Studies on the Pharmacology and Cytokinetics of 2,3-Dihydro-1H-imadazo[1,2-b]pyrazole (NSC 51143) with P815 Mastocytoma Cells

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ABSTRACT

A study has been made of the biochemical, cytokinetic, and pharmacological effects of pyrazole-imidazole (NSC 51143) (IMPY) on P815 mastocytoma ascites cells maintained in mice and of cells maintained in culture.

The distribution phase of IMPY equivalents from the peritoneal fluid of the mouse was found to be two hr, with an elimination phase of 69 hr. No consistent alteration in the ribonucleotide pools of the ascites tumor cells in vivo was observed by high-pressure liquid chromatography using i.p. doses of IMPY up to 1000 mg/kg (25% increase in survival). Correspondingly, no significant alteration occurred in the proportion of cells in G0, G1, S, or G2 + M in vivo by flow cytometric analysis. This is in contrast to the in vitro data which showed a significant blockage in S phase (50% effective dose, 1.6 x 10^-4 M). Using Dowex 1 chromatography of extracts from ascites tumor cells treated with IMPY in vivo, several intracellular drug metabolites were detected, and their proportion was noted to change with time. No such metabolism was detected in vitro. Some radiolabeled drug was detected in RNA and DNA from the cold, acid-insoluble fraction of ascites tumor cells. Analysis of alkaline sucrose sedimentation indicated that part of the radiolabeled IMPY was in the heavy-sedimenting DNA fraction.

INTRODUCTION

IMPY markedly and selectively inhibits DNA synthesis without significantly inhibiting RNA or protein biosynthesis in a variety of experimental tumor systems both in vivo and in vitro (7). The drug has been used to synchronize human lymphoid and HeLa cells in culture (3, 11) and P388 cells in vivo (9). There is clinical interest in this compound because IMPY is able to inhibit ribonucleotide reductase in cell-free systems and to cause prolonged in vivo inhibition of thymidine incorporation into the DNA of tumor cells (3, 4). Recently, IMPY has been shown to markedly stimulate 1-beta-arabinofuranosylcytosine incorporation into total cell material of Novikoff rat hepatoma cells in culture (14). The long-term action of this drug is in contrast to other ribonucleotide reductase inhibitors, such as guanazolane and hydroxyurea, which have shown only short-lived inhibition. In conjunction with a Phase I clinical investigation of IMPY at our institution, therefore, we have undertaken in vitro and in vivo studies on the pharmacology and cytokinetics of this agent with P815 murine mastocytoma cells. A preliminary report of this work has been presented (1).

MATERIALS AND METHODS

Ribonucleotide standards were obtained from Sigma Chemical Co. (St. Louis, Mo.). IMPY was provided by Dr. R. Engle, Drug Research and Development, National Cancer Institute, NIH, Bethesda, Md.

HPLC. A Varian Model 8500 high-pressure liquid chromatography system (Varian Associates, Palo Alto, Calif.) with autosampler, variable wavelength detector, and CDS-11 data system was used to analyze the ribonucleotide pools extracted from tumor cells. The analysis was done with a Partisil-10 SAX column (Whatman, Inc., Clifton, N. J.), 25 x 0.39 cm, using a linear gradient from 5 mm potassium dihydrogen phosphate (pH 2.8) to 250 mm potassium dihydrogen phosphate (pH 4.4) plus 500 mm potassium chloride. The flow rate was 60 ml/hr, and detector sensitivity was 0.016 absorbance full scale at 260 nm. Analysis of individual ribonucleotides was done by internal standard and quantitative calibration. The internal standard was xanthosine diphosphate added to the perchloric acid extract of tumor cells. The ribonucleotides were obtained from 2 x 10^7 cells by the addition of 0.2 ml of cold (4°) perchloric acid (0.5 M). This extract with internal standard (xanthosine diphosphate) was allowed to sit for 60 min at 4° and then centrifuged at 2000 rpm for 15 min at 4°. The supernatant was transferred to 1.5-ml Brinkman microcentrifuge tubes (Brinkman Instruments, Inc., Westbury, N. Y.). The acid extract was neutralized by the addition of 20 µl of 5 N KOH, and the insoluble perchlorate salts were allowed to precipitate overnight. The supernatant was obtained after microcentrifugation at 10,000 x g for 10 min at 4°. The final adjustment of the pH was carried out by titration with 1 N phosphoric acid. Samples were frozen and stored at -20° for brief periods until analyzed.

