9-Norbornyl-6-chloropurine Is a Novel Antileukemic Compound Interacting with Cellular GSH

PAVLA PLAČKOVÁ, NELA ROZUMOVÁ, HUBERT HŘEBABECKÝ, MICHAL ŠÁLA, RADIM NENCKA, TOMÁŠ ELBERT, ALEXANDRA DVOŘÁKOVÁ, IVAN VOTRUBA and HELENA MERTLÍKOVÁ-KAISEROVÁ

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Abstract. *Aim: 6-Chloropurines substituted at position 9 with bicyclic skeletons represent promising chemotherapeutic agents. We explored the metabolism and membrane transport of 9-norbornyl-6-chloropurine (NCP) aiming to understand its mechanism of action. Materials and Methods: The metabolism of NCP was studied in vitro in whole cells (CCRF-CEM), cellular extracts, subcellular fractions and purified enzymes. Transport experiments were conducted in Caco-2 cell monolayers. Results: Three metabolites were identified, a glutathione conjugate (NCP-GS), NCP-cysteinylglycine and NCP-cysteine. Both glutathione-S-transferase inhibition and glutathione (GSH) depletion prevented metabolite formation and increased the cytotoxicity of NCP. Transepithelial transport (Caco-2) indicated good permeability, with* P_{app} *(12.6±0.3)* $\times 10^{-5}$ *cm/s. Importantly, the drug induced glutathione depletion in treated cells and affected the activity of several GSH-dependent enzymes. Conclusion: The novel nucleoside analog NCP represents a promising orally available antileukemic agent, acting through lowering of GSH levels in tumor cells.*

6-Chloropurines substituted at position 9 with variously modified bicyclic skeletons represent analogs of carbocyclic nucleosides, where the bicyclic part mimics the sugar moiety. Many of these compounds, previously synthesized in our laboratories, have been reported as efficient antivirals against Coxsackieviruses (*Picornaviridae*) (1, 2). These (+)ssRNA viruses cause a wide spectrum of diseases (3) while there is a lack of approved antipicornaviral treatment (4). Importantly, these compounds also revealed considerable cytotoxicity to CCRF-CEM and HL-60 cell lines and could be considered as candidates for the development of new antileukemic drugs. 9-[(1*R**,2*R**,4*S**)-Bicyclo[2.2.1]hept-2-yl]-6-chloro-9*H*-purine (9-norbornyl-6-chloropurine, NCP; Figure 1) represents the simplest structure from the series, possessing both antiviral and cytostatic activity. The mechanism of action of NCP is completely unknown, as is its cellular uptake and metabolism.

Previously published metabolic studies with 6-chloropurine base (6-CP) demonstrated the oxidation of 6-CP by xanthine oxidase, however, large quantities of the enzyme were used (5). Hwang and Elfarra (6) later presented hepatic and renal glutathione-*S*-transferase (GST)-dependent transformation of 6-CP to *S*-(purinyl)glutathione and its further metabolites, 6-mercaptopurine, 6-methylmercaptopurine and 6-thiouric acid. On the other hand, norbornane is known to be a substrate for cytochrome P450-mediated hydroxylation to produce *exo*- and *endo*-2-norborneol (7). The question remains whether any of the metabolic routes described for 6-chloropurine or norbornane also apply to NCP.

The aim of this study was to investigate the metabolism and membrane permeability of the novel (pseudo)nucleoside analog NCP to extend our understanding of the usefulness and limitations of the therapeutic use of the compound. Most experiments were conducted using the CCRF-CEM cell line, as these leukemia cells represent one potential target tissue. Since the metabolic capacity of immortalized cell lines is limited, subcellular fractions from rat liver and purified enzymes were also employed to validate our findings.

Materials and Methods

Materials. NCP, the glutathione conjugate of NCP, NCP-cysteinylglycine and NCP-cysteine were synthesized as described previously (8, 9). 9-[(1*R**,2*R**,4*S**)-Bicyclo[2.2.1][5'$_{exo}$,6'$_{exo}$-$^{3}H_2$]hept-2-yl]-6-chloro-9*H*-purine ([^{3}H]NCP) with specific activity 47.5 Ci/mmol was prepared by the catalytic tritiation of the corresponding 5',6'-unsaturated precursor at the Laboratory of

Correspondence to: Helena Mertlíková-Kaiserová, Institute of Organic Chemistry and Biochemistry Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic. Tel: +420 220183114, Fax: +420 220183560, e-mail: kaiserova@uochb.cas.cz

Key Words: Substituted 6-chloropurines, carbocyclic nucleoside analogs, glutathione-*S*-transferase, glutathione depletion.

