of compounds **1**-**3** has been reported previously [22].

6-Ethoxycarbonyl-3,3-diethyl-1,5-diazabicyclo[3.1.0] hexane-2,4-dione (**1**). Analytical results were reported previously [22].

6-Ethoxycarbonyl-3-ethyl-3-phenyl-1,5-diazabicyclo [3.1.0]hexane-2,4-dione (**2**). Theoretical yield was 40%: m.p. 110–120° (dec); IR (Nujol) 1757, 1755 $cm^{-1;}$ ^1H NMR ($CDCl_3$) broad unresolved peaks at δ 0.3–1.1 (3 H), 1.1–1.4 (3 H), 1.8–2.4 (2 H), 3.9–4.4 (3 H), 7.1–7.7 (5 H); ^{13}C NMR ($CDCl_3$) broad peaks at δ 8–10, 13–14.4, 26–32, 58–59, 62.4–64, 126–127.2, 127.8–129.6 (two peaks), 133.6–135.4, 162.2–164.6, 172–173.6; MS (rel int) *m/z* 146 [85] (ethyl phenylketene). Anal. Calc. for $C_{15}H_{16}N_2O_4$: C, 62.5; H, 5.6; N, 9.7. Found: C, 62.7; H, 5.7; N, 9.9.

6-Ethoxycarbonyl-3-ethyl-3-(4-methylphenyl)-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**3**). Theoretical yield was 35%: m.p. 150–160° (dec); IR (Nujol) 1738, 1731 $cm^{-1;}$ ^1H NMR ($CDCl_3$) broad unresolved peaks at δ 0.4–1.0 (3 H), 1.0–1.4 (3 H), 1.75–2.4 (5 H), 2.3 (s, 3 H), 6.6–7.5 (4 H); MS (rel int) *m/z* 160 [60] (ethyl 4-methylphenylketene). Anal. Calc. for $C_{16}H_{18}N_2O_4$: C, 63.6; H, 6.0; N, 9.3. Found: C, 63.7; H, 6.17; N, 9.2.

2.3. Cell proliferation assay

Compounds **1**-**3** were tested for antiproliferative activity by homogenizing drugs as a 1 mg/mL solution in 0.05% Tween 80/H_2O. These solutions were sterilized by passage through an acrodisc (45 μm) and were tested serially from 10^{-4} to 10^{-10} M against each cell line. The following cell lines were maintained by literature techniques [23] and the growth mediums and conditions were according to protocols of the American Type Culture Collection: murine L_{1210} lymphoid leukemia and P_{388} lymphocytic leukemia, human $Tmolt_3$ and $Tmolt_4$ acute lymphoblastic leukemia, HL-60 promyelocytic leukemia, HUT-78 lymphoma, THP-1 acute monocytic leukemia, HeLa-S^3 suspended cervical carcinoma, HeLa solid cervical carcinoma, KB epidermoid nasopharynx, SkMel-2 malignant melanoma, colorectal adenocarcinoma SW 480, HCT-8 ileocecal adenocarcinoma, lung bronchogenic MB-9812, A549 lung carcinoma, Saos-2 osteosarcoma, breast MCF-7, clear cell renal Caki-1, A-431 skin epidermoid carcinoma, glioma U87MG, and normal lung fibroblast WI-38. The protocol of Geran *et al.* [23] was used to assess the antiproliferative effects of the compounds on cell suspensions and standards for each cell line. Cell viability was determined by the trypan blue exclusion technique after incubation for 72 hr at 37° in a 5% CO_2 atmosphere. Cell proliferation in solid tumors was determined by the method of Leibovitz *et al.* [24], utilizing crystal violet/MeOH, and read at 562 nm (Molecular Devices) after a 4- to 5-day incubation when the controls had grown to confluency. Values for cytotoxicity were expressed as EC_{50} (μg/mL and μM), i.e. the concentration of the compound that inhibits cell growth by 50%, where values of less than 4 μg/mL were required for significant inhibition of cell growth by NIH standards. Standard antineoplastic agents 6-MP, VP-16, and MPA also were examined.

2.4. Incorporation studies

The effects of agents **1**-**3** at 25, 50, and 100 μM on the incorporation of radiolabeled [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine into DNA, RNA, or protein, respectively, for 10^6 human $Tmolt_4$ cells were determined for 60-min incubations. The acid-insoluble labeled DNA, RNA, or protein was collected on Whatman filters, which were counted in a Packard beta counter. The incorporation of [^{14}C]glycine

(53.0 mCi/mmol) into purines and of [^{14}C]formate (53.0 mCi/mmol) into pyrimidines were obtained as previously described [25].

