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Renal Cellular Transport, Metabolism, and Cytotoxicity of *S*-(6-Purinyl)glutathione, a Prodrug of 6-Mercaptopurine, and Analogues

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ABSTRACT. The disposition of *S*-(6-purinyl)glutathione (6-PG) and its metabolites, including the antitumor agent 6-mercaptopurine (6-MP), was characterized in freshly isolated renal cortical cells from male F344 rats to assess the ability of the kidney to convert 6-PG to 6-MP. The intracellular transport and accumulation of 6-PG and 6-MP, the metabolism of 6-PG to 6-MP, and the potential cytotoxicity of 6-MP, 6-thioxanthine (6-ThXan), and 6-thioguanine (6-ThGua) were determined. 6-PG and 6-MP were accumulated by renal cortical cells by time- and concentration-dependent processes, reaching maximal levels of 14.2 and 1.52 nmol/10^6 cells, respectively, with 1 mM concentrations of each compound. Treatment with acivicin, an inhibitor of 6-PG metabolism by γ-glutamyltransferase, increased accumulation of 6-PG, and treatment with α-keto-γ-methiolbutyrate, a keto acid cosubstrate that stimulates activity of the cysteine conjugate β-lyase (β-lyase), which generates 6-MP, decreased accumulation of 6-PG. Incubation of renal cells with 10 mM 6-PG generated 6-MP at a rate of 2.4 nmol/min per 10^6 cells, demonstrating that the β-lyase pathway forms the desired product from the prodrug within the intact renal cell. Preincubation of cells with acivicin or aminooxyacetic acid, an inhibitor of the β-lyase, decreased the net formation of 6-MP, demonstrating further the function of the β-lyase. 6-MP, 6-ThXan, and 6-ThGua exhibited approximately equivalent cytotoxicity (45–55% release of lactate dehydrogenase with 1 mM at 2 hr) in isolated renal cells. Based on the known antitumor potency of these agents, this suggests that cytotoxicity and antitumor activity occur by distinct mechanisms. The high amount of accumulation of 6-PG and its subsequent metabolism to 6-MP, as compared with the relatively low amount of accumulation of 6-MP, in renal cells suggest that 6-PG can function as a prodrug and is a more effective delivery vehicle for 6-MP to renal cells than 6-MP itself. Administration of 6-PG may be an effective means of treating renal tumors or suppressing renal transplant rejection. BIOCHEM PHARMACOL **54**;12:1341–1349, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. kidney; prodrug; 6-mercaptopurine; antitumor agent; glutathione conjugate; cysteine conjugate β-lyase; kidney transplant rejection

Biologically active therapeutic agents may be chemically modified to generate a new, inactive chemical that, after metabolism within a target tissue, may become active. If transport and metabolic processes are selectively localized to the target tissue, this strategy may be effective in delivering the therapeutic agent to the target tissue with minimal side-effects in nontarget tissues. The modified therapeutic agent is known as a prodrug [1], and this approach has been used for the delivery of drugs to several tissues.

There is a need for development of drugs to treat kidney cancer and for more efficient means to deliver therapeutic agents to the kidneys to minimize extrarenal side-effects, thereby improving treatment. The use of prodrugs may provide such an improvement, as it would be a noninvasive means to deliver therapeutic agents to a target organ. The kidneys are ideally suited to be target organs for various classes of prodrugs for the following reasons: (1) they receive an exceptionally high rate of blood flow per gram of tissue, thereby hastening delivery of blood-borne chemicals; (2) glomerular filtration concentrates chemicals in the tubular lumen; (3) the kidneys, in particular the renal proximal tubules, contain a large array of plasma membrane transport systems that mediate uptake and intracellular accumulation of both filtered chemicals and those in the renal plasma; and (4) renal cells, in particular the proximal tubules, contain a large number of drug metabolism enzymes, some of which are uniquely present in the kidneys, that can bioactivate prodrugs.

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Cysteine conjugates of numerous halogenated alkanes and alkenes are selective nephrotoxicants [2]. This target organ selectivity is due to the presence of plasma membrane transport systems for GSH§ conjugates [3] and cysteine conjugates [4, 5], the presence of high amounts of GGT (EC 2.3.2.2) and dipeptidases on renal proximal tubular brush-border membranes, and the presence of β-lyase (EC 4.4.1.13), which metabolizes cysteine conjugates to thiols, some of which are reactive electrophiles that may produce nephrotoxicity or nephrocarcinogenicity.

6-MP is an antitumor agent with immunosuppressive properties [6], and selective delivery of 6-MP to the kidneys by a surgically implanted infusion pump has been a strategy to avoid extrarenal toxicity [7, 8]. In a series of studies, Elfarra and colleagues [9–13] employed a different strategy to selectively deliver 6-MP to the kidneys, taking advantage of renal-specific biochemistry. They showed that rats given 6-CP form 6-PG *in vivo*, and that 6-PG and the corresponding cysteine conjugate 6-PC form 6-MP by a β-lyase-dependent mechanism. The known presence of the appropriate transport and enzymatic processes in the kidneys supports the suggestion that 6-PG can serve as a prodrug for 6-MP. However, the ability of 6-PG to generate 6-MP within renal cells and the role of specific steps of metabolism have not been directly assessed.

A scheme summarizing the hypothesized pathways of 6-PG metabolism is shown in Fig. 1, and it illustrates potential strategies that can be used to establish the bioactivation pathway. Requirement for the metabolism of 6-PG by renal GGT can be demonstrated by showing that acivicin, a specific inhibitor of GGT [14], should enhance intracellular accumulation of 6-PG and inhibit intracellular generation of 6-MP. The critical step, metabolism of 6-PC by β-lyase, to generate 6-MP can be inhibited by AOAA [10, 11]. This same strategy was used previously to demonstrate the function of GGT and β-lyase in the bioactivation of *S*-(1,2-dichlorovinyl)glutathione and DCVC *in vivo* [15] and in isolated renal cells [16]. Additionally, 6-MP may be metabolized by XO to 6-ThXan, and allopurinol should inhibit this reaction.

