Quantitation of Intracellular Metabolites of $[35S]$-6-Mercaptopurine in L5178Y Cells Grown in Time-Course Incubates

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ABSTRACT

6-Mercaptopurine (6MP) metabolism was quantitatively determined in L5178Y murine lymphoma. Cells grown in time-course incubates with $[35S]$-6MP were extracted with cold perchloric acid, and the buffered extracts were subjected to high-performance liquid cation-exchange chromatography prior to and after hydrolysis with alkaline phosphatase. Free sulfate, 6-thiouric acid, 6-thioxanthosine, 6-thioguanosine, 6-thioinosine, free 6MP, and 6-methylthioinosine were separated from each other; identified in the radiochromatograms by elution volume, UV spectroscopic data, and enzymatic peak-shifting analyses with purine nucleoside phosphorylase; and quantitatively determined by means of $35S$ radioactivity. Gross intracellular $35S$ concentrations remained constant at $5 \times 10^{-5}$ M after 1 hr of incubation. 6MP metabolism in L5178Y cells was distinguished into an early phase (to 1 hr of incubation) in which 6MP was predominantly catabolized to 6-thiouric acid and free sulfate, into an intermediate phase (to 8 hr) in which substantial amounts of free 6MP and of ribonucleotides of 6-thiopurine bases and ribonucleosides were present while the concentrations of nonnucleotide oxidation products sharply decreased, and into a late phase (to 24 hr) in which the ribonucleotides of 6MP, of 6-thioguanosine and, in particular, of 6-methylthioinosine were the most abundant metabolites.

INTRODUCTION

Many methods have been applied to determine, as a measure of therapeutic efficiency or as a possible guide to more effective therapy, the concentrations of 6MP and its most abundant metabolites and catabolites in biological materials. The separation from various constituents of the biological materials of 6MP and its derivatives formed in mammalian cells was achieved either by paper (4, 13), thin-layer (19), or liquid (28, 34, 40, 41) and gas (3) column chromatography and by thin-layer electrophoresis (38). The detection and quantitation of the compounds was carried out by (low-temperature) fluorimetry with (34) and without (38) oxidation of the 6-thiopurines to 6-sulfopurines, by colorimetry (22), by UV detection (5, 6, 9, 40, 41), by radioactivity measurements (4, 7, 13), and by mass spectrometry (32).

In general, it has been established that the efficiency of 6MP is closely related to its intracellular transformation to the ribonucleoside monophosphate, 6MPRP (17, 23), a reaction catalyzed by hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8). Thus, in contrast to other antineoplastic agents, the plasma concentrations or the excreted amounts of 6MP and its catabolites are not correlated to its antitumor effects (31), giving these parameters less importance than intracellular concentrations.

In several reports, intracellular concentrations of 6-thiopurines have been determined by using radioactively labeled drug with subsequent conventional chromatography (33, 37). Little information has, however, been presented with quantitative data on the time course of intracellular modifications which occur at the 6MP base, the stages of phosphorylation in which these modifications exist, and the exact biochemical mode of action which causes antitumor and, in particular, immunosuppressive effects.

Only a few column chromatographic methods have been reported for the predominant separation of 6-thiopurines and their ribonucleotides (2, 28, 34, 40, 41). However, these give poor resolution for the phosphorylated compounds.

Recently, we reported a separation method by HPLC for 6-thiopurine bases and (deoxy)ribonucleosides and for some common purines (5, 6) and demonstrated the advantage of using a variable-wavelength HPLC detector for the identification of these compounds on the pmol level (9). The methods proved useful for the quantitative determination of time-dependent intracellular base modifications of 6MP. Cell extracts were hydrolyzed with APase (and PNPase) yielding the ribonucleosides (and the free bases for peak-shifting analyses). The hydrolysates were easily processed with the HPLC cation-exchange procedures. On the basis of these methods, we determined the distribution of $8^{-14}$C-labeled 6-thiopurines in L5178Y cells after a 24-hr incubation period with [8-$14$C]-6MP (7).