2.5. Enzyme assays

The effects of compounds **1-3** on Tmolt$_4$ nucleic acid metabolism were determined at 25, 50, and 100 μM after 60-min incubations. The activity of DNA polymerase α (EC 2.7.7.7) was determined in cytoplasmic extracts utilizing the protocol of Sawada *et al.* [26] with [^{3}H]TTP. Messenger-, ribosomal- and transfer-RNA polymerase nuclear enzymes (EC 2.7.7.6) were isolated with different concentrations of ammonium sulfate; individual RNA polymerase activities were determined using [^{3}H]UTP. The following enzyme activities were determined using Tmolt$_4$ homogenates. Ribonucleoside reductase (EC 1.17.4.1) activity was measured using [^{14}C]CDP with dithioerythritol [25]. [^{14}C]2′-Deoxyribocytidine-5′-diphosphate was separated from the [^{14}C]CDP by TLC on polyethyleneimine cellulose (PEI) plates. Carbamyl phosphate synthase II (EC 6.3.5.5) activity was determined as described previously [25]. Aspartate transcarbamylase (EC 2.1.3.2) activity and the product carbamyl aspartate were determined colorimetrically [25]. Thymidylate synthase (EC 2.1.1.45) activity was analyzed as described previously [25]. Thymidine, TMP and TDP kinase (EC 2.7.1.21) activities were determined using [^{3}H]thymidine (58.3 mCi/mmol) in the medium of Maley and Ochoa [27] and separated by TLC. Dihydrofolate reductase (EC 1.5.1.3) activity was determined by monitoring the disappearance of NADH at 340 nm using the spectrophotometric method [25]. Amidophosphoribosyltransferase (EC 2.4.2.14) activity was determined by the method of Martin using [U-^{14}C]glutamine [28], and IMPDH activity was analyzed with [8-^{14}C]IMP (54 mCi/mmol) (Amersham) after separating [^{14}C]XMP on PEI plates by TLC [25]. Radioactivity in the samples was assessed by scintillation counting. Effects of compounds **1-3** on IMPDH enzymatic activity were determined using whole cell Tmolt$_4$. The activity of ribavirin was determined on a cellular homogenate. Protein content was determined for the enzymatic assays by the Lowry technique.

2.6. DNA studies

After deoxyribonucleoside triphosphates (dNTP) were extracted, dNTP levels were determined by the method of Hunting and Henderson [29] with calf thymus DNA, *E. coli* DNA polymerase I, non-limiting amounts of the three dNTPs not being assayed, and either 0.4 mCi of [*methyl*-^{3}H]dTTP or [5-^{3}H]dCTP. Thus, dATP, dGTP, dCTP, and dTTP levels were determined after incubation with the drugs for 60 min at 100 μM. The effects of compounds **1, 2** and **3** on DNA strand scission were determined by methods described previously [25]. Briefly, Tmolt$_4$ lymphoid leukemia cells (10^7) were incubated with 10 μCi of [*methyl*-^{3}H]thymidine (84.0 Ci/mmol) for 24 hr at 37°, and then were harvested and centrifuged at 600 *g* for 10 min at room temperature in PBS. ^{3}H-Labeled cells were resuspended in PBS, and 0.2 mL of this suspension was gently layered onto 0.5 mL of a lysis solution overlay (0.5 M NaOH, 0.02 M EDTA, 0.01% Triton X-100, 0.4 M NaCl, and 2.5% sucrose) on an alkaline 5–20% sucrose gradient (5 mL; 0.3 M NaOH, 0.7 M KCl, and 0.01 M EDTA). After the gradient was incubated for 2.5 hr at room temperature, it was centrifuged at 12,000 *g* for 17 hr at 8°. Fractions (0.2 mL) were collected from the bottom of the gradient, neutralized with 0.2 mL of 0.3 N HCl, and measured for radioactivity. Thermal calf thymus DNA denaturation studies, changes in DNA UV absorption from 220 to 340 nm, and DNA viscosity studies were conducted after incubation of compounds **1-3** at 100 μM, 37°, for 24 hr [30].

2.7. Guanosine recovery studies

Cytotoxicity and DNA synthesis studies were conducted in Tmolt$_4$ cells as indicated above at the respective EC_{50} values for each compound. Exogenous guanosine from 5 to 50 μM was co-incubated in the assays [20,21]. Viable cells were counted by trypan blue exclusion after 72 hr, and the percent inhibition of DNA synthesis after 60 min was determined with and without the addition of guanosine to the medium.

2.8. Recombinant IMPDH types I and II enzyme assay

Recombinant human Types I and II IMPDH were prepared as previously described [22], and specific activities were calculated to be 1.1 and 0.9 U/mg, respectively, in the standard assay buffer [50 mM Tris/Cl (pH 8.0), 100 mM KCl, 3 mM EDTA, 1 mM dithiothreitol (DTT), 10–200 μM IMP, 30–500 μM NAD, and 0.2 to 0.4 μM purified enzyme] at 37°. IMPDH activity was determined spectrophotometrically by monitoring the formation of NADH at 340 nm (ϵ_{340} = 6200 M^{-1} cm^{-1}) with a Beckman DU-640 UV-VIS spectrophotometer equipped with a Peltier electronic temperature control. Reactions initiated by the addition of purified enzyme were performed in 1-cm cuvettes. Steady-state apparent kinetic parameters were evaluated by fitting the initial velocity data and substrate concentration to the Michaelis–Menten equation using a weighted non-linear regression method in the program "Enzyme Kinetics" by Exeter.

2.9. IMPDH type I and type II enzyme inhibition

The IC_{50} values for compounds **1-3** were determined using purified recombinant Type I and Type II IMPDH [22]. 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 to 0.4 μM Type I IMP dehydrogenase enzyme, and inhibitors. With the Type II enzyme, 50 μM IMP and 170 μM NAD were used. The IC_{50} values were estimated from a semilog plot of inhibitor concentration versus percent inhibition of enzyme activity for Type I or Type II IMP dehydrogenase.

2.10. Kinetic analysis for type II inhibition

Kinetic studies were conducted at 37° using the standard assay buffer but with variable concentrations of inhibitor and one of the substrates, while the second substrate concentration was either saturating or below saturation [22]. The type of inhibition (e.g. competitive, uncompetitive, or noncompetitive) was determined from double-reciprocal plots of 1/*v* versus 1/[IMP] at saturating and subsaturating concentrations of the non-variable 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 non-variable substrate and NAD at various concentrations in the presence of increasing concentrations of the inhibitors. In the same manner, kinetic values for MPA inhibition of recombinant Types I and II IMPDH were determined with IMP as the non-variable substrate and NAD as the variable substrate. For the determination of K_i values, the apparent K_m and V_{max} values at each inhibitor concentration were determined by non-linear regression assuming Michaelis–Menten kinetics. These values were replotted against inhibitor concentration, and the K_i was taken as the negative of the x-intercept of the least squares fit line. Calculations were carried out using "Enzyme Kinetics" from Exeter. MPA and ribavirin were utilized as positive controls for all inhibition studies. Reactions for K_i determinations of MPA were performed at 14 nM enzyme.