In Vivo Chemotherapy. Groups of male DBA/2J mice (6/group, 20 to 22 g/mouse) received 10^6 cells by i.p. injection of P815 ascites cells maintained by weekly passage into mice. Twenty-four hr after tumor implant, IMPY dissolved in 0.85% NaCl solution was injected into 3 groups of mice (8/group) at 200, 400, and 1000 mg/kg, respectively. A fourth group of mice received only 0.85% NaCl solution. Each animal was weighed daily. The doses chosen are comparable to those used by other investigators (4, 15).

Isolation of Cells from Mice for in Vitro Studies. Male DBA/2J mice received weekly i.p. injections of 5 x 10^6 mastocytoma cells. The cells were harvested 4 to 5 days later. The animals were killed by cervical dislocation, and the cells were removed from the peritoneum by lavage with 5 ml of cold RPMI with 25 mm 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid and

1 Support of this work has been in part from the Biological Handling Program (PROPHET Computer system) of the Division of Research Resources, NIH, Center Grant CA-14395, and in part from the Women's Cancer Association of Miami.
2 Present address: College of Medicine, Department of Biophysics, East Tennessee State University, Johnson City, Tenn. 37601.
3 The abbreviations used are: IMPY, 1H-imidazo[1,2-b]pyrazole (NSC 51143); HPLC, high-pressure liquid chromatography; RPMI, Roswell Park Memorial Institute Medium 1640; FC, flow cytometric; TCA, trichloroacetic acid; DAPI, 4',6-diamidino-2-phenylindole hydrochloride.
4 R. Engle, personal communication.
5 Received December 12, 1979; accepted July 28, 1980.
was added to the column and eluted stepwise starting with 4°C and then counted for cell number and viability using trypan blue dye exclusion (0.2%) in phosphate-buffered saline [2.68 mM KCl-1.47 mM KH₂PO₄-0.14 mM NaCl-8.06 mM Na₂HPO₄-7H₂O]. The viability of the cells used in these studies exceeded 95%.

Drug Analysis in Peritoneal Fluid. IMPY was administered i.p. in 0.3 ml at 250 to 600 mg/kg mixed with 0.8 ml of ¹⁴C-labeled drug. The animals were sacrificed as described above, except that the cells were removed without dilution of peritoneal fluid in order to measure drug level. The ascites tumor cells were harvested, washed, and counted. The determination of intracellular drug level was carried out with 10⁷ cells; 2 x 10⁶ cells were used for FC analysis, and 2 x 10⁷ cells were used for HPLC analysis. Drug level in the peritoneum was measured by counting 0.05 to 0.1 ml of peritoneal fluid. The intracellular drug level was determined by digesting an aliquot of cells equivalent to 10⁷ cells with 0.2 ml of 0.5 N NaOH at 37°C for 24 hr. The digest was diluted to 1 ml with water and transferred to 10 ml of scintillation fluid (Hydromix; Yorktown Research, Hackensack, N. J.). In order to eliminate chemiluminescence, the samples were placed in the dark for 18 to 24 hr and then analyzed for radioactivity by liquid scintillation counting.

DNA and RNA Extraction. For DNA analysis, the pellets from cold acid extraction (1.6 N O₂) were suspended in 0.5 ml of 1.6 N HClO₄ and heated at 95°C for 120 min. The digest was cooled to 4°C and neutralized with 4 N KOH (4°C). The supernatant was removed after allowing the reaction mixture to set overnight at 4°C. An aliquot of the supernatant was analyzed for DNA (17) and for radioactivity by liquid scintillation counting. The pellet from the neutralized acid hydrolysis step was digested with 0.2 N KOH at 37°C overnight. The cooled alkaline digestions were diluted with water, and an aliquot was used for RNA analysis (5).

Dowex 1 Chromatography. A 0.5- x 30-cm column was packed by gravity with washed and regenerated Dowex 1 resin. A neutralized, cold HClO₄ extract of 5 x 10⁷ radiolabeled cells was added to the column and eluted stepwise starting with water (10 ml) and increasing in formic acid from 5 mM to 1 M in steps of 5 mM, 50 mM, and 1 M (10 ml each). [³H]Uridine (0.5 μCi) injected into mice was used as an internal standard for nucleoside-nucleotide elution. Fractions of 4 ml were collected from the column by a microfractionation apparatus (Gilson Medical Electronics, Inc., Middleton, Wis.). A 0.5-ml fraction from each tube was counted for radioactivity.

