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A Novel Series of Non-nucleoside Inhibitors of Inosine 5′-Monophosphate Dehydrogenase with Immunosuppressive Activity

Trevor J. Franklin, William P. Morris,* Vivien N. Jacobs,* Eric J. Culbert,* Christine A. Heys,* Walter H. J. Ward,*† Peter N. Cook,* Frédéric Jung‡ and Patrick Plé‡*

*Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG, U.K.; and ‡Centre de Recherches, Chemin de Vrilly, Zeneca Pharma, Reims, France

ABSTRACT. Inhibitors of inosine 5′-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205) are effective immunosuppressive drugs that may also have additional potential applications as antitumour and antimicrobial agents. The clinical value of the most potent and specific inhibitor of IMPDH, mycophenolic acid, is limited by its rapid metabolism *in vivo* to an inactive glucuronide derivative. There is, therefore, a considerable incentive to develop structurally novel, preferably non-nucleoside, inhibitors with greater metabolic stability than mycophenolic acid. Here, we describe a high throughput screen for inhibitors of IMPDH, which facilitated the discovery of a single novel non-nucleoside inhibitor from a collection of approximately 80,000 compounds. The inhibitor is a pyridazine, which, like mycophenolic acid, exerts uncompetitive inhibition of IMPDH. Analysis of the enzyme kinetics suggests that the inhibitory action of the pyridazine is similar to that of mycophenolic acid, which involves trapping of a covalent intermediate formed during the conversion of IMP to xanthosine monophosphate. Chemical modification of the lead compound resulted in pyridazine derivatives with enhanced potency against IMPDH and guanine nucleotide synthesis in cultured cells *in vitro* and also against guanine nucleotide synthesis in the mouse spleen *in vivo*. One of the compounds was available in sufficient quantity to demonstrate highly effective immunosuppressive activity in a model of delayed type hypersensitivity in mice. To our knowledge, the novel pyridazines described in this report represent the first non-nucleoside uncompetitive inhibitors of IMPDH with immunosuppressive activity since the discovery of the inhibitory activity of mycophenolic acid and its derivatives thirty years ago. BIOCHEM PHARMACOL **58**;5:867–876, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. IMP dehydrogenase; mycophenolic acid; GTP; guanine nucleotides; immunosuppression; pyridazines

IMPDH§ (EC 1.1.1.205) is believed to be the rate-limiting enzyme for the biosynthesis of guanine nucleotides [1]. Inhibition of IMPDH underlies the antiviral action of ribavirin and 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide [2], the antileukemic action of tiazofurin [3] and, perhaps, most significantly, the immunosuppressive activity of MPA [4]. When administered as the prodrug MFT, MPA, in combination with cyclosporin and prednisolone, is effective in reducing the number of acute rejection episodes in patients with kidney grafts [4]. However, rapid metabolism of MPA to the biologically inactive 7-*O*-glucuronide and subsequent rapid clearance necessitates relatively high doses of 2–3 g per day, which may be linked to the unpleasant gastrointestinal side effects experienced by a substantial proportion of patients [4]. Despite considerable effort within the pharmaceutical industry, there has been no significant progress in devising analogues of MPA with sufficiently improved pharmacokinetics to permit smaller doses. All of the inhibitors of IMPDH mentioned above are nucleoside analogues, with the exception of MPA, which has a unique, uncompetitive kinetic of mechanism of inhibition [5], the molecular details of which have recently been revealed by an x-ray crystallographic analysis of the IMPDH–MPA complex [6]. In an attempt to discover novel, non-nucleoside structures with uncompetitive or non-competitive modes of inhibition, we screened some 80,000 compounds from the Zeneca compound library. As a result of that screening programme and subse-

† Corresponding author: Dr. Walter H. J. Ward, ZENECA Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG, U.K. Tel. (44)-(0)1625-515998; FAX (44)-(0)1625-583074; E-mail: walter.ward@alderley.zeneca.com

§ *Abbreviations:* β, coefficient of interaction; DTH, delayed-type hypersensitivity; FBS, foetal bovine serum; IMP, inosine 5′-monophosphate; IMPDH, IMP dehydrogenase; K'_i, apparent inhibition constant for compound I; K_{ii}, inhibition constant for effect on intercept of Lineweaver–Burk plots; K'_x, apparent inhibition constant for compound X; MFT, mycophenolate mofetil; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MPA, mycophenolic acid; TCA, trichloroacteic acid (10% w/v); v, velocity; v_0, uninhibited velocity; and XMP, xanthosine monophosphate.

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quent medicinal chemistry, we now describe a series of novel pyridazines that inhibit IMPDH *in vitro* and *in vivo* and which also have significant immunosuppressive activity. A representative member of the series is shown to be an uncompetitive inhibitor of the recombinant Type II human IMPDH *in vitro*.

MATERIALS AND METHODS

Materials

The gene for Type II human IMPDH was cloned and expressed in *Escherichia coli* as previously described [7]. Amersham International supplied [8-^{14}C]hypoxanthine (53 mCi/mmol), [8-^{3}H]hypoxanthine (10 Ci/mmol), and [methyl-^{3}H]thymidine (5 Ci/mmol). Microbial diaphorase (D-5540), MTT, IMP (monosodium salt), XMP (monosodium salt), NAD, NADH, allopurinol, and ovalbumin were from Sigma Chemical Co. The novel pyridazines and MPA were all prepared at Zeneca Pharmaceuticals, U.K.