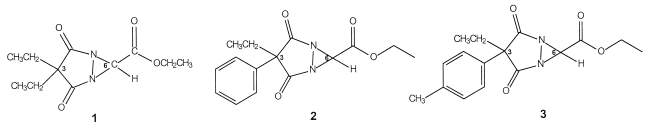

Fig. 1. Chemical structures: 6-ethoxycarbonyl-3,3-diethyl-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**1**), 6-ethoxycarbonyl-3-ethyl-3-phenyl-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**2**), and 6-ethoxycarbonyl-3-ethyl-3-(4-methylphenyl)-1,5-diazabicyclo[3.1.0]hexane-2,4-dione (**3**).

2.11. Statistical analysis

Data displayed in the tables and figures are representative of the mean of 4–6 experimental values with the standard deviation reported in the figure legend or as a table footnote. For Table 3, data are displayed as the means ± SEM, expressed as a percentage of the control. N is the number of samples per group. Student's *t*-test was used to determine the probable level of significance (*P*) between test samples and control samples.

3. Results

3.1. Synthesis of inhibitors

Compounds **2** and **3** were successfully synthesized and purified as derivatives of the parent compound **1** (Fig. 1). The identity and purity of these compounds were confirmed by NMR spectroscopy, IR, MS, and elemental analysis.

Table 1
Antiproliferative activity of the 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-diones in suspended tumor cell lines

Compounds	EC_{50} values[a]							
	Murine		Human					
	L_{1210}	P_{388}	THP1	$Tmolt_3$	$Tmolt_4$	HUT-78	HL-60	HeLa-S^3
1	**3.10**	**2.28**	**4.41**	**1.12**	**0.79**	**1.63**	**2.25**	**1.89**
	(12.9)	(9.5)	(18.4)	(5.2)	(3.3)	(6.8)	(9.4)	(7.9)
2	**2.68**	**3.19**	**2.78**	**3.44**	**1.85**	**3.38**	**3.89**	**3.16**
	(9.3)	(11.1)	(9.7)	(12.0)	(6.4)	(11.8)	(13.5)	(11.0)
3	**3.23**	**2.60**	**1.56**	**3.44**	**2.77**	**2.50**	**4.32**	**1.89**
	(10.6)	(8.5)	(5.1)	(11.3)	(9.1)	(8.2)	(14.2)	(6.2)
6-MP	**2.43**	**2.04**	**3.03**	**1.62**	**2.67**	**1.63**	**3.35**	**2.12**
	(14.3)	(12.0)	(17.8)	(9.5)	(15.7)	(9.6)	(19.7)	(12.4)
VP-16	**1.83**	**0.99**	**3.27**	**1.00**	**1.92**	**1.33**	**4.43**	**1.89**
	(3.1)	(1.7)	(5.6)	(1.7)	(3.3)	(2.3)	(7.5)	(3.2)
MPA	**2.32**	**0.92**	**2.29**	**2.44**	**2.86**	**1.63**	**4.27**	**1.47**
	(7.2)	(2.9)	(7.1)	(7.6)	(8.9)	(5.1)	(13.3)	(4.6)

[a] The EC_{50} values, expressed as **μg/mL** (μM), were calculated as the mean of 4 independent experiments. An EC_{50} value < 4 μg/mL is required for significant cytotoxic activity by NIH standards. Standard deviations were within 4% of the reported value. Incubations were conducted with 5×10^4 cells in a final volume of 1 mL for 72 hr at 37° in a 5% CO_2 atmosphere.

Table 2
Antiproliferative activity of the 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-diones in human solid tumor cell lines

Compounds	EC_{50} values[a]											
	MCF-7 Breast	SkMel-2 Melanoma	SW 480 Colon	HCT-8 Ileum	KB Naso	A-549 Lung	MB-9812 Lung	HeLa Solid	A-431 Skin	U87MG Glioma	Saos-2 Bone	Caki-1 Kidney
1	**1.39**	**6.74**	**8.24**	**3.85**	**6.32**	**4.03**	**3.78**	**8.80**	**7.15**	**2.17**	**3.48**	**5.78**
	(5.8)	(28.1)	(34.3)	(16.0)	(26.2)	(16.8)	(15.7)	(36.7)	(29.8)	(9.0)	(14.5)	(24.1)
2	**0.20**	**6.71**	**4.81**	**7.31**	**3.33**	**5.80**	**6.70**	**10.69**	**7.28**	**1.23**	**10.40**	**6.54**
	(0.69)	(23.4)	(16.7)	(25.5)	(11.6)	(20.2)	(23.3)	(37.2)	(25.4)	(4.3)	(36.2)	(22.8)
3	**1.83**	**7.87**	**3.49**	**8.12**	**3.64**	**8.17**	**5.76**	**13.64**	**0.49**	**3.94**	**5.26**	**8.19**
	(6.02)	(25.9)	(11.5)	(26.7)	(12.0)	(26.9)	(18.9)	(44.9)	(1.6)	(13.0)	(17.3)	(26.9)
6-MP	**8.84**	**6.86**	**3.61**	**1.15**	**5.74**	**4.71**	**4.29**	**5.61**	**2.92**	**4.46**	**5.07**	**9.35**
	(51.9)	(40.3)	(21.2)	(6.7)	(33.7)	(27.7)	(25.2)	(33.0)	(17.2)	(26.2)	(29.8)	(54.9)
VP-16	**11.00**	**3.53**	**0.93**	**1.13**	**3.32**	**4.74**	**3.50**	**3.05**	**0.71**	**2.44**	**8.61**	**7.01**
	(18.7)	(6.0)	(1.6)	(1.9)	(5.6)	(8.0)	(5.9)	(5.2)	(1.2)	(4.1)	(14.6)	(11.9)
MPA	**5.27**	**3.85**	**4.56**	**2.65**	**3.60**	**7.75**	**6.89**	**NA**	**0.31**	**6.61**	**7.73**	**5.65**
	(16.4)	(12.0)	(14.2)	(8.3)	(11.2)	(24.2)	(21.5)	NA	(0.97)	(20.6)	(24.1)	(17.6)