The goals of the present study were to demonstrate uptake and accumulation of 6-PG and 6-MP by isolated renal cells, to demonstrate intracellular metabolism of 6-PG to 6-MP, and to explore the potential cytotoxicity of 6-MP and analogues. Selective inhibitors of several of the critical steps in this pathway were used to provide support for these reactions in the function of 6-PG as a renal prodrug of 6-MP.

§ *Abbreviations:* acivicin, L-(αS, 5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; AOAA, aminooxyacetic acid; β-lyase; cysteine conjugate β-lyase; 6-CP, 6-chloropurine; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; GGT, γ-glutamyltransferase; GSH, glutathione; KMB, α-keto-γ-methiolbutyrate; LDH, lactate dehydrogenase; 6-MP, 6-mercaptopurine; 6-PC, *S*-(6-purinyl)-L-cysteine; 6-PG, *S*-(6-purinyl)glutathione; 6-ThGua, 6-thioguanine; 6-ThXan, 6-thioxanthine; and XO, xanthine oxidase.

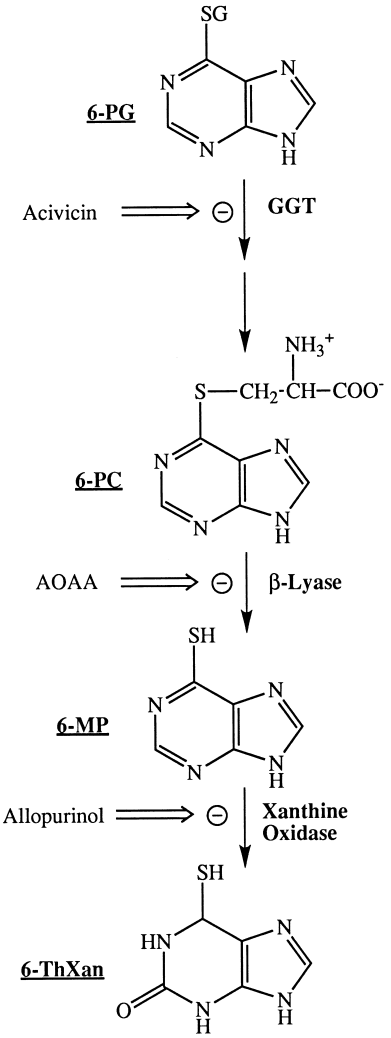

FIG. 1. Scheme of 6-PG metabolism in rat kidney. Abbreviations: 6-PG, *S*-(6-purinyl)glutathione; 6-PC, *S*-(6-purinyl)-L-cysteine; 6-MP, 6-mercaptopurine; 6-ThXan, 6-thioxanthine; GGT, γ-glutamyltransferase; β-lyase; cysteine conjugate β-lyase; and AOAA, aminooxyacetic acid.

MATERIALS AND METHODS

Materials

6-PG and DCVC were synthesized as previously described [11, 15]. Purity was > 95%, as assessed by HPLC, FAB-MS, and ^{1}H NMR. 6-MP, 6-ThXan, 6-ThGua, acivicin, AOAA, allopurinol, KMB, and collagenase (type I) were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity available and were obtained from commercial sources. Solutions of acivicin, AOAA, allopurinol, KMB, or 6-PG were prepared in Krebs–Henseleit buffer or saline. Solutions of 6-MP, 6-ThXan, or 6-ThGua were prepared by first dissolving in a minimal volume (0.1 to 0.2 mL/10 mL final volume) of 1 M NaOH and then Krebs–Henseleit buffer.

Isolation of Rat Renal Cortical Cells

Isolated renal cortical cells were obtained by collagenase perfusion [17] from male F344 rats (200–300 g; Charles

River Laboratories, Wilmington, MA). Animals were housed in the Wayne State University vivarium, were allowed access to food and water *ad lib.*, and were kept in a room on a 12-hr light–dark cycle. Prior to surgery, rats were anesthetized with i.p. injections of sodium pentobarbital (50 mg/kg body weight). Renal cells were suspended in Krebs–Henseleit buffer, pH 7.4, supplemented with 25 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, and 5 mM glutamine. Cortical cells are predominantly of proximal tubular origin [17, 18], and the yield from two kidneys was approximately 50×10^6 cells. Viability, as estimated by either trypan blue exclusion or release of LDH, was greater than 90% at the time of isolation. All incubations for transport, metabolism, or cytotoxicity assays were performed in 25-mL polyethylene Erlenmeyer flasks on a Dubnoff metabolic shaking incubator (60 cycles/min) under an atmosphere of 95% O_2/5% CO_2.

Transport Assays

Uptake and intracellular accumulation of 6-PG or 6-MP were measured by preincubating 9 vol. of renal cells ($3–5 \times 10^6$ cells/mL) with 1 vol. of either buffer or 10-fold concentrated solutions of either acivicin (0.25 mM final) or KMB (10 mM final) for 15 min at 37°. Preincubated cells (9 vol.) were then incubated with 1 vol. of 10-fold concentrated solutions of either 6-PG or 6-MP at 37° for up to 60 min. At indicated times, 0.25-mL aliquots of cells were layered on 1 mL of 20% (v/v) Percoll in saline in 1.5-mL microcentrifuge tubes. After centrifugation for 30 sec at $10,000 \times g$, supernatants were removed, and pellets were resuspended in 0.3 mL saline. After addition of 0.05 mL of 30% (w/v) trichloroacetic acid, mixing, and centrifugation, protein-free supernatants were used for analysis of 6-PG or 6-MP by HPLC (see below).