DNA (Alkaline Sucrose Gradient) Sedimentation. Cells were harvested from 3 mice given 1 μCi of [³¹⁴C]IMPY 24 hr before sacrifice as described above. After a washing in cold 0.85% NaCl solution the cells were diluted to 3 x 10⁶ cells/ml. An alkaline sucrose gradient (8) was prepared in SW 27 rotor centrifuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.). The gradient was overlaid with 0.2 ml of 1 N NaOH followed by 0.2 ml of mastocytoma cells (10⁷). After 30 min, the gradients were centrifuged for 3 hr at 24,000 x g. One-ml fractions from the gradients were collected, and to each fraction were added 0.1 ml of calf thymus DNA (1 mg/ml), 0.1 ml of 0.01 M sodium pyrophosphate, and 1 ml of 5% TCA. The fractions were set aside for 2 hr, and the precipitate in each tube was collected on glass fiber filters previously soaked in 5% TCA and 0.01 M sodium pyrophosphate. The filters were washed successively with 5 ml of 5% TCA plus 0.01 M sodium pyrophosphate, 5 ml of ethyl alcohol, and then 5 ml of ether. The dried filters were counted for radioactivity as described below.

Tissue Culture. After sterile removal of the P815 mastocytoma cells from the peritoneal cavity of the DBA/2J mice, the cells were washed once in RPMI. Mastocytoma cells were cultured in 50-ml spinner flasks at a density of 2 x 10⁵ cells/ml and incubated for 24 hr at 37°C in a humid, 5% carbon dioxide incubator. A 0.5-ml aliquot of IMPY (100X), which was dissolved in the RPMI solution and sterilized by Millipore filtration, was added to each spinner flask. At various times, 1-ml aliquots were removed for cell counts, viability (0.2% trypan blue exclusion), and FC analyses. Duplicate spinner flasks were set up for cytotoxicity, FC, and biochemical studies. An initial assessment of the sensitivity of the mastocytoma cells in culture to IMPY was carried out by exposing duplicate spinner culture to drug (10⁻³ to 10⁻¹ M) for 36 hr. After the incubation period, cell counts and viability were determined.

DNA-fluorescent FC Analysis. Cell suspensions for FC analysis were centrifuged as described previously and resuspended in a solution of the DNA-specific fluorochrome, DAPI (Serva Company, New York, N. Y.), dissolved in a special nuclear isolation medium (19). This solution simultaneously isolated single nuclei and stained the DNA with DAPI. After 5 min at 21°C, the stained nuclei were filtered through a 50-μm mesh and placed at 4°C until ready for FC analysis.

High-resolution DNA distribution analysis was accomplished by utilizing an ICP-22 flow cytometer (Ortho Instruments, Westwood, Mass.). A UG1 exciter filter and TK400 dichroic mirror were used to isolate the 365 nm mercury emission line for exciting the DAPI fluorochrome. In order to detect the 450 nm fluorescence emission peak, a TK580 dichroic mirror and a LP395 filter were placed in front of the photomultiplier tube. Digital data processing and analysis utilized a SUE Lockheed computer system (Lockheed Electronics Co., Inc., Northridge, Calif.).

Radioactivity Analysis. Radioactivity was measured utilizing a Beckman Model 9000 microprocessor-controlled liquid scintillation counter, using quench standard calibration for the determination of the dpm. Instrument efficiency was 83% for ¹⁴C. A toluene-based scintillation fluid (Hydromix) was used throughout.

Data Analysis. In the DNA histograms, regions of interest were integrated by setting the boundaries of integration with a user-interactive vertical cursor line on the Tektronix 4010 terminal (Tektronix, Inc., Beaverton, Oreg.). The G₀ + G₁ boundaries of integration started just before the start of the base line of the G₀ + G₁ peak and ended on the right side of this peak at a point tangent to the curve. The next region of integration was the S phase which stopped at the point tangent to the left side of the G₂ + M peak (second peak). The remaining part of the curve encompassed the G₂ + M region. This simple integration procedure was sufficient due to the high resolution of the flow cytometer in which coefficients of variation in the 1 to 2% range were routinely obtained.