High Throughput Screening Assay for Inhibitors of IMPDH

A lysate prepared from *E. coli* carrying the gene for the human Type II enzyme [7] was used in the assay. The recombinant *E. coli* were grown with vigorous shaking in L-broth at 37° until the culture reached early stationary phase. The bacteria were harvested by centrifugation at 4°, resuspended in 40 mM Tris–HCl buffer, pH 8.0 containing Triton X100 (1.5%), dithiothreitol (1 mM), and lysozyme (0.2 mg/mL) and incubated at 37° for 10 min. Cellular lysis was completed by subjecting the suspension to ultrasound for 6 periods of 15 sec at 0° separated by 1-min intervals at 0°. The lysate was then centrifuged for 30 min at 30,000 *g* to remove unbroken cells and debris and the supernatant stored in aliquots of 1 mL at −80°. Under these conditions the activity of IMPDH was stable for at least one month. In order to measure the activity of IMPDH in the bacterial lysate, we developed a procedure which quantitatively determines the NADH formed in the reaction by coupling the diaphorase-catalyzed oxidation of NADH to the reduction of MTT. The reaction was carried out at 25° in 96-well plates, with each well containing: Tris–HCl (100 mM, pH 7.5), KCl (30 mM), IMP (2.6 mM), NAD (1.1 mM), MTT (0.12 mM), dithiothreitol (0.16 mM), diaphorase 40 μg and recombinant bacterial lysate, 10 μg of total protein, in a total volume/well of 250 μL. The optical density of the reduced MTT generated in the reaction was monitored at 540 nm. Compounds tested for inhibitory activity were dissolved in DMSO, which was diluted to give a final concentration in the assay of 0.25%. For compounds giving inhibition in the assay, it was necessary to determine whether they inhibited the diaphorase phase of the assay. Compounds were assayed in a mixture (total volume 250 μL) containing the following: Tris–HCl (100 mM, pH 7.5), KCl (30 mM), MTT (0.12 mM), NADH (5.1 mM) and diaphorase (40 μg), and the reaction monitored at 540 nm. Compounds which were specific inhibitors of IMPDH (which did not inhibit diaphorase) were subjected to IC_{50} determinations and the values calculated by fitting a four-parameter logistic plot to the data.

Preparation and Assay of Purified IMPDH

The enzyme was purified to homogeneity from the bacterial lysate by affinity chromatography on IMP coupled to Sepharose 6B as previously described [7]. The purified enzyme, which was essentially homogeneous on polyacrylamide gel electrophoresis, was stored at −20° at a concentration not exceeding 1 mg/mL. The activity of the purified enzyme was followed by monitoring either the absorbance at 340 nm or fluorescence at 460 nm (excitation at 340 nm). Both methods measure the concentration of NADH. An extinction coefficient of 6300 M^{-1} cm^{-1} was assumed for the absorbance method. Typical active site concentrations used were 40 nM for the spectrophotometric assays and 4 nM for the spectrofluorometric assays, approximately 10 and 1 nM enzyme, respectively. Active site concentration was estimated from analysis of tight-binding inhibition by MPA [8]. The assays were performed at 37° in Tris/HCl (100 mM), pH 8.0, containing KCl (100 mM), ethylenediaminetetraacetic acid (3 mM) and dithiothreitol (1 mM). The increased sensitivity of the fluorimetric assay was required in assays involving low substrate concentrations and high inhibitor concentrations. Rate equations were fitted to measured velocities by unweighted non-linear regression using *GraFit* Version 3.01, (Erithacus Software Ltd.). When substrate and inhibitor, or two different inhibitors, were varied in the same experiment, multivariate regression was used. Identification of the most suitable rate equation was assisted by an *F*-test [9]. The following additional criteria were used to help selection of the best rate equation: reasonable parameter values and standard errors, and the residual differences between observed and calculated rates following a random distribution [9].

Cell Proliferation Assay for Inhibitors of IMPDH Detected by the High Throughput Screen

Compounds that specifically inhibited IMPDH in the high throughput screen were evaluated for their ability to inhibit the proliferation of mouse mammary carcinoma cells (EMT6) over 48 hr in the presence or absence of guanosine (35 μM). Cultures were initiated with 10^4 cells in Eagle's MEM (GIBCO-BRL) medium supplemented with FBS (8.6% w/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (1% w/v). The cultures (in 96-well plates) were incubated overnight at 37° in an atmosphere of 95% air and 5% CO_2. Compounds under investigation were dissolved in DMSO and added to the cultures to give a final concentration of 0.25% (v/v) of DMSO and the incubation continued for 48 hr. Parallel incubations were carried out with guanosine (35 μM). The cell densities were determined by the cellular reduction of MTT. MTT was added

to the wells to give a final concentration of 0.48 mM and the incubation continued for 4 hr at 37°. The medium was removed and the cell density determined by extracting the reduced MTT from the cells in DMSO (0.15 mL/well) and measuring its optical density at 540 nm. The concentrations of compounds required to inhibit cell proliferation by 50% (IC_{50}) in the absence of guanosine were calculated by fitting a 4-parameter logistic plot to the data.

Biochemical Activity of Inhibitors of IMPDH in EMT6 Cells In Vitro

EMT6 cells (2×10^5) were seeded into flasks and grown overnight at 37° in the medium described above and in an atmosphere of 95% air and 5% CO_2. The effects of pyridazine on the incorporation of [8-^{14}C]hypoxanthine into the cellular pools of GTP and ATP were determined by incubating the cultures grown overnight with radiolabel (0.24 μCi/mL) and a range of concentrations of the compounds for 2 hr at 37°. All incubations were carried out in triplicate. The medium was then removed and the cell sheets rinsed twice with ice-cold PBS, followed by extraction of the acid-soluble nucleotide pool with ice-cold TCA (10% w/v) for 1 hr at 0°. Precipitated proteins and nucleic acids were removed by centrifugation and the supernatants mixed with an equal volume of Freon 11 containing 0.5 M tri-*N*-octylamine to neutralize and extract the TCA. Measurement of the content and radioactivity of GTP and ATP in the neutralized extracts was carried out by HPLC as previously described [10].