Metabolism Assay

Metabolism of 6-PG to 6-MP was measured by preincubating 9 vol. of renal cells (8×10^6 cells/mL) with 1 vol. of either buffer, acivicin (0.25 mM final), AOAA (0.1 mM final), or allopurinol (1 mM final) for 15 min at 30°. Then preincubated cells (9 vol.) were mixed with 0.1% (v/v) Triton X-100 to solubilize the cells and 1 vol. of 100 mM 6-PG (10 mM final) for up to 60 min at 30°. At indicated times, 0.5-mL aliquots of cells were removed and placed in 1.5-mL microcentrifuge tubes containing 0.1 mL of 70% (v/v) perchloric acid. Protein-free supernatants were then analyzed for 6-PG and 6-MP by HPLC (see below). The reported rate of metabolism of 6-PG to 6-MP was obtained by taking the earliest time point (1 min) and expressing product formation as nanomoles per minute per 10^6 cells. Formation of 6-MP rapidly becomes non-linear, so that the earliest time points are needed to estimate a rate of formation.

HPLC Assay of 6-PG and 6-MP

6-PG and 6-MP were measured by HPLC using a reversed-phase, μBondapak C_{18} 10-μm cartridge (8 mm × 10 cm) (Waters Associates, Milford, MA) with a Waters model 600E multisolvent delivery system using a mobile phase of 50% aqueous acetonitrile (solvent A) and 1% aqueous acetonitrile (solvent B), each adjusted to pH 2.5 with a few drops of 99% (v/v) trifluoroacetic acid, and gradient elution at a flow rate of 1 mL/min. 6-PG and 6-MP were detected by absorbance at 288 and 320 nm, respectively, and were quantified with respect to standards using a Waters model 745B data module. 6-PG was detected by running the following program: Initial conditions = 15% A; at 8 min, gradient to 60% A over 2 min; hold at 60% A until 23 min; gradient back to 15% A over 2 min. 6-PG eluted at approximately 10 min. 6-MP was detected by running the following program: Initial conditions = 8% A; at 8 min, gradient to 60% A over 2 min; hold at 60% A until 23 min; gradient to 15% A over 2 min. 6-MP eluted as two peaks at approximately 6 and 8 min, and the latter peak was used for quantitation. Limits of detection for both chemicals were approximately 50 pmol/0.1 mL HPLC injection.

Xanthine Oxidase/Xanthine Dehydrogenase Assay

Activity of xanthine oxidase (EC 1.2.1.37)/xanthine dehydrogenase (EC 1.2.3.2) was assayed by either a spectrophotometric method [19] that measures uric acid formation with xanthine as substrate at 292 nm or a fluorometric assay [20] that measures isoxanthopterin formation with pterin as substrate. The spectrophotometric assay has a limit of detection of 10 pmol urate formed/min per mL, whereas the fluorometric assay has a 100-fold lower limit of detection (i.e. 0.1 pmol/min per mL). The assays were validated by use of purified XO (Sigma Chemical Co.).

Cytotoxicity Assay

Renal cortical cells (9 vol., $3–5 \times 10^6$ cells/mL) were preincubated at 37° with 1 vol. of either buffer or 10 mM allopurinol (1 mM final) for 15 min. Preincubated cells (9 vol.) were then incubated at 37° with 1 vol. of either buffer or 10-fold concentrated solutions of 6-MP, 6-ThXan, 6-ThGua, or DCVC as a positive control. At the indicated times, 50-μL aliquots of cells were removed for determination of LDH release [18].

Data Analysis

All values are means ± SEM, unless otherwise indicated, of measurements from the indicated number of separate cell preparations. Significant differences between means were first assessed by a one-way analysis of variance. When significant "F-values" were obtained with the analysis of variance, the Fisher's protected least significant difference *t*-test was performed to determine which means were