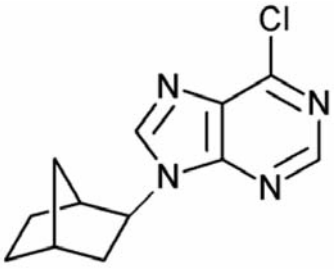

Figure 1. *Structure of 9-[(1R*,2R*,4S*)-bicyclo[2.2.1]hept-2-yl]-6-chloro-9H-purine (9-norbornyl-6-chloropurine, NCP).*

Radioisotopes (IOCB, Czech Republic). Its chemical identity was confirmed by ^{3}H and ^{1}H Nuclear magnetic resonance (NMR); specific activity was assayed by a combination of radio-high-performance liquid chromatography and liquid scintillation counting. Allopurinol, ethacrynic acid (GST inhibitors), 1-aminobezotriazole, buthionine sulfoximine (BSO), sulfosalicylic acid, streptomycin, penicillin G, phosphate buffered saline (PBS), RPMI-1640 medium and Minimum Essential Medium Eagle's (Eagle's MEM) were purchased from Sigma–Aldrich (St. Louis, MO, USA); fetal calf serum was obtained from PAA Laboratories GmbH (Pasching, Austria). Subcellular fractions from rat liver homogenate were isolated using previously published procedures (10). Soluene 350® was from PerkinElmer (Waltham, MA, USA), salts for buffer preparations and NADPH were from Serva (Heidelberg, Germany). HUVEC-2 cells, Endothelial Cell Culture Medium, BD BioCoat™ HTS Caco-2 Assay System, Seeding Basal Medium™ and Enterocyte Differentiation MediumTM were from BD Biosciences (Heidelberg, Germany). All other cell lines and Fibroblast Basal Medium were from LGC standard (Teddington, UK).

Cell culture. CCRF-CEM, HL-60, HeLa-S3, HepG2, HUVEC-2, NHDF-Ad and Caco-2 cells were cultured under a humidified atmosphere containing 5% CO_2 at 37˚C. CCRF-CEM, HL-60, HeLa-S3 and HepG2 cells were grown in RPMI-1640 or Eagle's MEM supplemented with 10% or 20% (Caco-2) fetal calf serum, 200 mg/ml of streptomycin, 200 U/ml of penicillin G and 4 mM glutamine. HUVEC-2 cells were grown in Endothelial Cell Culture Medium. NHDF-Ad cells were grown in Fibroblast Basal Medium. 1% Non-essential amino acid solution, 1 mM sodium pyruvate and 1.5 g/l $NaHCO_3$ was added to the medium for Caco-2 cells.

Preparation of crude cellular extract. Cells (1.5×10^{9}) were washed with PBS and suspended in 50 mM Tris–HCl buffer, pH 7.4 containing 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, protease inhibitor cocktail (Sigma–Aldrich) and 0.4% NP-40. The cells were sonicated for 5 s three-times and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 30,000 ×*g* for 30 min. The supernatant was incubated with streptomycin sulfate for 1 h to remove nucleic acids and centrifuged at 30,000 ×*g* for 30 min. The resulting crude cell extract was desalted on PD-10 columns (GE Healthcare, Freiburg, Germany), aliquoted and stored at –80˚C. The protein content was determined by the BCA kit (Sigma–Aldrich), according to the manufacturer's instructions.

Non-radioactive in vitro metabolic assays and HPLC analysis of the metabolites. NCP (100 μM) was incubated with crude CCRF-CEM cell extract (5.4 mg of protein per ml) with/without the inhibitors of xanthine oxidase, GST and cytochrome *P450* (0.1 and 1 mM allopurinol, 0.05 and 1 mM ethacrynic acid and 0.1 and 1 mM 1-aminobenzotriazole, respectively) in 0.05 mM phosphate buffer, pH 7.4, for 30 min at 37˚C. For the study of the inhibition of cytochrome *P450*, NADPH (1 mM) was included in the reaction. The reactions were stopped and extracted with 100% methanol and chilled at –20˚C for 1 h. Then the samples were centrifuged at 8,000 ×*g* for 2 min in centrifugal filter units (Ultrafree-MC dura 0.22 μm, Millipore, Billerica, Massachusetts, USA). The filtrates (50 μl) were injected onto a Supelcosil LC-18-S HPLC column (150 mm × 4.6 mm I. D., 5 μm; Sigma Aldrich) and eluted in a non-linear gradient of 10-30% acetonitrile in 50 mM phosphate buffer. The flow rate was 0.9 ml/min. The absorption spectra of the eluate were recorded by a Photodiode array detector (PDA; Waters Corporation, Milford Massachusetts, USA). The data were analyzed at 260 nm.