Effect of Inhibitors of IMPDH on DNA Synthesis in T Cells In Vitro

HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS. Mononuclear cell suspensions were prepared from freshly drawn heparinised human blood by layering 10 mL blood over 10 mL lymphoprep (Nycomed), followed by centrifugation at 250 *g* for 20 min at room temperature. The interfaces were recovered and washed three times in cold PBS. The cells were suspended at a concentration of 10^6 cells/mL in RPMI 1640 medium (GIBCO) containing 5% (v/v) heat-inactivated pooled human serum, and 100 μL added to each well of a 96-well round-bottom tissue culture tray (Costar). Inhibitors, initially dissolved in DMSO, were added in 50 μL of RPMI containing 5% human serum and incubated for 30 min at 37°, followed by the addition of anti-CD3 monoclonal antibody (ATCC) in 50 μL of RPMI containing 5% human serum to give a final antibody concentration of 1 μg/mL. Trays were incubated for 48 hr at 37° in a humidified atmosphere of 5% CO_2 in air. For the last 6 hr of culture, 1 μCi per well of [^{3}H]thymidine was added. Trays were harvested and the incorporated tritium determined by scintillation counting. The concentrations of compounds required to inhibit DNA synthesis by 50% (IC_{50}) were calculated by fitting a 4-parameter logistic plot to the data.

MOUSE SPLEEN CELLS. Spleens were removed by aseptic dissection from young adult (6–9 weeks) Balb/C female mice and minced into cold PBS. The cell suspensions were passed through a nylon mesh filter (Becton Dickinson), and pelleted. Cells from 5 spleens were resuspended in 5 mL PBS and layered onto 5 mL Lympho-sep Separation Media-Mouse (Harlan Sera-Lab) in a plastic universal container and centrifuged at 250 *g* for 20 min. The interfaces were recovered into ice-cold RPMI-1640 (GIBCO) supplemented with 2 mM glutamine and 1% (v/v) FBS (GIBCO), and washed twice in RPMI containing 5% FBS. The cells were suspended at a concentration of 10^6 cells/mL in RPMI/5% FBS, and 100 μL added to each well of a 96-well flat-bottom tissue culture tray (Costar). Inhibitors, initially dissolved in DMSO, were added in 50 μL of RPMI/5% FBS and incubated for 30 min at 37°, following which concanavalin A was added in 50 μL of RPMI/5% FBS to give a final concentration of 4 μg/mL. Trays were incubated for 48 hr at 37° in a humidified atmosphere of 5% CO_2 in air. For the last 6 hr of culture, 1 μCi per well of [^{3}H]thymidine was added. Trays were harvested and the incorporated tritium determined by scintillation counting. The concentrations of compounds required to inhibit DNA synthesis by 50% (IC_{50}) in the absence of guanosine were calculated by a 4-parameter logistic plot to the data.

Biochemical Activity of Inhibitors of IMPDH In Vivo

Compounds with IC_{50} values in the EMT6 cell proliferation assay equal to, or less than, 20 μM were considered sufficiently interesting to administer to mice in order to assess their ability to inhibit nucleic acid and guanine nucleotide synthesis *in vivo*. Compounds suspended in aqueous 5% (v/v) Tween 80 were dosed orally or by i.p. injection to adult male albino mice (Alderley Park strain) at intervals prior to the i.p. injection of 10 μCi of either [^{14}C]hypoxanthine (for guanine nucleotide synthesis) or [^{3}H]hypoxanthine (for total nucleic acid synthesis) together with allopurinol (50 mg/kg) to suppress xanthine oxidase activity. The animals were humanely killed 30 min later and the spleens removed for the determination of the incorporation of radiolabel into GTP and ATP as previously described [11], or into total nucleic acids. For the latter measurements, the spleens were homogenized in TCA (10% w/v) and left for 60 min at 0°. The precipitates were harvested by centrifugation and washed twice with ice-cold TCA. Nucleic acids were then solubilized by heating the precipitates with TCA at 90° for 60 min. The precipitates were sedimented by centrifugation and the radioactivity in the supernatant fluids determined by liquid scintillation counting after removal of TCA as previously described [11].

Assay of Immunosuppressive Activity of Compounds in Mice

Immunosuppressive activity was assessed using a model of DTH. Young adult (6–9 weeks old) female Balb/C mice were immunised by injection into the left flank with 100

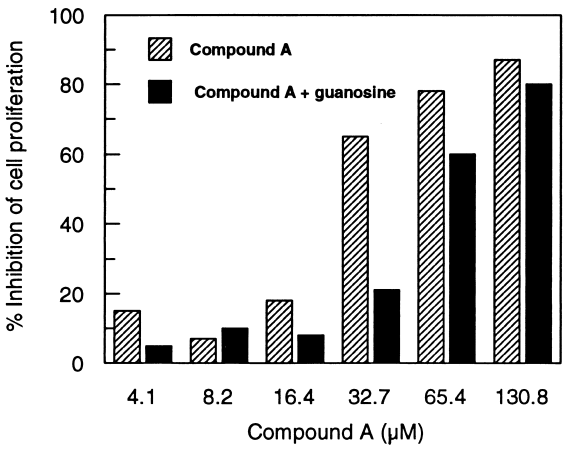


FIG. 1. Effect of supplementation of culture medium with guanosine on the antiproliferative action of compound A on EMT6 cells. Preconfluent cultures of EMT6 cells were incubated for 48 hr with the indicated concentrations of compound A and with, or without, supplementary guanosine (35 μM). The cell densities at the end of the incubation period were determined as described in Materials and Methods. In this MTT colourimetric method, uninhibited cultures gave an O.D. of 0.8 to 1.0 at 540 nm.

μL of an emulsified mixture of equal parts of 2% (w/v) heat-denatured ovalbumin (60° for 30 min), dissolved in sterile physiological saline and Freund's complete adjuvant (Difco). Between 7 and 10 days later, mice were challenged in the right rear paw by intraplantar injection of 20 μL of 1% (w/v) heat-denatured ovalbumin solution in sterile physiological saline. The thickness of the footpad was measured with a micrometer immediately before antigen challenge, and again at 24 hr after challenge. The degree of inflammation in each individual mouse was indicated by the increase in footpad thickness at 24 hr after antigen challenge compared with the thickness before challenge. Inhibitors of IMPDH were suspended in Tween 80, 5% (v/v), and given 1 hr before challenge and again 6 hr after challenge at the indicated doses and routes.