The results of labeling experiments with $[35S]$-6MP during short- and long-term incubation of L5178Y cells are reported in this paper. A possible metabolic mechanism is outlined which might explain the characteristic distribution patterns of 6-thiopurines found in L5178Y cells after different incubation times with $[35S]$-6MP. The application of the method for quantitative determinations of unlabeled 6MP metabolites in biological materials is discussed.

MATERIALS AND METHODS

Chemicals. Common purine bases and ribonucleosides, 6-thiopurine bases and ribonucleosides, and all basic chemicals were purchased from Papierwerke Waldhof-Aschaffenburg, Mannheim, West Germany; EGA-Chemie, Weinheim, West Germany; E. Merck, Darmstadt, West Germany; P-L Biochemicals,
the requirements for reliable identification by peak-shift analyses of all 6-thiopurines mentioned thus far. Considerable amounts of $^{35}$S radioactivity, however, remain after hydrolysis with PNPase in the positions of 6-thioxanthine and 6-thioguanosine. These are assumed to be due to 6TX and an unidentified $^{35}$S-labeled compound.

**DISCUSSION**

L5178Y cells growing at low concentrations of 6MP are expected to metabolize the drug in a characteristic series of enzyme-catalyzed reactions which can be followed quantitatively by determining the actual intracellular distribution of the various intermediary products after different incubation intervals. Experiments on this line provide a source of biological material containing minute amounts of 6MP metabolites and catabolites during early incubation periods and also give characteristic patterns of more or less strongly changing intracellular concentrations of 6-thiopurines and of free sulfate.

**Quantitative Determination of $[^{35}S]$-6-Thiopurine Bases and Ribonucleosides.** According to the specific radioactivity of the $[^{35}S]$-6MP lot, the total amounts of 6-thiopurine bases and ribonucleosides have been determined quantitatively from HPLC radiochromatograms of cell extracts which had been incubated with APase and PNPase for enzymatic peak-shifting analyses (Extracts I,a, I,b, and I,c) (Table 4). According to these data, however, remain after hydrolysis with PNPase in the positions of 6-thioxanthine and 6-thioguanosine. These are assumed to be due to 6TX and an unidentified $^{35}$S-labeled compound.

**RESULTS**

ED$_{50}$ of $[^{35}S]$-6MP on L5178Y Cells. In the dose-response experiment with $[^{35}S]$-6MP, an ED$_{50}$ of 7.7 $\mu M$ was determined for concentrations of 100,000 cells/ml.

**Proliferation of L5178Y Cells.** Cells grown with $[^{35}S]$-6MP for different time intervals initially proliferated slowly. After 24 hr, the cell number had been diminished seriously as compared to the control incubates (Table 1). Cell viability as measured by dye exclusion methods was greater than 90%.

**Incorporation of $[^{35}S]$ Radioactivity into L5178Y Cells.** The incorporation rate of $[^{35}S]$-6MP into L5178Y cells was 0.48% of the total amount of $[^{35}S]$-6MP which was initially dissolved in the culture medium. Five hundred million cells grown with 8.1 $\mu M$ $[^{35}S]$-6MP were extracted, and the intracellular $^{35}$S concentration was determined to 60 $\mu M$ (Table 2).

**Identification of $[^{35}S]$-6MP Metabolites.** 6MP and its metabolites which were contained in the perchloric acid extracts were identified by means of radioactive label, elution volumes in the HPLC system, and behavior in enzymatic peak-shifting analyses using stepwise incubation of the extracts with APase and PNPase for 16 hr(7,9),(994,992)
specific activity of the radioactive compound should be used if reliable quantitative determinations are desired, in incubates comprising less than 10 million cells. At an average cell volume of 1.2 pl, intracellular 6-thiopurines in 10 million cells equal a total amount of 1 nmol. When working with 6MP, a 35S label can be introduced into the mercapto group. The specific activity of [35S]-6MP reaches about 1 to 30 Ci/mol (1, 4, 35). Provided there is a specific radioactivity of 10 Ci/mol and a counting efficiency of 80%, 1 nmol of intracellular 6-thiopurines represents only 18,000 cpm.