[^{3}H]NCP metabolism whole CCRF-CEM cells and in vitro. CCRF-CEM cells (5×10^{5} cells/ml) were preincubated with or without 50 μM BSO in a CO_2-incubator at 37˚C. After 24 h, [^{3}H]NCP was added to a final concentration of 25 μM (10 μCi/ml). After another 24 h of incubation, the cells were washed with PBS and pelleted by centrifugation at 250 ×*g* for 5 min. The pellets were extracted with 100 μl of 70% methanol and chilled at –20˚C for 1 h. The equivalents of RPMI-1640 medium (200 μl) were extracted with 583 μl of 100% methanol and chilled at –20˚C for 1 h. Alternatively, crude CCRF-CEM cell extract (5.4 mg of protein per ml) or rat liver subcellular fractions (protein content 4.6-5.4 mg/ml) were incubated with 25 μM (1 μCi/ml) NCP in 0.05 mM phosphate buffer, pH 7.4 for 30 min at 37˚C. Where indicated, 1 mM ethacrynic acid was added. The reactions were extracted with 100% methanol and chilled as described above. The HPLC analysis was carried out as described in the previous section. Fractions of 0.23-ml of the HPLC-eluate were collected and the radioactivity was counted in Aquasafe® scintillation cocktail (Zinsser Analytic, Maidenhead, UK). Radioactivity (CPM) was plotted against retention time to obtain a high-sensitivity [^{3}H]NCP metabolite profile.

Glutathione (GSH) determination. Cells preserved in sulfosalicylic acid were thawed, sonicated for 5 min on ice, centrifuged for 5 min at 5,000 ×*g* and the supernatants were used for the assay. Total GSH content was determined using the enzymatic recycling method with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and GSH reductase in a microplate format. The rate of TNB formation was recorded at 405 nm for 2 min and the slope was compared to that of the standard curve of GSH disulfide (GSSG). The concentration of total GSH was calculated using the method of linear regression. The results were then protein-correlated and expressed as relative values, taking the total GSH in control sample as 100%.

Cytotoxicity evaluation. The cytotoxicity of the tested compounds was assessed with the XTT sodium salt cell proliferation kit II (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. CCRF-CEM, HL-60, HeLa-S3, HepG2, HUVEC-2 and NHDF-Ad cells were seeded in a 96-well plate at a density of 13,500, 18,000, 2,700, 19,800, 4,000 and 4,000 cells per well, respectively. After 24 h, the tested compounds were added to the culture media and incubated for 72 h before the XTT dye was