Drug decay (peritoneal fluid) or accumulation (intracellular) was analyzed by nonlinear squares regression using the NIH PROPHET Computer System (16).

RESULTS

The increases in life span for groups of mice receiving IMPY...
Studies of IMPY with Mastocytoma

Studies of IMPY with Mastocytoma at 200, 400, and 1000 mg/kg were 0, 10, and 25%, respectively. There was no statistically significant change of animal weight between treated and control groups of mice. The 50% effective dose for the cytotoxicity of IMPY in vitro was $1.6 \times 10^{-4}$ M.

The lack of significant in vivo antitumor activity of IMPY compared to its in vitro activity could be due to several factors such as uptake, metabolism, and excretion. These may be important in understanding the general lack of hematological activity clinically (20). Therefore, some aspects of the in vivo pharmacology, cytokinetics, and biochemistry of IMPY with mastocytoma ascites tumor cells have been studied.

IMPY was biphasically eliminated (Chart 1A) from the peritoneal cavity of the mouse with half-lives of 2 and 69 hr, respectively. Concomitant with this drug elimination, IMPY or IMPY metabolites could apparently accumulate in the face of exponentially declining extracellular drug levels (Chart 1B). The ratio of intracellular to extracellular drug radioactivity remained linear (slope, 0.009; $r$, 0.97) from 200 to 1000 mg/kg.

Table 1 shows the relative distribution of ribonucleotides from mastocytoma cells 1 to 24 hr after IMPY administration. A typical ribonucleotide analysis chromatogram is shown in

<table>
<thead>
<tr>
<th>Ribonucleotide</th>
<th>Control</th>
<th>1 hr</th>
<th>6 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>0.005</td>
<td>0.020</td>
<td>0.046</td>
<td>0.028</td>
</tr>
<tr>
<td>UMP</td>
<td>0.060</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>0.480</td>
<td>0.520</td>
<td>0.974</td>
<td>0.393</td>
</tr>
<tr>
<td>GMP</td>
<td>0.060</td>
<td>0.039</td>
<td>0.086</td>
<td>0.076</td>
</tr>
<tr>
<td>IMP</td>
<td>0.033</td>
<td>0.061</td>
<td>0.040</td>
<td>0.030</td>
</tr>
<tr>
<td>CDP</td>
<td>0.002</td>
<td>0.028</td>
<td>0.040</td>
<td>0.025</td>
</tr>
<tr>
<td>UDP</td>
<td>0.110</td>
<td>0.086</td>
<td>0.134</td>
<td>0.090</td>
</tr>
<tr>
<td>ADP</td>
<td>1.094</td>
<td>0.830</td>
<td>1.079</td>
<td>0.869</td>
</tr>
<tr>
<td>GDP</td>
<td>0.176</td>
<td>0.150</td>
<td>0.195</td>
<td>0.173</td>
</tr>
<tr>
<td>CTP</td>
<td>0.158</td>
<td>0.117</td>
<td>0.087</td>
<td>0.034</td>
</tr>
<tr>
<td>UTP</td>
<td>0.241</td>
<td>0.104</td>
<td>0.153</td>
<td>0.064</td>
</tr>
<tr>
<td>ATP</td>
<td>2.828</td>
<td>2.216</td>
<td>1.902</td>
<td>1.452</td>
</tr>
<tr>
<td>GTP</td>
<td>1.202</td>
<td>0.457</td>
<td>1.094</td>
<td>0.555</td>
</tr>
</tbody>
</table>

* All values within 10% of mean on duplicate and 25% of mean on replicate experiments.

In vitro studies indicated a reasonable dose-response relationship between IMPY and an interruption of the cells in the cell cycle relative to control. However, in vivo studies at doses which gave drug concentrations equivalent to or higher than extracellular in vitro drug levels did not show the same perturbation of DNA distribution content as occurred from IMPY in vitro (Chart 3, upper versus lower curves). Interestingly, about 12 hr after drug exposure, the cells in vitro appeared to escape blockade by IMPY as seen by a decline in the proportion of cells in S phase and a concomitant increase in cells with G$_2$ + M DNA content.