RESULTS

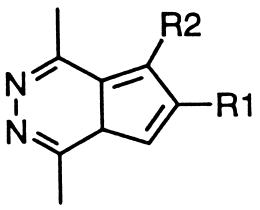
Detection and Characterization of Lead Compound from High Throughput Screen

Several inhibitors of IMPDH were detected after screening approximately 80,000 compounds from the Zeneca compound library. In assays following proliferation of EMT6 cells, guanosine causes partial reversal of inhibition by only one compound, which is a pyridazine derivative (compound A, Fig. 1). This was taken as an indication of a degree of specificity for the inhibition of IMPDH. The IC_{50} of compound A against recombinant IMPDH in the bacterial lysate was 1.93 ± 0.1 μM (SEM, N = 14), and the IC_{50} values against the proliferation of EMT6 cells in the presence and absence of guanosine were 57.7 μM and 39.7 ± 2.5 μM (SEM, N = 6), respectively.

Structure–Activity Relationships among Analogues of Compound A

Although compound A showed significant activity against isolated IMPDH and against cell proliferation, it was insufficiently potent and specific in the cell proliferation assay to justify evaluation *in vivo*. A programme of chemical synthesis, therefore, was initiated to discover more potent compounds. The *in vitro* activities of the most active derivatives are summarized in Table 1. Compounds B, C, and D were all more potent than compound A against IMPDH (in the bacterial lysate) and against the prolifera-

TABLE 1. *In vitro* structure–activity relationships of pyridazine inhibitors of IMP dehydrogenase



			IC_{50} (μM) vs:			
					DNA synthesis	
Compound	R1	R2	IMPDH*	EMT6†	Human T cells‡	Mouse spleen cells§
A	NO_2	H	1.93 ± 0.10	39.7 ± 2.5	ND	ND
B	Cl	Cl	1.15 ± 0.03	16.0	0.44	2.0
C	NO_2	Cl	0.78 ± 0.03	13.5	0.37	1.7
D	CN	Cl	0.76 ± 0.07	10.4	1.63	8.5

*Mean values ± SEM (N ≥ 6) versus recombinant Type II human IMP dehydrogenase in bacterial lysate.
†Against the proliferation of EMT6 cells over 48 hr.
‡Human peripheral blood mononuclear cells stimulated by anti-CD3 antibody.
§Mouse spleen cells stimulated by concanavalin A. ND: not determined.

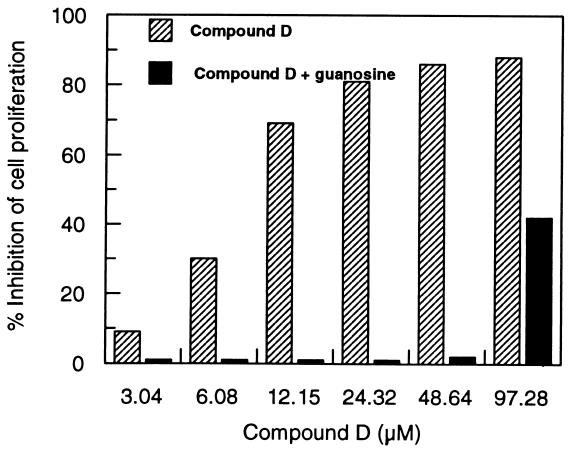


FIG. 2. Effect of supplementation of culture medium with guanosine on the antiproliferative action of compound D on EMT6 cells. Preconfluent cultures of EMT6 cells were incubated for 48 hr with the indicated concentrations of compound D and with, or without, supplementary guanosine (35 μM). The cell densities at the end of the incubation period were determined as described in Materials and Methods. In this MTT colourimetric method, uninhibited cultures gave an O.D. of 0.8 to 1.0 at 540 nm.

tion of EMT6 cells. In the latter assay, the antiproliferative action of compounds B, C, and D was more effectively reversed by guanosine than in the case of compound A (exemplified by compound D in Fig. 2). IC_{50} values could not be obtained for compounds B, C, and D in the presence of guanosine because their potencies were too low. Compounds B, C, and D also inhibited the synthesis of DNA in cultures of human T cells and mouse spleen cells (Table 1). The inhibitory potencies of compounds B, C, and D against both human T cells and mouse spleen cells were generally substantially higher than against EMT6 cells. The introduction of a chlorine atom vicinal to certain substituents at position 6, including NO_2, CN, and Cl, enhanced inhibitory activity (Table 1). The effects of a range of other modifications to either the 5 or 6 membered rings were either neutral or disfavoured activity compared with that of compound A (not shown).

Kinetics of Inhibition of IMPDH by Compound A

The enzymic reaction rates were measured in the presence of compound A at various concentrations of either IMP or NAD, above and below their respective K_m values. This enabled a comparison of the binding of the inhibitor before and after the association of the two substrates. The kinetics of inhibition by compound A were uncompetitive with respect to both substrates, giving parallel lines on Lineweaver–Burk plots which are characteristic of this mechanism (Fig. 3). The measured rates followed the relationship:

$$v = v_0 \Big/ \left(1 + \frac{I}{K_{ii}} \cdot \left(\frac{S}{K_m + S}\right)\right) \qquad (1)$$