[a] EC_{50} values, expressed as **μg/mL** (μM), were calculated as the mean of 6 experimental values obtained. An $EC_{50} < 4$ μg/mL is required for significant activity. Standard deviations were within 2.1% of the reported value. Tumor cytotoxicity was determined in 96-well plates utilizing crystal violet/methanol and read at 562 nm after untreated cells had grown to confluency.

3.2. Antiproliferation and cellular enzyme inhibition by compounds **1–3**

Since it had been determined previously by this laboratory that three compounds from the chemical class 1,5-diazabicyclo[3.1.0]hexane-2,4-diones selectively inhibit IMPDH Type II activity leading to a reduction in human $Tmolt_4$ cell growth [22], two new compounds have been synthesized to study in detail the nature of IMPDH isoform selectivity in the hopes of designing new approaches for cancer chemotherapy. To examine further the effects of the 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0] hexane-2,4-diones (**1-3**) on cancer cell proliferation and cellular enzymatic activities, a number of tumor cell lines were investigated along with an assortment of enzymes involved with cell metabolism, specifically nucleic acid synthesis. Agents **1-3** were all significantly active according to NIH standards with EC_{50} values < 4 μg/mL in the majority of the murine and human tumor cell lines examined in suspension (Table 1). The agents were highly active in the human $Molt_4$ acute lymphoblastic T cell leukemia screen (EC_{50} 3.3 to 9.1 μM). Table 2 summarizes the antiproliferative effects of agents on human solid tumor cell growth. All three agents were selective toward the inhibition of MCF-7 breast and U87MG glioma cell growth, yielding EC_{50} values of 0.7 to 6.0 and 4.3 to 13.0 μM, respectively. In comparing the antiproliferative activity of compound **1** in all human solid tumor screens, this agent was most effective in reducing the growth of MCF-7 and U87MG cells but also reduced the growth of ileum HCT-8, lung MB-9812, and bone Saos-2 cells, with EC_{50} values ranging from 5.8 to 16.0 μM. Compound **2** was the most potent antiproliferative agent in the MCF-7 (0.7 μM), U87MG (4.3 μM), and KB nasopharynx (11.6 μM) screens, whereas compound **3** demonstrated the broadest range of activity in solid tumor screens by also inhibiting the growth of colon SW 480 (11.5 μM) and epidermoid carcinoma A-431. Specifically in the A-431 tumor screen, compound **3** was highly active, yielding an EC_{50} of 1.6 μM. Most noteworthy was the antiproliferative activity of these agents in normal human lung fibroblast, WI-38. None of the 6-ethoxycarbonyl-substituted compounds demonstrated antiproliferative effects over the 5-day growth period where untreated cells had grown to confluency. On the contrary, treatment with these agents led to enhanced cell growth as compared with untreated WI-38 cells, exhibiting a 10–15% increase in cell number. The standard IMPDH inhibitors MPA and ribavirin were examined for their antiproliferative effects on the normal cell line, WI-38. Both compounds exhibited similar activity with

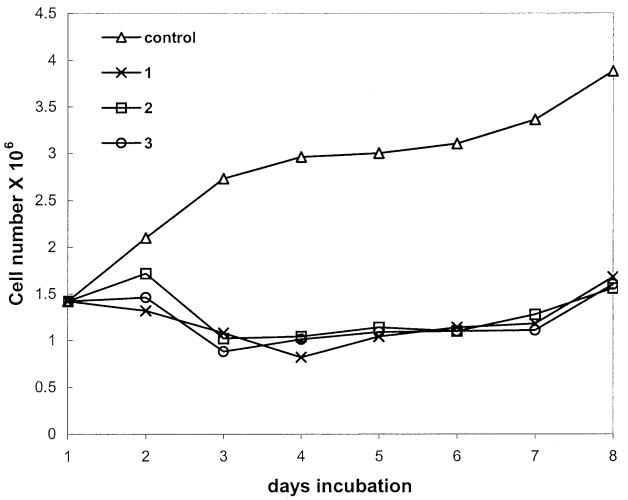

Fig. 2. Effects of compounds **1-3** on human $Tmolt_4$ lymphoblastic leukemia cell growth over 8 days. Cells (100 μL) were removed from incubated tubes at each time point, stained with trypan blue to determine cell viability, and counted using a hemocytometer. Points on the lines are representative of the mean of four experimental values. For each value point, the SD was within 2.8%.