The ED50 for [35S]-6MP in L5178Y cell incubates with 100,000 cells/ml was 7.7 μM. Since experiments with [8-14C]-6MP gave identical results with less than 10% of the specific radioactivity (7), the low ED50 rather seems to be characteristic for the cell line than due to radiation effects.

During the initial period of incubation (0 to 8 hr) with 8.1 μM [35S]-6MP, the cell concentrations in the time-course incubates and hence the number of doubling steps increased steadily (Table 1). Probably due to effects of 6MP on nucleic acid and nucleotide metabolism (10, 12, 39), a significant inhibition of nucleotide metabolism (10, 12, 39), a significant inhibition of intracellular 6-thiopurine metabolism, however, remained constant after 1 hr of incubation, suggesting steady-state conditions in uptake and excretion of [35S]-6MP and 6-thiopurines, respectively, by the L5178Y cells after this time.

Despite constant intracellular concentrations, the distribution patterns of intracellular 6-thiopurines strongly changed within the 24-hr incubation period. These changes might represent a highly important characteristic of intracellular 6MP metabolism.

From the data presented in Table 3, 3 phases of 6MP metabolism in L5178Y cells become likely: Phase 1, an early phase (0.5 to 1 hr of incubation) in which intracellular 6MP is rapidly oxidized to 6TUA and high levels of free sulfate are generated, this change might represent a specific 24-hr incubation period. These changes might represent a highly important characteristic of intracellular 6MP metabolism.
oxidation of 6MP slows down, the intracellular concentrations of 6MPRP and 6TXRP rise significantly, even small amounts of 2A6MP ribonucleotides are already detectable, and 6TUA and free sulfate reach constant levels which might represent approximate measures for basic oxidation and desulfurization of 6MP; and Phase 3, a late phase (24 hr) in which large amounts of 6MPRP and of base-modified 6MP metabolites, in particular 6MeMP ribonucleotides, cumulate within the cells.

A strong objection to this scheme of timely events should be the possibility of insufficient or to a certain extent varying enzymatic hydrolysis. No significant variations of 35S distributions were, however, found using different incubation periods for enzymatic hydrolyses (4 to 48 hr) (data not shown). On the contrary, the complete hydrolysis of mono-, di-, and triphosphates to ribonucleosides by APase has since long been proven (15, 16, 24), and the action of PNase on common and modified ribonucleosides has been studied extensively (21, 30). The data on enzymatic peak-shifting analyses (Table 4) still support the reliability of the quantitative methods. Thus, it seems unlikely that variations of the 35S distribution patterns in the HPLC radiochromatograms should be caused by insufficient sample processing. Furthermore, the concentrations of free sulfate, 6TUA, and free 6MP in the cell extracts are generally independent from any enzymatic processing with APase.

The results obtained from the evaluation of HPLC radiochromatograms of (enzymatically hydrolyzed) cell extracts from time-course incubates seem to support the following sequence of events in 6MP metabolism. 6MP reaching the inner compartments of the cell during the early phase of incubation is rapidly oxidized at a very high extent. High intracellular levels of 6TUA and free sulfate are thus generated. Probably, a minute amount only of the intracellular 6MP is converted to 6MPRP and, if so, is rapidly processed into various 6-thiopurine ribonucleotides. Delayed cytotoxic effects becoming apparent in the cells within 2 to 6 days after even short incubation periods with 6MP are supposedly due to these metabolites (35, 36).