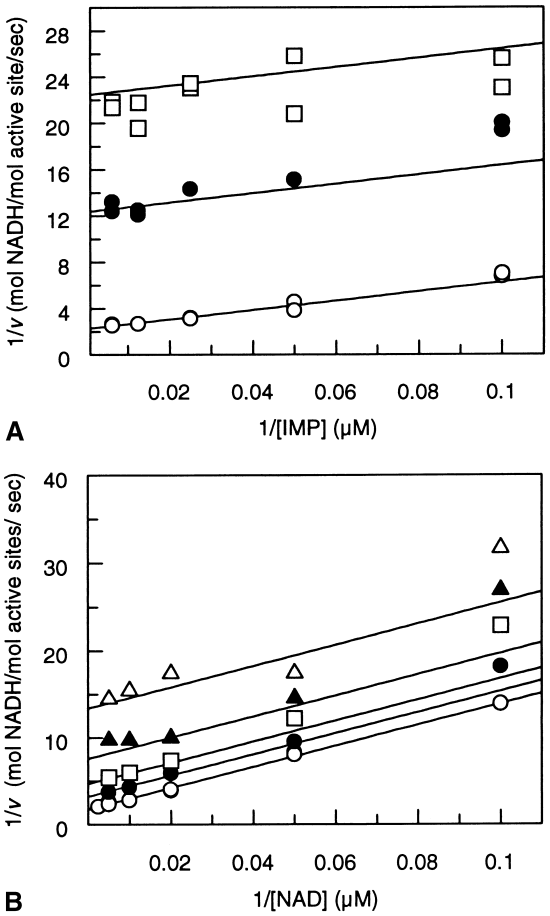


FIG. 3. Kinetics of inhibition by compound A. Rates were measured as described in the Methods section. The data are presented as Lineweaver–Burk plots of $1/v$ versus $1/S$ at different inhibitor concentrations. The rates were calculated using Eqn 1 and the parameter values given in Table 1. (A) IMP is varied at 0.4 mM NAD^+. Compound A concentrations are 0 (○), 5 μM (●), and 10 μM (□). (B) NAD^+ is varied at 0.15 mM IMP. Compound A concentrations are 0 (○), 1.25 μM (●), 2.5 μM (□), 5 μM (▲), and 10 μM (△). Data at 0.3125 and 0.625 μM inhibitor are omitted for clarity.

where S is the substrate concentration, I is the concentration of inhibitor, K_{ii} is the inhibition constant for the effect on the intercept of Lineweaver–Burk plots, and v_0 is the rate at a saturating concentration of the varied substrate in the absence of inhibitor. The best fit parameter values are shown in Table 2. The observed kinetics indicate that compound A binds most tightly after the enzyme has associated with IMP and NAD^+. The value of K_{ii} is the apparent dissociation constant for the inhibitor when the varied substrate is at saturation. Table 2 shows that similar K_{ii} values are obtained when the concentrations of either of the two substrates are varied. This suggests that both experiments measure binding of compound A to the same intermediate (Fig. 4).

The kinetics were also studied in the presence of several concentrations of compound A, together with varying concentrations of either MPA or XMP. The measured reaction rates were analysed by fitting the relationship

TABLE 2. Inhibition of human Type II IMPDH by compound A

Fixed substrate	Varied substrate	K_{ii} (μM)	v_0*	K_m (μM)
0.4 mM NAD^+	IMP	1.1 ± 0.1	0.44 ± 0.01	18 ± 2
0.15 mM IMP	NAD^+	1.5 ± 0.2	0.57 ± 0.02	69 ± 6

Reaction rates were measured as described in Materials and Methods. Parameter values were calculated using Eqn 1.
*mol product/mol active site/sec.

$$v = v_0/(1 + I/K_i' + \mathcal{X}/K_x' + \beta I\mathcal{X}/K_i'K_x') \quad (2)$$

for compound A and XMP. This was modified in order to allow for tight binding of MPA, giving

$$v = \frac{0.5v_0}{(1 + I/K_i')}\left[- \frac{K_x'(K_i' + I)}{E_0(K_i' + \beta I)} - \frac{\mathcal{X}}{E_0} + 1 - \sqrt{\left(\frac{K_x'(K_i' + I)}{E_0(K_i' + \beta I)} + \frac{\mathcal{X}}{E_0} - 1\right)^2 + \frac{4K_x'(K_i' + I)}{(K_i' + \beta I)E_0}}\right] \quad (3)$$

where K_i' is the inhibition constant for compound A (concentration, I), K_x' is the inhibition constant for MPA or XMP (concentration, $\mathcal{X}$), E_0 is the total enzyme concentration, and β is the interaction coefficient [12] (Fig. 5). β is zero when there is kinetic competition. Independent binding gives β = 1. Equation 3 is related to the relationship for a single tight-binding inhibitor $\mathcal{X}$ [8]. It allows for a second inhibitor, I, to change the rate in the absence of $\mathcal{X}$ from v_0 to $v_0/(1 + I/K_i')$, and to change the apparent inhibition constant of $\mathcal{X}$ from K_x' to $K_x'(K_i' + I)/(K_i' + \beta I)$. These data are shown as Yonetani–Theorell [13] plots (Fig. 6), where parallel lines indicate kinetic competition, and intersection at a single point indicates independent binding. The values of v_0 from the inhibitor study (Table 3) are approximately 2-fold higher than those from the substrate study (Table 2). This is reasonable agreement, because the v_0 values are influenced by experimental uncertainty, not only in the measured rate, but also in the estimated concentration of active sites. The values of K_i' in the kinetic competition experiments (Table 3) are very similar to the K_{ii} values from the studies of mechanism of inhibition (Table 2), implying that the compound interacts with the same intermediate in each experiment. The inhibition constants for MPA and XMP are similar to those previously published [5]. The coefficient of interaction, β, is effectively zero for both experiments, implying that the inhibition by compound is mutually exclusive with respect to both XMP and MPA. The slight curvature observed in Fig. 6(A) is due to significant depletion of the free concentration of MPA by binding to IMPDH (tight-binding kinetics). The weak divergence in Fig. 6(B) reflects the best fit of β being close to zero (−0.03 ± 0.03), rather than precisely zero.

Inhibition of GTP Synthesis in EMT6 Cells by Pyridazines

Inhibition of IMPDH characteristically suppresses the biosynthesis of guanine nucleotides in cultured cells, resulting in partial depletion of the cellular pools of these nucleotides [14]. We therefore examined the effects of compounds A, B, C, and D on the incorporation of radiolabel into the cellular GTP and ATP of EMT6 cells and also on the size of the GTP and ATP pools. Table 4 shows that the compounds markedly depressed the specific radioactivity of GTP. The specific radioactivity of ATP was unaffected by compounds B, C, and D at concentrations five times their antiproliferative IC_{50} values. After 2-hr incubation, all four compounds had reduced the intracellular levels of GTP, but had no effect on the size of the ATP pool.