Table 3
Effects of agents **1–3** on human $Tmolt_4$ lymphoid leukemia cell metabolism over 60 min

A. Compound **1**	Percent of control[a]			
Assay	Control	25 μM	50 μM	100 μM
DNA synthesis[b]	100 ± 2.0	79 ± 2.8*	50 ± 2.0*	34 ± 1.6*
RNA synthesis[c]	100 ± 2.4	120 ± 2.4*	65 ± 1.6*	59 ± 1.2*
Purine *de novo* synthesis[d]	100 ± 4.0	55 ± 2.0	10 ± 1.5*	9 ± 1.0*
PPRP amido-transferase[e]	100 ± 2.5	100 ± 1.5	100 ± 2.0	100 ± 1.0
IMPDH[f]	100 ± 3.5	65 ± 2.5*	60 ± 2.0*	52 ± 2.0*
dATP[g]	100 ± 2.4	ND[i]	ND	104 ± 2.0
dGTP[h]	100 ± 2.4	ND	ND	84 ± 1.6*
Compound **2**	Percent of control[a]			
Assay	Control	25 μM	50 μM	100 μM
DNA synthesis[b]	100 ± 2.0	98 ± 2.4	62 ± 2.0*	49 ± 1.2*
RNA synthesis[c]	100 ± 2.4	103 ± 2.0	102 ± 3.0	96 ± 1.6
Purine *de novo* synthesis[d]	100 ± 2.5	54 ± 1.5*	48 ± 2.0*	46 ± 2.0*
PPRP amido-transferase[e]	100 ± 3.0	100 ± 3.0	98 ± 2.5	95 ± 2.5
IMPDH[f]	100 ± 2.5	36 ± 2.0*	24 ± 1.5*	17 ± 1.5*
dATP[g]	100 ± 2.0	ND	ND	92 ± 1.6*
dGTP[h]	100 ± 2.4	ND	ND	30 ± 2.0*
C. Compound **3**	Percent of control[a]			
Assay	Control	25 μM	50 μM	100 μM
DNA synthesis[b]	100 ± 2.0	104 ± 2.0	82 ± 2.4*	70 ± 1.2*
RNA synthesis[c]	100 ± 2.4	100 ± 2.4	91 ± 1.6	83 ± 1.6
Purine *de novo* synthesis[d]	100 ± 2.5	58 ± 2.0*	36 ± 1.5*	33 ± 1.0*
PPRP amido-transferase[e]	100 ± 2.5	108 ± 2.5	106 ± 2.5	91 ± 2.5
IMPDH[f]	100 ± 2.5	31 ± 1.5*	23 ± 1.5*	21 ± 1.0*
dATP[g]	100 ± 2.0	ND	ND	75 ± 2.0*
dGTP[h]	100 ± 2.4	ND	ND	61 ± 2.5*

[a] Mean values ± SEM ($N \geq 4$) versus specified $Tmolt_4$ cellular synthesis, enzyme activity, or nucleotide pool levels.
[b-h] $Tmolt_4$ cells incubated for 60 min: [b] = 12,719 dpm; [c] = 11,261 dpm; [d] = 27,621 dpm; [e] = 0.1236 O.D. units; [f] = 15,578 dpm; [g] = 17.23 pmol; and [h] = 16.38 pmol.
[i] ND = value not determined at this concentration point.
* $P \leq 0.0001$ (Student's *t*-test).

EC_{50} values of 18 and 31 μM, respectively (data not shown). When compounds **1-3** were co-incubated at their respective EC_{50} values for inhibition of $Tmolt_4$ cell growth over an 8-day period, they caused a reduction in leukemia cell proliferation that appeared cytostatic versus cytolytic in nature (Fig. 2), suggesting that these agents are acting as antimetabolites.

Mechanism studies in $Tmolt_4$ lymphoid leukemia cells over 60 min indicated that DNA synthesis was suppressed preferentially by 30–66% by agents **1–3** at 100 μM. RNA synthesis was reduced 4–41% by the agents at 100 μM, and protein synthesis (data not shown) was not inhibited significantly over this time period. Table 3 summarizes the effects on enzymes and pathways that were specific targets of the agents. All three compounds inhibited DNA synthesis significantly, leading to the observed tumor cell death by specifically targeting the *de novo* purine biosynthetic pathway. Suppression of this pathway by 54–91% was due to the potent inhibition of the key regulatory enzyme, IMPDH, by 48–83% at 100 μM after 60 min, not amidophosphoribosyltransferase. None of the other enzymes examined showed any significant degree of inhibition by compounds **1-3**. Effects of the agents on IMPDH activity compared well with effects on *de novo* purine biosynthesis. In addition, a significant reduction in dGTP pool levels was incurred over 60 min at 100 μM (16–70%). Inhibition of IMPDH characteristically suppresses the biosynthesis of guanine nucleotides in cultured cells, resulting in partial depletion of the cellular pools of these nucleotides [31]. Effects of the agents on dATP pool levels were examined, and only slight reductions in these levels occurred over the same time period, 8 and 25%, by **2** and **3**, respectively. The observed levels correlate with published results on the effects of MPA and ribavirin on ATP pool levels over various time periods [20, 32]. Furthermore, the DNA molecule itself was not a target of the agents since there was no alkylation of DNA bases, intercalation between DNA base pairs, or cross-linking of DNA strands. However, incubation of the agents with $Tmolt_4$ cells for 24 hr at 100 μM resulted in minor amounts of DNA fragmentation (data not shown).