Increasing amounts of 6-thiopurine ribonucleotides which are formed during the intermediate phase inhibit de novo purine synthesis, salvage pathway reactions, and purine nucleotide interconversions. This reportedly causes severe depletion of purine nucleotide pools with concentrations declining most sharply about 2 to 6 hr after the admission of 6MP to the culture medium (11, 35).

PRPP, normally used for de novo purine synthesis or salvage pathway reactions and known to control the salvage pathway reactions more extensively than do the involved purine phosphoribosyl-transferases (18, 20), then becomes available within the cells in large amounts. Thus, 6MP becomes more and more rapidly converted to 6MPRP, and again increasing levels of 6MPRP inhibit purine nucleotide metabolism and support further increase in PRPP concentration.

Recently, it was reported that the availability of PRPP in human leukemic leukocytes is extremely low as compared to L1210 cells which contain about 160 times as much PRPP (20). According to our data, the low availability of PRPP might thus be the cause for the very high oxidation rates of 6MP in humans during an initial period (14). Minute intracellular PRPP concentrations cause very low conversion rates of 6MP to 6MPRP and thus high oxidation rates of 6MP to 6TUA.

6TX, which is also a substrate for phosphoribosyltransferases, might be converted to the ribonucleotide at high intracellular PRPP concentrations, and 6TXRP concentrations dramatically increase in cells of the 2- to 8-hr incubates. There exists, however, the possibility that 6TXRP is mainly formed from 6MPRP by the action of inosine dehydrogenase since free 6TX has not been shown to be present in the cell extracts. To the same extent as 6MPRP and 6TXRP levels rise during the intermediate phase of 6MP metabolism, nonnucleotide oxidation products of 6MP drop to a low level which is maintained through the 24-hr incubation period.

The formation of 6TXRP in L5178Y cells is comparable to the time course of 6TXRP formation in Ehrlich ascites cells (2). 6TXRP as the major 6-thiopurine compound remains in these cells with its maximum concentration about 2 hr after completion of a 6-hr administration period of 6MP. 6TXRP concentrations were reported to be initially as high as the 6MPRP levels. After 6 to 8 hr, however, 6TXRP concentrations rose to a 3- to 4-fold excess over 6MPRP concentrations. The ratio found by us in L5178Y cells far exceeds these ratios.

During the late phase (24 hr), a typical distribution pattern of 6MP metabolites is generated in the cells. 2A6MP and 6MeMP ribonucleotides are the most abundant 6-thiopurine compounds besides 6MPRP. The high intracellular concentrations of 6MeMP ribonucleotides are most probably due to the inability of PNase to split 6-methylthioinosine which is generated in catabolic pathways. Kinase action then converts 6-methylthioinosine to 6-methylthioinosinate, and even higher levels of the di- and triphosphate have been reported (29, 40, 41). 6MeMP ribonucleotides strongly inhibit the first step of the de novo purine synthesis, and 6MPRP preferentially inhibits purine nucleotide interconversions, whereas 2A6MP ribonucleotides are incorporated into the nucleic acids, DNA as well as RNA (13, 36).

[35S]-6-Thiopurines which were extracted from L5178Y cells in amounts of about 100 pmol/million cells were easily detected and quantitatively determined in HPLC radiochromatograms. In the human body, however, 6MP is rapidly metabolized to 6TUA and excreted. Thus, intracellular concentrations of unlabeled 6-thiopurines hardly reach values of 10 μM if 6MP is administered in daily p.o. doses of less than 200 mg/sq m or 2.5 mg/kg body weight. Highly efficient separation devices together with sensitive variable-wavelength HPLC detectors working at 0.005 absorbance unit (full scale) in the 290 to 350 nm range might be capable of detecting even less than 100 pmol of 6MP metabolites in a single run. Nevertheless, the determination of unlabeled 6-thiopurines in biological materials of 6MP-treated patients still strongly depends on the amount of tissue or cells available for single extraction procedures.

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