Anion-exchange analysis of intracellular IMPY metabolites indicated that several fractions behaved chromatographically as nucleotides or strong anions (Chart 4). Furthermore, the proportion and intracellular composition of the metabolites changed over time.
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The alkaline sucrose gradient in Chart 5 indicated that some IMPY metabolite(s) possibly entered DNA or became tightly associated with DNA at a low level and could sediment mainly with the higher-molecular-weight DNA fractions.

PER CENT
CONTROL

PER CENT
CONTROL

IN VITRO

IN VIVO

Chart 3. The proportion of cells in each cell cycle phase as percentage of control at each time point after IMPY administration. In vitro analysis: percentage of cells in S phase relative to control (●, 0.2; △, 0.8 mM IMPY) and percentage of cells relative to control in G2 + M phase (○, 0.2; △, 0.8 mM IMPY); in vivo FC analysis: percentage of cells in S phase (●) and G2 + M phase (○) relative to control, respectively (400 mg/kg).

DISCUSSION

It has been shown previously that, in vitro, IMPY is able to synchronize HeLa and human lymphoid cells, inhibit the reduction of ribonucleotides, and augment the metabolism of 1-β-D-arabinofuranosylcytosine (3, 4, 7, 12, 14). Recently, it has been shown that IMPY is extensively metabolized in dogs and humans (2, 11).

In contrast to the effect of IMPY on the DNA distribution content of mastocytoma cells in culture, we have found little influence from IMPY on the same cells in vivo. Furthermore, HPLC ribonucleotide analysis did not indicate any effect of IMPY on ribonucleotide pools of mastocytoma cells in vivo. We have shown that this lack of significant in vivo effect of IMPY on DNA distribution content and ribonucleotide pools probably was not due to a low extracellular in vivo concentration of IMPY relative to in vitro studies, since intracellular and peritoneal cavity drug levels were high (0.4 mM at 24 hr). A time-sequential study of intracellular IMPY on an anion-exchange column indicated the presence of several strongly binding metabolites, the proportions of which vary with time after drug treatment. From 0.2 to 0.8 mM, IMPY in vitro leads to a maximum accumulation of P815 cells in S phase after about 10 hr of drug exposure. This blockade then declines to control level in the next 14 to 22 hr. This indicates that drug catabolism may have occurred or that biochemical pathways capable of circumventing IMPY inhibition may have been activated. At a 400-mg/kg dose of IMPY, the accumulation of P815 cells in S phase in vivo slowly increases from control level to 120% of control (control, 100%) after 24 hr. This slow accumulation of cells with S-phase DNA content in excess of control cells would be consistent with the long terminal half-life of IMPY in the peritoneal fluid. This would not explain the rise and fall in S-phase DNA content of P815 cells in vitro which does not occur in vivo, despite an intracellular level of IMPY equivalents of 0.4 mM. It is highly likely that the extent and type of metabolites generated in vivo (for example, via the liver) may be qualitatively or quantitatively different from the metabolites of IMPY generated in vitro. As with fluorouracil metabolism, an analysis of IMPY metabolism may be an important pharmacological deter-
ominant of antitumor drug action in vivo. It has been reported that at least one urinary metabolite of IMPY obtained from dogs is active biologically (11). This metabolite has been identified as a ribonucleoside (13). Chromatography of intracellular mastocytoma IMPY metabolites on Dowex 1 gave an elution pattern similar to that of uridine ribonucleotides. RNA and DNA fractions from cold acid-insoluble extracts of cells harvested in vivo had about 300 and 700 dpm IMPY per µg RNA or DNA, respectively. We have shown that a small amount of IMPY is apparently able to be incorporated into DNA of mastocytoma cells growing in vivo. Recent studies on the metabolism of other nucleoside analogs indicate that these compounds are capable of undergoing extensive metabolism leading to metabolic products with diverse toxicity and biochemical effects (6–8, 10, 18).

For example, fluorafur and 3-deazauridine are prodrugs which are transformed to their ultimate in vivo cytostatic components by riboside conjugation reactions. Therefore, compounds like IMPY should be considered to exert their pharmacological action as a riboside, a deoxyriboside, a deoxyribonucleotide, or a ribonucleotide, and attempts should be made early in drug development to determine the relative contributions of such conjugates to host toxicity and tumor growth.

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