Table 4
Summary of kinetic and inhibition constants (μM) for compounds **1**, **2** and **3**: Effects on cellular IMPDH and recombinant Type I and Type II activities

Inhibitors	Whole cell IC_{50}[a]	Type I IC_{50}	Type II IC_{50}	Type II K_i	Mode of inhibition[b]
1	52	NO[c]	32	11.7	Competitive
2	21	NO	71	33.4	Competitive
3	18	NO	48	ND[d]	ND
MPA	ND	0.1	0.082	0.037	Uncompetitive
Ribavirin	81	121	79	37	Competitive

Results are expressed as the mean of 3–6 experimental values obtained, and the standard deviations in values are within 4.8%.

[a] Whole cell extracts were used to determine IMPDH inhibition by compounds **1–3** as described in "Materials and methods." Ribavirin was incubated in a cellular homogenate of $Tmolt_4$ cells to determine the IC_{50} value.

[b] The type of inhibition was determined from Lineweaver-Burk plots of steady-state reactions with IMP as the varied substrate except for the studies with MPA. K_i values were determined by a weighted non-linear regression method from replots of K_m/V_{max} versus substrate concentration. Inhibitors that are competitive with IMP interact with the IMP binding site, and inhibitors uncompetitive with respect to IMP interact with the NAD site.

[c] NO = no inhibition was observed at the highest concentration permitted by compound solubility. The highest concentration tested for **1** was 500 μM, and **2** and **3** were tested at 200 μM.

[d] ND = not determined.

3.3. Specific effects on cellular IMPDH activity by compounds **1–3** *and reversal of these effects by guanosine*

To test whether IMPDH is the primary target for the cytotoxic effects of agents **1-3**, studies were performed on cellular $Tmolt_4$ IMPDH and purified recombinant isoforms. The IC_{50} values for inhibition of whole cell IMPDH activity over 60 min were determined to be 52, 21 and 18 μM for agents **1**, **2** and **3**, respectively (Table 4). A time–course study monitoring the effects of these agents on cellular IMPDH activity over 120 min indicated that the highest degree of IMPDH inhibition for agents **1** and **3** occurred at the 30-min time point (Fig. 3). This prompt reduction in IMPDH activity supports the rapid diminishment of dGTP pool levels observed over the 60-min time period.

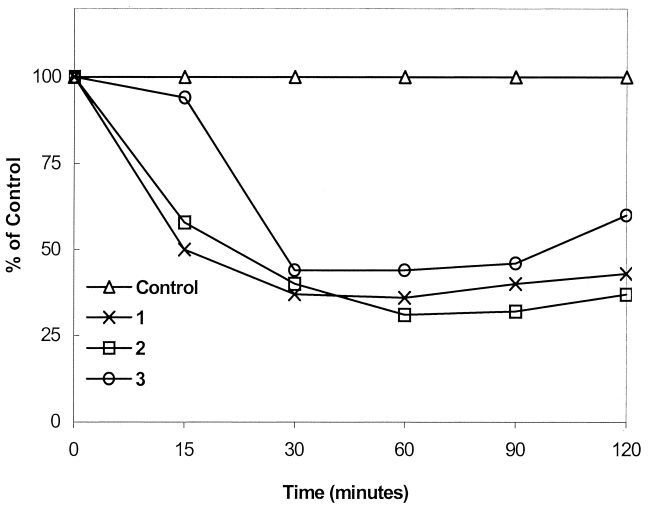

Fig. 3. Effects of compounds **1-3** on the inhibition of whole cell $Tmolt_4$ IMPDH activity over 120 min. Agents were incubated with $Tmolt_4$ cells, and activity was measured by the radioisotope method described in "Materials and methods." Values are represented as percent of the control, and each point on the line was calculated as the mean of four experimental values. Standard deviations were within 3.1% of each other, and an absolute value of 21,435 dpm corresponds to the control at 100%.

The metabolic block in *de novo* purine synthesis incurred by the agents, due to inhibition of IMPDH activity, should be circumvented by the addition of exogenous guanosine to the medium. This level of examination is an indication of the degree of specificity for inhibition of IMPDH activity. The effect can be seen when 5 μM guanosine was co-incubated with agents **1-3** while monitoring cell growth over 3 days and DNA synthesis over 60 min (Fig. 4). Specifically, compounds **1** and **2** reversed the levels of $Tmolt_4$ cell growth back to control levels, whereas inhibitor **3** provided only a 65% repletion upon co-incubation with guanosine, suggesting that **3** may have additional targets that have yet to be identified. In addition, the reduction in levels of DNA synthesis attributed to the effects of the agents could be reversed by co-incubation with guanosine. All three agents restored DNA synthesis in $Tmolt_4$ cells back to levels seen with guanosine alone. The effects of compounds **1-3** on DNA synthesis were very similar to those demonstrated by MPA (Fig. 4B).

3.4. Selectivity of type II IMPDH over the type I isoform

Studies were undertaken to examine the effects of IMPDH inhibitors, **1-3**, on human recombinant Type I and II activities in order to determine whether the reduction in cellular IMPDH activity attributed to the agents was due to a specific selectivity between isoforms. The enzymatic reaction rates for Types I and II IMPDH were measured in the presence of compounds **1-3** at various concentrations of either IMP or NAD, above and below their respective K_m values, to determine interactions at both binding sites. Inhibition followed first-order kinetics, as demonstrated by the exponential loss of activity. Replots of K_m/V_{max} versus concentration of inhibitor demonstrated that the agents act as competitive inhibitors of IMPDH Type II with respect to the IMP substrate (Fig. 5). The compounds did not display inhibition when NAD was used as the variable substrate, indicating that the compounds do not interact with the