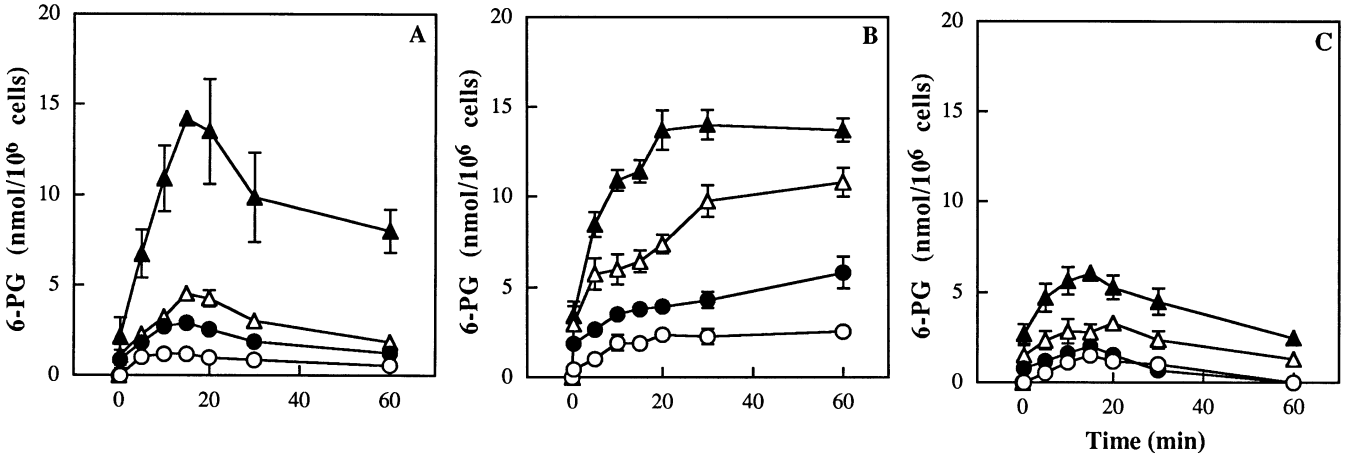

FIG. 2. Time and concentration dependence of 6-PG uptake and accumulation by isolated renal cells. Isolated renal cortical cells (3–5 × 10^6 cells/mL) were preincubated for 15 min at 37° with either buffer (A), 0.25 mM acivicin (B), or 10 mM KMB (C) and then were incubated at 37° with the indicated concentrations of 6-PG. At indicated times, aliquots were removed for analysis of 6-PG by HPLC. Results are means ± SEM of measurements from 8 separate experiments. *Statistical analyses:* (1) Significantly different ($P < 0.05$) from 0.1 mM 6-PG uptake at the same time point: Fig. 2A—All times for 0.25 (●), 0.5 (△), and 1 (▲) mM; Fig. 2B—All times for 0.25 (●), 0.5 (△), and 1 (▲) mM; Fig. 2C—0.3, 5, and 10 min for 0.25 mM and all times for 0.5 (△) and 1 (▲) mM. (2) Significantly different ($P < 0.05$) from 0.25 mM 6-PG uptake at the same time point: Fig. 2A—15, 20, and 30 min for 0.5 mM and all times for 1 mM; Fig. 2B—all times except 0.3 min for 0.5 mM and all times for 1 mM; Fig. 2C—5, 20, 30, and 60 min for 0.5 mM and all times for 1 mM. (3) Significantly different ($P < 0.05$) from 0.5 mM 6-PG uptake at the same time point: Fig. 2A, 2B, and 2C—all except 0.3 min for 1 mM. (4) Significantly different ($P < 0.05$) from 6-PG uptake in cells incubated with buffer at the same 6-PG concentration and time point: *0.1 mM 6-PG*—acivicin and KMB at 0.3 and 60 min, acivicin at 10, 15, 20, and 30 min; *0.25 mM 6-PG*—acivicin and KMB at all times except 0.3 min; *0.5 mM 6-PG*—acivicin at 0.3, 5, 10, and 30 min, acivicin and KMB at 15, 20, and 60 min; *1 mM 6-PG*—KMB at 10, 20, and 30 min, acivicin and KMB at 15 and 60 min.

significantly different from one another with two-tail probabilities of less than 0.05 considered significant. For Figs. 2–4, indication of statistical significance is given in the legends for clarity.

RESULTS

Accumulation of 6-PG and 6-MP

Uptake and net intracellular accumulation of 0.1 to 1 mM 6-PG over a 60-min time course was measured in suspensions of renal cortical cells preincubated with either buffer (Fig. 2A), 0.25 mM acivicin to inhibit GGT (Fig. 2B), or 10 mM KMB to stimulate the β-lyase (Fig. 2C). Under control conditions with buffer, 6-PG accumulated to a maximum intracellular level of 14.2 nmol/10^6 cells at 15 min with 1 mM 6-PG and decreased thereafter to reach a level of 8.02 nmol/10^6 cells at 60 min. Net accumulation was both time and 6-PG concentration dependent. Preincubation of renal cells with 10 mM KMB, which stimulates β-lyase activity by converting the enzyme to the pyridoxal form that can accept amino-containing substrates [21], resulted in a significant decrease in 6-PG accumulation at most time points, particularly at the higher concentrations of 6-PG. Maximal net accumulation of 6-PG in the presence of KMB was 6.02 nmol/10^6 cells, which is only 42% of that in the cells incubated with buffer. In contrast, preincubation of renal cells with acivicin, which inhibits GGT [14], had little effect on the maximal intracellular level of 6-PG but prevented the decrease in intracellular 6-PG content that occurred in control cells after the 15-min time point.

Cells incubated with 6-MP (0.25, 0.5, or 1 mM) exhibited time- and concentration-dependent accumulation, with maximal intracellular 6-MP contents occurring after 20 min of incubation (Fig. 3). Intracellular contents at 20 min were 0.83, 1.19, or 1.43 nmol/10^6 cells with 0.25, 0.5, or 1 mM 6-MP, respectively, and these were maintained during the entire 60-min incubation. Interestingly, intracellular accumulation of 6-MP was only about 10% of that of 6-PG, suggesting that the GSH conjugate precursor is transported and accumulated more rapidly by renal cells than 6-MP.

Metabolism of 6-PG to 6-MP

Permeabilized cells incubated with 10 mM 6-PG rapidly generated 6-MP at an estimated rate of 2.4 nmol/min per 10^6 cells (Fig. 4). The maximal total content of 6-MP (20.3 nmol/10^6 cells) was obtained after 20 min of incubation, and this level decreased by approximately 50% over the remaining 40 min of the incubation. In contrast, pretreatment of cells with 0.25 mM acivicin significantly decreased formation of 6-MP by as much as 75% at all time points after the initial 1 min, and pretreatment of cells with 0.1 mM AOAA significantly reduced cellular formation of 6-MP at 5, 30, and 60 min by as much as 50%. These results support a role for metabolism of 6-PG by GGT and subsequent metabolism of 6-PC by the β-lyase to form

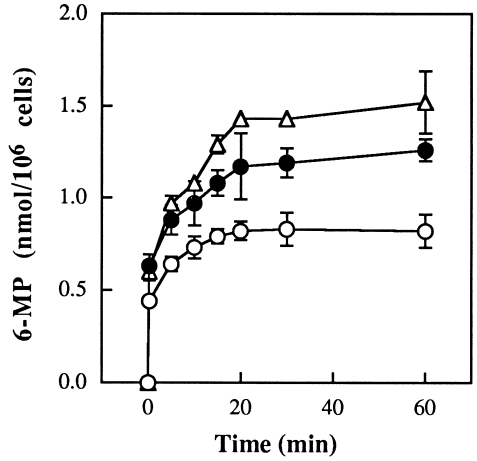

FIG. 3. Time and concentration dependence of 6-MP uptake and accumulation by isolated renal cells. Isolated renal cortical cells (3–5 × 10^6 cells/mL) were incubated at 37° with the indicated concentrations of 6-MP. At indicated times, aliquots were removed for analysis of 6-MP by HPLC. Results are means ± SEM of measurements from 4 separate experiments. *Statistical analyses:* (1) Significantly different ($P < 0.05$) from 0.25 mM (○) 6-MP at the same time point: 0.5 (●) and 1 mM 6-MP (△) at all time points. (2) Significantly different ($P < 0.05$) from 0.5 mM 6-MP at the same time point: 1 mM 6-MP at 15, 20, 30, and 60 min.