Biochemical Activity of Selected Pyridazines In Vivo *in Mice*

Table 5 summarizes the effects of compounds B, C, and D on the incorporation of [^{3}H]hypoxanthine into splenic nucleic acids in three separate experiments in which animals were dosed either orally or by intraperitoneal injection. For the purposes of comparison, some groups of animals in experiments 1 and 2 were given MPA. The three pyridazines all produced significant inhibition of incorporation of radiolabel when given by i.p. injection. Although compound D exhibited activity when dosed orally at 100 mg/kg (Table 5), no activity was detectable when the compound was given orally at 50 mg/kg (not shown). Because the pyridazines have low aqueous solubility, compound C was milled to reduce particle size before injection as a suspension in experiment 3. This resulted in an acceptable dose–response relationship (Table 5), although milling of the compound did not improve its activity following oral dosing (not shown).

The effect of compound C on the incorporation of [^{14}C]hypoxanthine into the acid-soluble pools of splenic GTP and ATP, as well as on the pool sizes, is shown in Table 6. The compound was milled to reduce particle size and given in suspension by i.p. injection to mice at intervals up to 6 hr before the administration of radiolabel and allopurinol, and then the animals were killed humanely 30 min later. For comparison, MPA was given to an additional group of mice 1 hr before the radiolabel. Compound C inhibited incorporation of radiolabel into GTP at all time points. Although there was also significant inhibi-

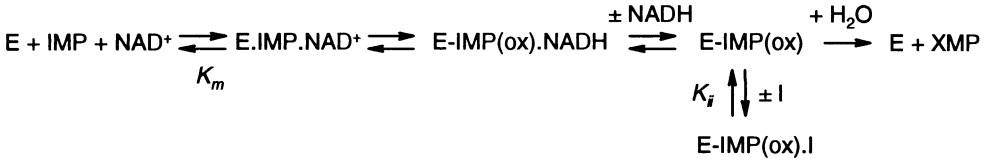


FIG. 4. Proposed kinetic mechanism. MPA functions as shown for I, where E-IMP(ox) is an intermediate, involving a covalent bond between carbon 2 of the purine moiety of IMP and the sulphur atom of Cys-331 [6, 19]. XMP follows competitive kinetics with respect to compound A and MPA, suggesting that each of these compounds associates with the E-IMP(ox) complex. Compound A now is proposed to follow the same mechanism as MPA. K_m is the apparent dissociation constant for the varied substrate (IMP or NAD^+) and K_{ii} is the apparent dissociation constant for the inhibitor.

tion of the radiolabeling of ATP, this was much less than for GTP. Compound C significantly reduced the size of the splenic pool of GTP 2 hr after dosing, reaching a maximum effect at 6 hr. In contrast, the ATP pool size was unaffected throughout the course of the experiment.

Immunosuppressive Action of Pyridazines in Mice

The inhibitory activities of compounds B, C, and D against DNA synthesis in mouse spleen cells *in vitro* and against guanine nucleotide and nucleic acid synthesis in the mouse spleen *in vivo* suggested that the compounds might have immunosuppressive activity. Although it was the least potent compound *in vitro*, only compound D was available in sufficient amounts for the initial immunosuppression studies described below. The model of immune responsiveness, DTH, involves the initial sensitisation of mice to ovalbumin followed, approximately one week later, by the eliciting of a paw-swelling response by intraplantar challenge with ovalbumin in saline. Compound D was dosed either orally or by i.p. injection 1 hr before and 6 hr after antigen challenge. Pooled results from three separate experiments indicate that a dose-dependent suppression of the DTH response was obtained following i.p. dosing of compound D (Table 7). Although compound D is much less potent than MPA against isolated IMPDH and against T cells *in vitro*, it was apparently more potent than MPA in the DTH model in the limited set of experiments performed. The potency of compound D given by the oral

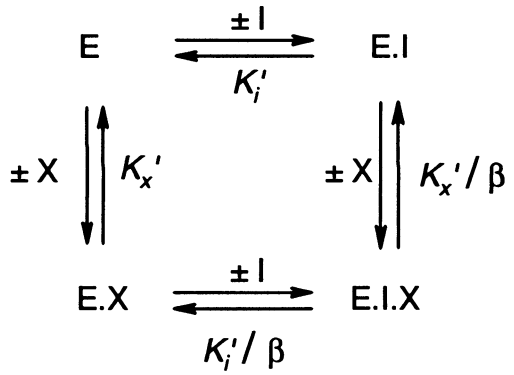


FIG. 5. Scheme for analysis of kinetic competition. The symbol, E, corresponds to free enzyme and all complexes that are catalytically active. E may bind inhibitor I (inhibition constant, K_i'), or inhibitor X (inhibition constant, K_x'). When a second inhibitor attempts to bind, the inhibition constants are perturbed by the inverse of the interaction coefficient, β. The magnitude of β is 1 if there is independent binding and zero if there is kinetic competition.