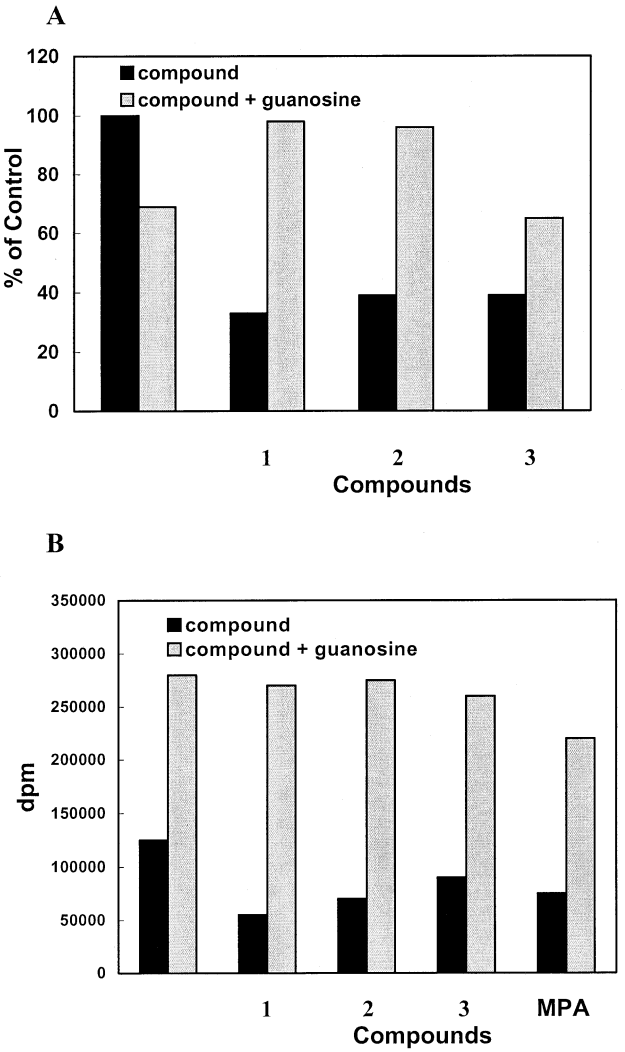

Fig. 4. Effects of exogenous guanosine on the inhibition of human $Tmolt_4$ cell growth and DNA synthesis by agents **1–3.** (A) Compounds were incubated at their EC_{50} values for growth inhibition, and guanosine was supplemented at 5 μM. The number of viable cells was determined at the end of the incubation period as described in "Materials and methods." The absolute value represented by 100% of control was 4.4×10^6 cells/mL. (B) Compounds were incubated at their EC_{50} values for inhibition of $Tmolt_4$ cell growth, and guanosine was added to obtain a final concentration of 5 μM. The amount of DNA synthesis was determined as described in "Materials and methods." For both panels A and B, results are expressed as the mean of four experimental values, and for each bar the SD was within 4.4%.

enzyme at the cofactor binding site. Type II IMPDH was inhibited competitively by both **1** and **2**, yielding IC_{50} values of 32 and 71 μM, respectively. Compound **3** gave an IC_{50} value of 48 μM, but the kinetics of inhibition were not determined due to a limited supply of purified inhibitor. K_i values for Type II IMPDH inhibition were determined to be 11.7 and 33.4 μM for compounds **1** and **2**, respectively. Compounds **1-3** were not inhibitors of Type I IMPDH activity. On the contrary, all three agents were able to increase Type I enzyme activity 12–18% over control levels at concentrations as high as 200 or 500 μM.

4. Discussion

The results of this study indicate that the 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-diones are specific inhibitors of human $Molt_4$ T cell leukemic IMPDH activity. The ability of these agents to inhibit IMPDH activity correlated well with the inhibition of DNA and purine synthesis along with the substantial depletion of dGTP pool levels, resulting in the suppression of tumor cell growth. The effects of agents on cellular IMPDH activity were due to the selective inhibition of the Type II IMPDH isoform. Compounds from this chemical class inhibited the Type II enzyme in a competitive manner with respect to IMP and were not found to interact at the NAD binding site. K_i values for compounds **1** and **2** were comparable to values reported for other IMPDH inhibitors [12]. In addition, these studies revealed that agents **1-3** were more effective than ribavirin in the inhibition of $Tmolt_4$ cellular IMPDH activity and inhibition of recombinant Type II enzyme. The reduction of $Molt_4$ T cell growth by agents **1** and **2** was completely reversible by the salvage substrate guanosine, whereas compound **3** demonstrated partial recovery by this method, suggesting that compounds **1** and **2** are more specific inhibitors of IMPDH activity. This greater specificity presumably would reduce the likelihood of non-specific, non-reversible inhibition of cell proliferation. It is of note to mention that effects produced by exogenous guanosine on $Tmolt_4$ cell proliferation and DNA synthesis correspond well with results published in the literature [33].

There are several potential applications of inhibitors of IMPDH activity, including immunosuppressive, antimicrobial, and antitumor. With regard to the antitumor clinical uses of IMPDH inhibitors, only tiazofurin has demonstrated significant activity in phase II clinical trials in patients with end-stage acute nonlymphocytic leukemia or in myeloblastic crisis of chronic myeloid leukemia [7,8]. However, lack of specificity remains a problem in the clinical use of tiazofurin. Efficacy achieved in phase II clinical trials of tiazofurin required hospitalization and aggressive treatment of neuro- and cardiovascular toxicities [7,8]. The findings reported herein suggest that new and novel inhibitors of IMPDH activity can be discovered for selective antineoplastic chemotherapeutic use. Inhibition of the growth of human MCF-7 breast tumor and U87MG glioma cell lines *in vitro* by the compounds, along with antiproliferative activities in the murine and human suspended tumor cell lines, provides encouragement for this area of study. In addition, as compared with the activity of 6-MP, VP-16, and MPA, a number of the agents demonstrating antiproliferative activity in the solid tumor screens were more effective than these known agents. In the suspended tumor cell line antiproliferation screens, results were less conclusive depending on the tumor line; yet, in most human screens, the inhibitors examined were more effective than the standard cytotoxic agents in reducing cell growth.