6-MP. Pretreatment of cells with 1 mM allopurinol had no significant effect on 6-MP formation, suggesting that minimal XO activity is present in renal cortical cells. This suggestion was supported by measurement of XO activity, which was barely detectable (data not shown).

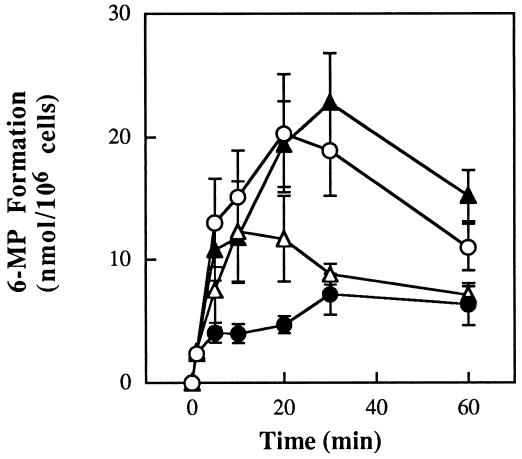

FIG. 4. Metabolism of 6-PG to 6-MP in renal cortical cells. Isolated renal cortical cells (8 × 10^6 cells/mL) were preincubated for 15 min at 30° with either buffer, 0.25 mM acivicin, 0.1 mM AOAA, or 1 mM allopurinol and then were incubated at 30° with 10 mM 6-PG in the presence of 0.1% (v/v) Triton X-100. At indicated times, aliquots were removed and processed for analysis of intracellular 6-MP by HPLC. Results are the means ± SEM of measurements from 8 separate experiments. *Statistical analyses:* Significantly different ($P < 0.05$) from 6-MP formation in cells incubated with buffer (= Control, ○) at the same time point: acivicin only at 10 and 20 min; acivicin and AOAA at 5, 30, and 60 min; allopurinol at no times.

TABLE 1. Time and concentration dependence of acute cytotoxicity of 6-MP, 6-ThXan, and 6-ThGua in isolated renal cortical cells

Incubation	LDH release (%) 1 hr	2 hr
I. 6-MP cytotoxicity (N = 6)		
Buffer	15.8 ± 3.8	22.1 ± 2.7
0.1 mM 6-MP	21.7 ± 3.7	32.7 ± 6.2
0.5 mM 6-MP	30.2 ± 9.1*	40.3 ± 4.8*
1 mM 6-MP	34.7 ± 3.6*	48.3 ± 7.5*
1 mM DCVC	50.7 ± 4.3*	69.2 ± 5.6*
II. 6-ThXan cytotoxicity (N = 5)		
Buffer	20.6 ± 1.4	26.3 ± 2.0
0.1 mM 6-ThXan	21.1 ± 3.1	26.4 ± 3.9
0.5 mM 6-ThXan	31.2 ± 5.5*	36.1 ± 3.5*
1 mM 6-ThXan	36.3 ± 6.6*	52.4 ± 4.7*
1 mM DCVC	44.3 ± 2.1*	63.4 ± 4.1*
III. 6-ThGua cytotoxicity (N = 4)		
Buffer	23.5 ± 6.5	28.7 ± 5.6
0.1 mM 6-ThGua	27.8 ± 7.6	33.3 ± 3.7
0.5 mM 6-ThGua	35.7 ± 9.6	46.2 ± 5.6*
1 mM 6-ThGua	41.1 ± 5.7*	48.4 ± 9.5*
1 mM DCVC	61.6 ± 2.2*	66.4 ± 2.1*

Isolated renal cortical cells (3–5 × 10^6 cells/mL) were incubated at 37° with either buffer, the indicated concentrations of 6-MP, 6-ThXan, or 6-ThGua, or with 1 mM DCVC as a positive control. At indicated times, aliquots were removed for determination of LDH release. The initial (i.e. Time-0) value for LDH release for cells incubated with buffer was 13.7 ± 3.4% (set I), 19.0 ± 2.0% (set II), and 20.5 ± 3.7% (set III). Results are the means ± SEM of measurements from 4 to 6 separate experiments.

* Significantly different ($P < 0.05$) from control incubations with buffer at the same time point.

Cytotoxicity of 6-MP and Analogues

A limiting factor in the utility of any chemotherapeutic agent is acute cytotoxicity in the target tissue. The presumed mechanism by which 6-MP could exert cytotoxicity is through metabolism by XO to 6-ThXan with concomitant generation of superoxide anion. To assess this possibility, isolated renal cells were incubated with 0.1, 0.5, or 1 mM 6-MP for up to 2 hr, and LDH release was measured (Table 1, set I). Additionally, LDH release due to 1 mM DCVC was determined as a positive control and for the purpose of comparison of cytotoxic potency. Cells incubated with 6-MP exhibited time- and concentration-dependent cytotoxicity; LDH release was modest, with maximal release of 48% as compared with 22% for control cells and 69% for cells incubated with 1 mM DCVC at 2 hr.

Cytotoxicity of 6-MP was explored further by studying the effect of 6-ThXan, the XO-derived metabolite of 6-MP, on LDH release (Table 1, set II). 6-ThXan is also a substrate for XO, which produces 6-thiourate and superoxide anion as the products. 6-ThXan caused significant increases in LDH release to an extent similar to that of 6-MP, with maximal LDH release with 1 mM 6-ThXan at 2 hr being 52% as compared with 26% for control cells and 63% for cells incubated with 1 mM DCVC.