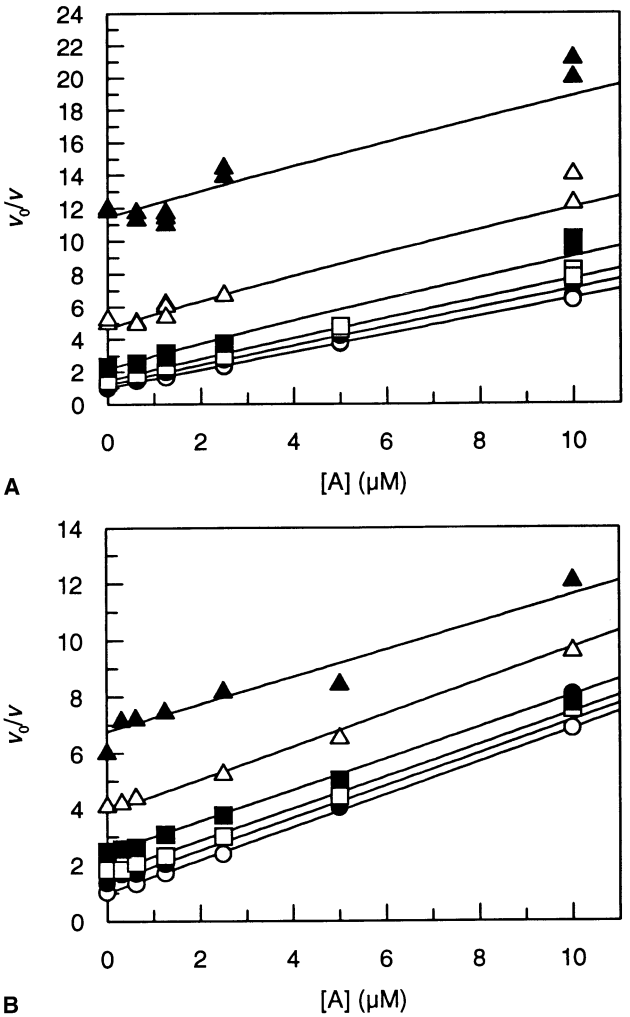


FIG. 6. Competition between inhibitors. Rates were measured at 400 μM NAD^+ and 150 μM IMP as described in Materials and Methods. Lines were calculated using Eqn 3 for MPA, or Eqn 2 for XMP, and the parameter values given in Table 2. (A) Compound A and MPA. MPA concentrations are 0 (○), 6.25 nM (●), 12.5 nM (□), 25 nM (■), 50 nM (△), and 100 nM (▲). (B) Compound A and XMP. XMP concentrations are 0 (○), 20 μM (●), 40 μM (□), 80 μM (■), 160 μM (△), and 320 μM (▲).

TABLE 3. Parameter values for competition between inhibitors

Compound A plus:	K_i' (μM) Compound A	K_x' (nM) MPA	K_x' (μM) XMP	v_o*	β†
MPA	1.8 ± 0.1	6.7 ± 0.6	—	1.00 ± 0.07	0.01 ± 0.06
XMP	1.8 ± 0.1	—	56 ± 2	0.93 ± 0.02	−0.03 ± 0.03

Rates were measured as described in Materials and Methods. Parameter values were calculated using Eqn 2 for XMP and Eqn 3 for MPA.

*mol product/mol active site/sec.

†Coefficient of interaction (a value of 0 indicates competitive kinetics).

route was markedly less than when dosed by i.p. injection (data not shown).

DISCUSSION

There are several potential applications of inhibitors of IMP dehydrogenase including antitumour, antimicrobial, and immunosuppressive therapies [7]. Of these, only the immunosuppressive potential has so far been fulfilled in the clinic. The prodrug of MPA, mycophenolate mofetil (MFT), has shown, in combination with cyclosporin and prednisolone, an impressive ability to reduce the number of acute renal graft rejections and, therefore, may represent a significant advance in immunosuppressive therapy [4]. However, the gastrointestinal side effects in graft recipients treated with MFT also experienced by patients receiving MPA for psoriasis and rheumatoid arthritis [15, 16], may limit the usefulness of the drug. Hitherto, MPA has been unique amongst the inhibitors of IMPDH as a non-nucleoside and in its highly specific, uncompetitive mechanism. Whereas these properties confer considerable advantages upon MPA as a therapeutic agent, the 7-OH function of the molecule, which is a major contributor to its biological activity, is also highly vulnerable to inactivation by *O*-glucuronidation *in vivo*. As a result, MPA is extensively converted to the biologically inactive glucuronide derivative in patients within 30 min of an oral dose [17]. Replacement of the 7-OH group by other, less vulnerable, groups has resulted in compounds with substantially inferior potency to MPA. There is, therefore, considerable interest in the possibility of discovering compounds that may be less susceptible to metabolic inactivation than MPA.

We have shown that a high throughput screen utilizing a bacterial lysate containing recombinant human Type II IMPDH, designed to detect non-competitive or uncompetitive inhibitors, yielded a single compound, from approximately 80,000 tested, that has an uncompetitive mode of inhibition. The inhibition of the growth of mammary carcinoma cells *in vitro* by the compound was partially reversible by the salvage substrate guanosine. The explanation for the greater reversibility by guanosine of the inhibitory actions of compounds B, C, and D on EMT6 cells compared with compound A is uncertain. The data suggest that compounds B, C, and D are more specific inhibitors of IMPDH than is compound A, despite their relatively modest increases in potency. This greater specificity presumably would reduce the likelihood of non-specific, non-reversible inhibition of cell proliferation. Compound A, a pyridazine, together with more potent derivatives (compounds B, C, and D), specifically inhibited the incorporation of radiolabeled hypoxanthine into the GTP of cultured cells and partially depleted the cellular pool of GTP without affecting ATP. Compounds B, C, and D inhibited the incorporation of radiolabeled hypoxanthine into the total splenic nucleic acids in mice *in vivo*. Further investigation of compound C demonstrated inhibition of the incorporation of hypoxanthine into splenic GTP *in vivo* and partial depletion of the GTP pool. The

TABLE 4. Effects of pyridazines on GTP and ATP synthesis and levels in EMT 6 cells *in vitro

Compound	Concn (μM)	Specific activity (cpm/μg) GTP	Specific activity (cpm/μg) ATP	Nucleotide concentration (μg/4 × 10^5 cells) GTP	Nucleotide concentration (μg/4 × 10^5 cells) ATP
Experiment 1					
No compound		10895	11531	1.01	5.05
A	105	3603	12784	0.48	5.68
C	15	6905	14684	0.54	5.34
C	75	1586	12833	0.38	3.86
D	10	6324	13816	0.63	5.64
D	50	898	15143	0.27	5.33
Experiment 2					
No compound		11833	14742	0.99	5.52
B	16	9709	16814	0.87	5.84
B	80	889	14100	0.52	5.32

*10^6 EMT6 cells were incubated for 2 hr with compounds and [^{14}C]hypoxanthine. The GTP and ATP pools were then extracted and analyzed for radioactivity and nucleotide content by HPLC as described under Materials and Methods.