The finding that compounds **1-3** do not inhibit the *in*

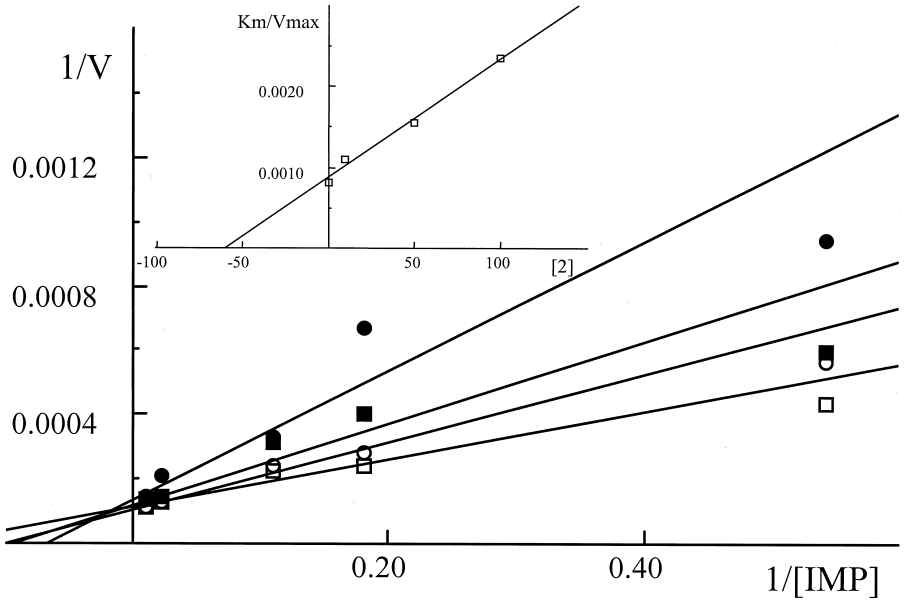

Fig. 5. Kinetics of inhibition for compound **2.** Data are presented as Lineweaver–Burk plots of $1/\nu$ (sec^{-1}) as a function of 1/[IMP] (μM^{-1}) at different inhibitor concentrations. Measurements were made as competitive inhibitors with respect to IMP in the IMPDH reaction unless noted, while NAD was held constant at 170 μM. The inset shows a replot of K_{m^-}, app./V_{max} ($\mu M \bullet sec^{-1}$) versus different concentrations of **2**: (□) 0 μM, (○) 10 μM, (■) 50 μM, and (●) 100 μM. Points on the lines are representative of the mean of six experimental values obtained, with the SD of each point within 4.4%.

vitro growth of the human normal lung fibroblast cell line, WI-38, suggests that specific targeting of the IMPDH Type II isoform by these agents could spare normal cells during chemotherapeutic treatment. When two non-isoform selective inhibitors of IMPDH activity, MPA and ribavirin, were examined for their ability to inhibit the growth of WI-38, both agents reduced cell growth similar to what was determined in the tumor cell antiproliferative screens. This observation would be invaluable in the development of antineoplastic agents since the main effort is to evade chemical disruption of normal proliferating cells, which are commonly affected by a number of clinically used antineoplastic agents, including tiazofurin. These studies suggest a correlation between the increase in Type I IMPDH activity and the increase in growth levels of WI-38 seen by the agents. Further studies on human normal cells would be necessary to correlate the enhanced cell growth with selective inhibition of Type II IMPDH. In addition, it would be significant to determine whether the agents are mimicking the IMP substrate, leading to increased Type I activity by binding to the enzyme active site, or whether they are activating the enzyme by binding to a different site. Information regarding the mechanism of activation is essential for determining the appropriate chemotherapeutic use.

Addition of a fused 3-membered ring to the parent chemical structure, 4,4-disubstituted-3,5-pyrazolidinedione [25], significantly enhanced specificity toward the enzymatic target, IMPDH. When substitution at the C-6 position of the diazabicyclic ring is an ethoxycarbonyl moiety, inhibition of Type II IMPDH activity is increased with diethyl (**1**) substitution at position 3,3 > ethyl-methylphenyl (**3**) ≫ ethylphenyl (**2**) substitution. The discrepancy between the EC_{50} values obtained in $Tmolt_4$ [3.3 μM (**1**), 6.4 μM (**2**), and 9.1 μM (**3**)] and the IC_{50} values obtained for recombinant Type II IMPDH inhibition indicates that metabolic processing may play a role in the potency of the drug. Results from studies on cellular IMPDH activity using whole cell $Tmolt_4$ and homogenized extracts show that these compounds are able to enter the cell efficiently in order to inhibit IMPDH activity to the levels discussed here (unpublished results). However, it is interesting to note that the activity of the agents could be increased up to 30% by using a homogenized extract to measure IMPDH activity [22]. These results suggest that more potent agents could be designed that would cross the cell membrane by either altering the chemical properties of the agents such as their hydrophobicity or by taking advantage of various cellular transport mechanisms. Taken together, all the data reported herein indicate that compound **1** is the most potent 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-dione inhibitor of IMPDH Type II activity, yielding the most effective inhibition of DNA synthesis and *de novo* purine biosynthesis, and thus, most active as a cytotoxic agent in the antiproliferative screens.

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