6-ThGua, which is a more potent antitumor agent than either 6-MP or 6-ThXan [13], also produced a similar

TABLE 2. Protection of renal cortical cells from 6-MP cytotoxicity by allopurinol

	LDH release (%)	
Preincubation/Incubation	**1 hr**	**2 hr**
Buffer/Buffer	14.0 ± 1.7	23.0 ± 1.0
0.5 mM Allopurinol/Buffer	20.0 ± 1.2*	25.8 ± 0.9
1 mM Allopurinol/Buffer	22.5 ± 1.6*	28.0 ± 2.5
2 mM Allopurinol/Buffer	22.8 ± 2.1*	30.3 ± 1.7*
Buffer/1 mM 6-MP	40.5 ± 1.8*	49.0 ± 7.1*
0.5 mM Allopurinol/1 mM 6-MP	26.5 ± 1.0*,†	32.3 ± 0.5*,†
1 mM Allopurinol/1 mM 6-MP	29.0 ± 1.3*,†	33.5 ± 2.3*,†
2 mM Allopurinol/1 mM 6-MP	28.0 ± 2.7*,†	36.3 ± 4.7*

Isolated renal cortical cells (3–5 × 10^6 cells/mL) were preincubated for 15 min at 37° with either buffer or allopurinol. Preincubated cells were then incubated with either buffer or 1 mM 6-MP for 1 or 2 hr. At the indicated time, LDH release was measured.

* Significantly different ($P < 0.05$) from "Buffer/Buffer" at the same incubation time.

† Significantly different ($P < 0.05$) from "Buffer/1 mM 6-MP" at the same incubation time.

degree of modest cytotoxicity in isolated renal cells (Table 1, set III). Maximal LDH release with 1 mM 6-ThGua at 2 hr was 48% as compared with 29% for control cells and 66% for 1 mM DCVC.

Although no measurable activity of XO was observed (see above), renal cells were also preincubated with various concentrations of allopurinol, which inhibits XO, and then were incubated with 6-MP to assess further any possible role for XO in the conversion of 6-MP to a cytotoxic metabolite (Table 2). Allopurinol by itself exhibited small, but statistically significant increases in LDH release. Nonetheless, preincubation of renal cells with allopurinol significantly decreased LDH release due to 6-MP, with the exception of cells preincubated with 2 mM allopurinol and incubated with 6-MP for 2 hr. These results suggested that the metabolism of 6-MP by XO plays a role in 6-MP induced cytotoxicity.

DISCUSSION

6-PG and 6-PC are metabolized both *in vivo* and *in vitro* to 6-MP in the kidneys of rats, and administration of 6-PG or 6-PC leads to the selective renal accumulation of 6-MP [9–11]. These data suggested that 6-PG and 6-PC can function as prodrugs of the antitumor agent 6-MP. Additional studies showed that 6-CP, which also has antitumor activity, is metabolized *in vivo* to 6-PG and 6-MP, suggesting that metabolism of 6-CP through the GSH conjugation and β-lyase pathways may play a role in the mechanism of 6-CP-induced antitumor activity. These previous studies, however, did not directly study the mechanism of the tissue selectivity of 6-PG. The present work, therefore, followed up on these studies by examining the intracellular accumulation, metabolism, and cytotoxicity of 6-PG and 6-MP in suspensions of freshly isolated rat renal cortical cells, which are the target cells for 6-MP.

The use of 6-MP as an antitumor agent has been limited by its severe bone marrow and liver toxicity [22, 23]. Strategies to circumvent this problem have included the use of azathiopurine (1-methyl-4-nitro-5-imidazolyl thiopurine), a prodrug of 6-MP that is readily converted to 6-MP in red blood cells [24–26]. The effectiveness of azathiopurine, which is widely used as an immunosuppressant drug in kidney transplantation, however, is also limited by myelotoxicity [27]. Gruber *et al.* [6–8] developed a renal allograft model for infusion of 6-MP directly into the kidneys to produce local immunosuppression without systemic effects. The advantage of this procedure is that extrarenal toxicity of 6-MP is avoided. However, this is an invasive procedure that carries risk that also limits its utility. The approach assessed in this study, that of administration of a prodrug that is selectively accumulated in the kidneys where it is metabolized to 6-MP, has the advantage of being noninvasive, as it uses the characteristic biochemistry of renal tissue to generate intracellular 6-MP with a high renal/extrarenal ratio.

6-PG was accumulated by renal cells in a time- and concentration-dependent manner and this was inhibited markedly by KMB and was enhanced by acivicin. These results support the proposed pathway of 6-PG metabolism (Fig. 1), which involves successive metabolism by GGT, dipeptidases, and β-lyase. The decrease in intracellular accumulation of 6-PG due to KMB is consistent with the ability of KMB to stimulate β-lyase [21], which metabolizes 6-PC to 6-MP, whereas enhancement of accumulation of 6-PG by acivicin is consistent with inhibition of 6-PG conversion to the cysteinylglycine conjugate by GGT. 6-MP was similarly accumulated by renal cells in a time- and concentration-dependent manner, although the process was much slower than that for 6-PG. In contrast to the results for 6-PG, the estimated rate for net accumulation of 6-MP was less than 30% of that for 6-PG, and maximal intracellular accumulation of 6-MP was only 10% of that of 6-PG. The efficient intracellular accumulation of 6-PG by isolated renal cells is consistent with previous work showing rapid uptake of other GSH conjugates by a Na^+-dependent system on the renal basolateral membrane [3]. This transport process for renal basolateral uptake of GSH conjugates is a catalytic function of the Na^+-dependent system for GSH uptake [28], and is distinct from the multidrug resistance pump that is an ATPase and catalyzes the efflux of GSSG from many tissues. Although transport processes for nucleosides and nucleotides across renal plasma membranes have been described, these are generally relatively slow as compared with the transport processes described here.