TABLE 5. Effect of inhibitors of IMP dehydrogenase on the incorporation of [^{3}H]hypoxanthine into total splenic nucleic acids in mice *in vivo

Compound	Dose (mg/kg)	Route	Incorporation of radiolabel into total splenic nucleic acids (cpm ± SEM)
Experiment 1			
Controls			21670 ± 1070
B	100	i.p.	7670 ± 1080†
C	100	i.p.	6320 ± 1110†
MPA	50	i.p.	6290 ± 1050†
Experiment 2			
Controls			20180 ± 1230
D	100	i.p.	8360 ± 1150†
D	100	oral	10350 ± 990†
MPA	50	oral	9460 ± 1250†
Experiment 3			
Controls			34260 ± 2540
C	100	i.p.	13910 ± 1270†
C	50	i.p.	14180 ± 1750†
C	25	i.p.	22020 ± 1560†
C	12.5	i.p.	30540 ± 2090‡

The spleens were removed and the radioactivity incorporated into total nucleic acids determined as in Materials and Methods.

*Mice, 10 animals per group, were given compounds as indicated 2 hr before [^{3}H]hypoxanthine and humanely killed 30 min later.

†$P < 0.001$.

‡Not significantly different from controls.

very low aqueous solubility of the pyridazines probably restricts their bioavailability, and this may underlie the lower efficacy of oral administration compared with intraperitoneal dosing.

A potentially immunosuppressive action of compounds B, C, and D was suggested by their ability to inhibit DNA synthesis in T cells from mouse and human and to inhibit both guanine nucleotide synthesis and total nucleic acid synthesis in the mouse spleen *in vivo*. The potency of the compounds was significantly higher in the human blood T cell assay compared to the assay using mouse spleen T cells. It is not clear whether this reflected the species difference (mouse vs man), or the different tissues (spleen vs blood), or possibly the different stimuli used (concanavalin A vs anti-CD3). Significantly, MPA showed little difference in potency in the two assays (not shown), suggesting that there are real differences in the potencies of the pyridazines against human and mouse cells. The increased potency of MPA for T cells over tumour cells has previously been noted by others [18]. In a model of delayed type hypersensitivity, compound D demonstrated immunosuppressive potency comparable with MPA. Since it was the least potent of the four compounds against human T cells and mouse spleen cells *in vitro*, it is likely that compounds B and C may have at least comparable immunosuppressive activity *in vivo*.

Compound A inhibits IMPDH by an uncompetitive mechanism with respect to both IMP and NAD^+ (apparent dissociation constant, $K_{ii} \approx 1.3$ μM) (Fig. 3, Table 2), indicating that it binds most strongly after association of both substrates and weakly, if at all, to either free enzyme or E.IMP complex. Uncompetitive kinetics imply that the IC_{50}

TABLE 6. Effect of Compound C on incorporation of [^{14}C]hypoxanthine into acid-soluble pools of GTP and ATP in mouse spleen *in vivo

Time compound C given before radiolabel (hr)	Radioactivity of nucleotide as % of total radioactivity ± SEM		Total nucleotide pool (μg) ± SEM	
	GTP	ATP	GTP	ATP
Controls (dosed with vehicle)	8.1 ± 0.3	49.1 ± 1.1	21.7 ± 1.6	200 ± 16
1	3.9 ± 0.4†	44.6 ± 2.2§	19.1 ± 1.1	205 ± 13
2	4.4 ± 0.3†	44.2 ± 1.4‡	17.7 ± 0.7§	222 ± 17
6	2.9 ± 0.3†	42.3 ± 0.8†	15.2 ± 0.5‡	230 ± 8
1 (MPA)	4.1 ± 0.4†	49.7 ± 0.7	20.2 ± 1.0	187 ± 17

Compound C was given as a suspension by i.p. injection at indicated intervals before i.p. injection of [^{14}C]hypoxanthine together with allopurinol (50 mg/kg). The animals (10 per group) were humanely killed 30 min later and the acid-soluble nucleotide pools extracted and assayed by HPLC as described in Materials and Methods.

*Total radioactivity was 43,026 cpm (SEM 1,728 cpm).

†$P < 0.001$.

‡$P < 0.01$.

§$P < 0.05$. Other values did not reach significant difference from controls.

TABLE 7. Inhibition of delayed type hypersensitivity in mice by Compound D*

Compound	Dose (mg/kg)	% Inhibition of paw swelling
D	6	23§
D	20	18 ± 5‡
D	60	47 ± 3†
D	200	93 ± 6†
MPA	60	22
MPA	200	54

Groups of 5 animals were given compounds by intraperitoneal injection 1 hr before and 6 hr after ovalbumin challenge. Increases in footpad thickness were measured as described in Materials and Methods.

*Mean and SEM of 3 separate experiments for Compound D; single experiment for MPA.

†$P \leq 0.001$.

‡$P \leq 0.01$.

§Not significantly different from excipient alone.

would not increase if the substrates accumulate *in vivo* as a result of inhibition. This uncompetitive inhibition profile is the same as that for MPA [5], which has been shown to trap reversibly a covalent intermediate in the IMPDH reaction [6, 19]. Further experiments indicate kinetic competition between compound A and both MPA and the product, XMP (Fig. 6, Table 3). These data are consistent with compound A and MPA trapping the same intermediate (Fig. 4). This hypothesis is supported by the observation of kinetic competition between compound A and XMP, since E.XMP complex is proposed to be unable to bind MPA [19].

To our knowledge, the novel pyridazines reported above represent the first non-nucleoside, uncompetitive inhibitors of IMPDH to be described since the discovery of MPA as an inhibitor of the enzyme 30 years ago [14]. In view of their relatively modest inhibitory activity *in vitro*, the biochemical and immunosuppressive activity *in vivo* was somewhat surprising. However, although direct evidence is not available, the expected resistance of the pyridazines to metabolic inactivation may contribute to their efficacy *in vivo*.

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