While the ultimate goal of this study is to provide supporting data for the *in vivo* use of 6-PG as a renal-selective chemotherapeutic agent for treatment of renal tumors or suppression of renal transplant rejection, we used *in vitro* methods to assess 6-PG and 6-MP transport and 6-PG metabolism to 6-MP. Comparison of cellular uptake data with tissue accumulation data from *in vivo* studies in rats, shows that the amounts of 6-PG (up to 15 nmol

6-PG/10^6 cells = 10 nmol 6-PG/mg protein = approximately 2 nmol 6-PG/mg tissue with 1 mM 6-PG; cf. Fig. 2) or 6-MP (up to 1.5 nmol 6-MP/10^6 cells = 1.0 nmol 6-MP/mg protein with 1 mM 6-MP; cf. Fig. 3) accumulated by isolated renal cortical cells are within an order of magnitude or less of the amounts of these metabolites that are accumulated by renal tissue 30 min to 2 hr after *in vivo* administration of 6-CP [12] or 6-PC [11] (0.2 nmol 6-PG/mg kidney tissue and 0.1 nmol 6-MP/mg kidney tissue with administration of 1200 μmol 6-CP/kg, i.p.). In both previous *in vivo* studies, doses of 6-CP or 6-PC that were administered to rats were shown to be those that were known to have antitumor activity. Hence, the values obtained in this *in vitro* study are reasonable, particularly in light of the fact that in the previous *in vivo* studies, 6-CP or 6-PC was administered i.p. and one can administer 6-PG by the i.v. route, which should produce higher tissue levels of the desired chemical. Additionally, since the 6-PG would be administered directly to the kidneys, such as by direct injection into the renal arteries, we would not be limited by physiological constraints and distribution into extrarenal tissue.

Renal selectivity of 6-PG as a prodrug for 6-MP is based, in part, on the selective tissue localization of transport processes for uptake of GSH conjugates. Although the liver has the highest activities of GSH *S*-transferase, GSH conjugates are not taken up by hepatocytes [29]. Rather, cellular uptake systems for GSH and GSH conjugates are restricted to basolateral membranes of epithelial tissues, such as kidney, small intestine, and lung [29, 30]. Once the GSH conjugates are formed in the liver, therefore, they undergo rapid efflux into bile or plasma and are either degraded to cysteine conjugates or mercapturates in bile and small intestine or are taken up efficiently by the kidneys, either by glomerular filtration or transport across the basolateral membrane into renal epithelial cells [29]. By this interorgan pathway, GSH conjugates and their metabolites are selectively delivered to the kidneys for further metabolism or excretion as mercapturates.

To provide further support for the hypothesis that 6-PG can function as a prodrug for 6-MP, metabolism of 6-PG to 6-MP was quantified in isolated renal cells. 6-PG (10 mM) was converted rapidly to 6-MP at an estimated rate of 2.4 nmol/min per 10^6 cells. 6-MP formation was inhibited by both acivicin and AOAA, providing further support for the proposed biotransformation pathway. Consideration of the relative rates of cellular uptake of 6-PG and 6-MP and of the rate of intracellular metabolism of 6-PG to 6-MP suggests that administration of 6-PG is a much more efficient means of increasing renal content of 6-MP than is administration of 6-MP directly. Addition of KMB or other keto acids that can serve as cosubstrates for β-lyase should enhance the conversion of 6-PG to 6-MP, as suggested by the marked decrease in 6-PG accumulation due to KMB (cf. Fig. 2). Furthermore, 6-MP will not exhibit renal selectivity *in vivo* as has been shown for 6-PG [12]. Although administration of 6-PC bypasses the first two steps of 6-PG metabolism and it might be concluded that this would provide an additional advantage, 6-PC is less chemically stable than 6-PG [31], indicating that formation of 6-MP from 6-PC will be less efficient than that from 6-PG. The kidney/liver ratio for 6-MP derived from 6-PG is higher than that for 6-MP derived from 6-PC [9, 11, 12], providing additional basis for use of 6-PG, rather than 6-PC, as a prodrug for 6-MP.

Further intracellular metabolism of 6-MP may be mediated by XO, which converts 6-MP to 6-ThXan (Fig. 1). Although barely detectable levels of XO activity were measured in the isolated renal cells and the XO inhibitor allopurinol had minimal effects on the intracellular formation of 6-MP derived from 6-PG (Fig. 4), a role for XO in the metabolism and toxicity of 6-MP was suggested by the protection by allopurinol of renal cells from 6-MP-induced LDH release (Table 2). Additionally, 6-ThXan was cytotoxic to renal cells, producing a similar degree of LDH release as 6-MP. Unlike 6-MP, however, 6-ThXan has no antitumor activity. The similar cytotoxic potency of 6-MP and 6-ThXan suggests that it is not the 6-ThXan or some reactive metabolite derived from it that is directly responsible for the toxicity. If that were the case, then one would expect 6-ThXan to be more potent than 6-MP. Hence, the probable mechanism of 6-MP- and 6-ThXan-induced cytotoxicity involves the generation of superoxide anion during metabolism by XO. This mechanism is consistent with the protection from 6-MP-induced cytotoxicity by the XO inhibitor allopurinol. Cytotoxicity of the analogue 6-ThGua was also assessed, and it was found to produce a degree of LDH release similar to that of the other two compounds. 6-ThGua exhibits an EC_{50} for killing of tumor cells of 0.5 to 1 μM, whereas 6-MP exhibits an EC_{50} of 5 to 10 μM [13]. Because 6-ThGua exhibits markedly greater antitumor activity than either 6-MP or 6-ThXan, the similarly moderate amounts of LDH release produced by the three chemicals suggest that the mechanisms of cytotoxicity and antitumor activity are distinct.

A potential limitation in the use of 6-PG as a renal prodrug of 6-MP is the modest renal cellular toxicity of 6-MP. In general, low toxicity of 6-thiopurines is expected in non-proliferating cells such as mature, renal epithelial cells, and nephrotoxicity is not a common problem with the use of these compounds [22–27]. Nonetheless, modest cytotoxicity with 6-MP was observed in the present study (cf. Table 1). However, it should be noted that significant increases in LDH release only occurred with 6-MP concentrations of 0.5 mM and higher. It is likely that the amounts of intracellular 6-MP generated from 6-PG will not equal this exposure level, making acute cytotoxicity less of a problem. Regarding potential adjustments to measurements of 6-PG or 6-MP uptake or 6-MP formation from 6-PG due to loss of viable cells, this was not done in the present studies and should not be necessary for most cases. In general, most of the samples for measurement of transport and metabolism are taken at earlier time points than those at which significant LDH release occurs, so that at these

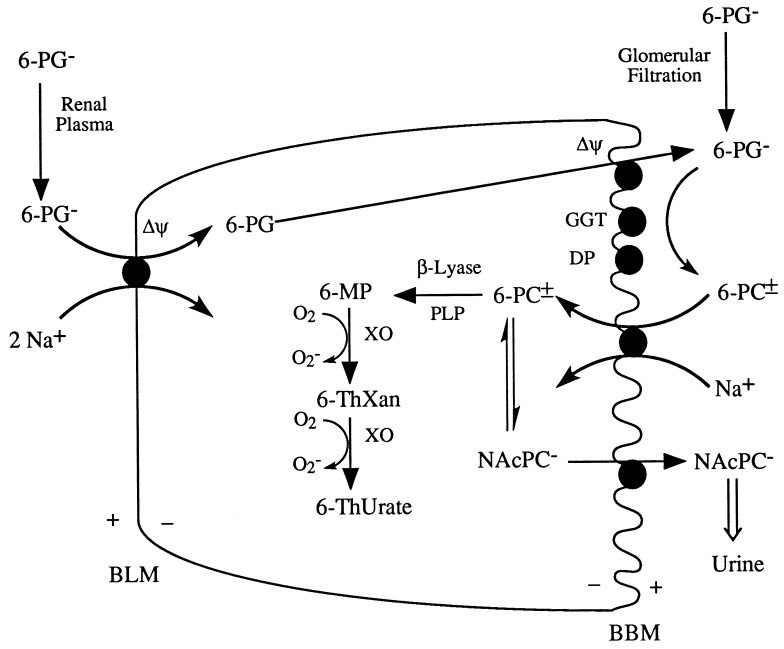

FIG. 5. Handling of 6-PG in renal proximal tubular cells. Abbreviations: BBM, brush-border plasma membrane; BLM, basolateral plasma membrane; 6-PG, *S*-(6-purinyl)glutathione; 6-PC, *S*-(6-purinyl)-L-cysteine; NAcPC, *N*-acetyl-*S*-(6-purinyl)-L-cysteine; GGT, γ-glutamyltransferase; DP, dipeptidase; β-lyase, cysteine conjugate β-lyase; PLP, pyridoxal phosphate; XO, xanthine oxidase; 6-ThXan, 6-thioxanthine; 6-ThUrate, 6-thiourate; and Δψ, membrane potential.

earlier time points, losses of cell viability are marginal. In terms of therapeutic applications, the *in vivo* equivalent of doses of 6-PG can be decreased significantly from those used in the present *in vitro* studies and should still provide therapeutic efficacy.

A schematic showing the localization of the key processes in the delivery of the prodrug 6-PG to the renal proximal tubule and subsequent generation of 6-MP is presented in Fig. 5. 6-PG, which is negatively charged at physiological pH, can either undergo glomerular filtration and enter the tubular lumen or it can enter the renal circulation. In the first case, lumenal 6-PG is rapidly degraded by GGT and dipeptidases on the proximal tubular brush-border membrane to 6-PC, which is transported into the renal cell by a Na^+-dependent transporter. Although transport of 6-PC was not measured in this or in previous studies, transport of other cysteine conjugates into renal cells has been documented [4, 5], and intracellular 6-MP has been recovered in kidneys of rats treated with 6-PC or 6-CP [9–12]. In the case of 6-CP, which is metabolized to 6-PG, and 6-PG itself, efflux of the GSH conjugate into the tubular lumen must occur to generate the cysteine conjugate, since the active site of GGT is extracellular [29]. Hence, transport of 6-PC back into the renal cell must occur for any further metabolism to occur. Intracellular 6-PC can then either undergo N-acetylation to the mercapturate, which can either be deacetylated or transported out into the lumen and excreted in the urine, or it can be metabolized by β-lyase to generate 6-MP. 6-MP can be a substrate for XO, thereby generating 6-ThXan and superoxide anion. In the second case, plasma 6-PG can be transported across the basolateral plasma membrane into the renal cell by a Na^+- and membrane potential-dependent transporter [3, 28, 29]. Then intracellular 6-PG is efficiently transported out of the cell into the lumen by a membrane potential-dependent transporter on the brush-border plasma membrane, where it is processed in the lumen as described above.

Other investigators recently have taken advantage of the tissue specificity of transport processes and the GSH conjugate and β-lyase pathways to design renal selective prodrugs. For example, Drieman *et al.* [32] showed that several *N*-acetyl-γ-glutamyl derivatives can function as renal prodrugs, and Andreadou *et al.* [33] synthesized several selenocysteine derivatives with aliphatic and benzylic selenium substitutions and showed that these compounds are excellent substrates for renal β-lyase. Taken together with the results reported in the present work, it appears that a large variety of chemicals that are precursors of therapeutic agents and that are either GSH or γ-glutamyl derivatives or are β-lyase substrates can function as renal selective prodrugs.

In conclusion, we have demonstrated that 6-PG is rapidly transported into and accumulated by rat renal cortical cells and undergoes successive metabolic conversions to produce the antitumor agent 6-MP. Although acute cytotoxicity may occur and could limit the therapeutic usefulness of these agents, cytotoxicity was modest as compared with that of a well-characterized nephrotoxicant, DCVC. Under the incubation conditions of renal cortical cells with 6-PG used in this study, intracellular 6-MP concentrations in the range of the ED_{50} were readily achieved, providing further support for the effectiveness of 6-PG as a renal selective prodrug of 6-MP. Use of 6-PG as a renal prodrug for 6-MP has the advantage of being a non-invasive method to direct a therapeutic agent to its target tissue. Administration of 6-PG may be useful for treatment of renal tumors or for suppression of renal transplant rejection. Since many tumor cells have high GGT activity and have β-lyase activity [13], 6-PG may also

be an effective prodrug for tumors derived from other tissues besides the kidneys.

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