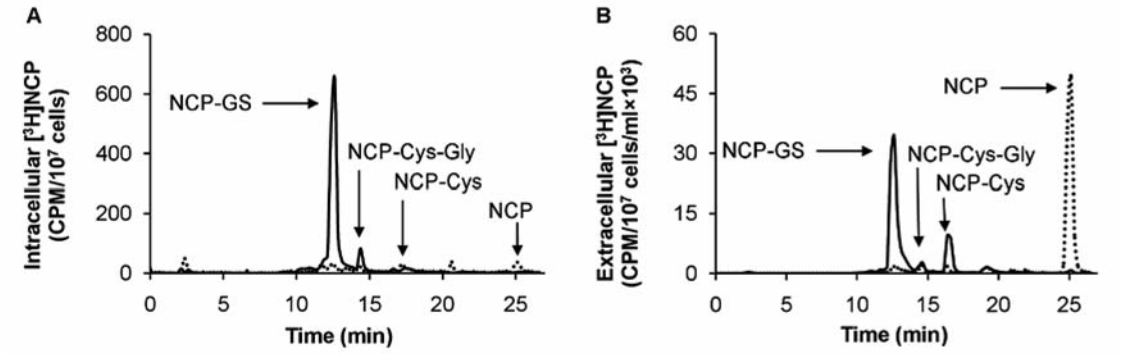

Figure 2. *High-performance liquid chromatography analysis of intra- and extracellular [^{3}H]9-norbornyl-6-chloropurine [(^{3}H]NCP)] metabolites. CCRF-CEM cells were incubated with 25 μM (10 μCi/ml) [^{3}H]NCP (–) for 24 h. In a parallel experiment, CCRF-CEM cells were preincubated with 50 μM buthionine sulfoximine for 24 h and after that 25 μM (10 μCi/ml) [^{3}H]NCP was added (····). The identity of all peaks in the cells (A) and the medium (B) was confirmed using the authentic standards. NCP-GS, Glutathione conjugate; NCP-Cys-Gly, NCP-cysteinylglycine; NCP-Cys, NCP-cysteine.*

Table I. *The effect of in vitro inhibition of xanthine oxidase (allopurinol), glutathione-S-transferase (ethacrynic acid) and cytochrome P450 (aminobenzotriazole) on 9-norbornyl-6-chloropurine (NCP) metabolism in CCRF-CEM cell extract.*

Inhibitor (mM)	Allopurinol		Ethacrynic acid		Aminobenzotriazole	
	0.1	1	0.05	1	0.1	1
NCP conversion† (%)	100±0	100±0	43±2	7±2	100±0	100±0

†Rate of disappearance of NCP from the reaction mixture, 30-min incubation, 100 μM NCP. Data are means from six independent experiments±SD.

added. The absorbance at 495 nm was recorded after a 1-h incubation with the dye. Half-maximal inhibitory concentration (IC_{50}) values were determined by GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA). Where indicated, cells were preincubated with 50 μM BSO for 24 h prior to the addition of NCP.

Transepithelial transport of NCP in Caco-2 monolayers. The transport of NCP across Caco-2 monolayers was studied using the BD BioCoat™ HTS Caco-2 Assay System according to the manufacturer's instructions. Immediately prior to the transport experiments, the monolayers were washed twice with HBSS containing 10 mM HEPES and 25 mM glucose, pH 7.4 and incubated for 30 min in a CO_2-incubator at 37˚C. A total of 2×10^5 cells in Seeding Basal Medium™ were seeded in each transwell insert, left to attach for 48 h and the medium was then changed for Enterocyte Differentiation Medium™ and cultured for the next 48 h. The integrity of cell monolayers was assessed by monitoring the transepithelial electrical resistance (TEER) using a Millicel ERS apparatus (Millipore) and paracellular flux marker, [^{14}C]mannitol (0.15 μCi/insert). Intact cell monolayers displaying TEER higher than 250 Ω cm^2 were used for the experiments. The transport was initiated by the addition of 1 ml of the transport medium to the basolateral side and 300 μl of the transport medium containing 10 μM [^{3}H]NCP (0.15 μCi), to the apical side. After a 3-h incubation, 80 μl aliquots were collected from the donor and acceptor sides. The radioactivity was counted in Aquasafe® scintillation cocktail. The apparent permeability coefficient (P_{app}) was calculated from the following formula: $P_{app}=(dQ/dt)/C_0\times A$ where dQ/dt is the rate of absorption of the drug across the cells, C_0 is the donor compartment concentration at time zero and A is the area of the monolayer.

Data analysis and statistical procedures. Unless otherwise indicated, the data are presented as the mean±SD from at least three independent experiments. Statistical evaluation was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software).

Results

NCP undergoes GST-mediated glutathione conjugation. To investigate metabolic pathways of NCP, we first performed HPLC analysis of *in vitro* incubation mixtures containing various enzyme inhibitors, crude cellular extract (CCRF-CEM) and NCP as a substrate (Table I). The concentration of NCP used in these pilot experiments (100 μM) was chosen with respect to the detection limit of the HPLC-PDA. The data presented in Table I point to GST as the key enzyme metabolizing NCP. Accordingly, in the whole cells the GSH conjugate (NCP-GS) was identified as the major metabolite of NCP (Rt=12.6 min) followed by NCP-cysteinylglycine (NCP-Cys-Gly, Rt=14.6 min) and NCP-cysteine (NCP-Cys, Rt=16.4 min) as minor products. The metabolism of NCP was

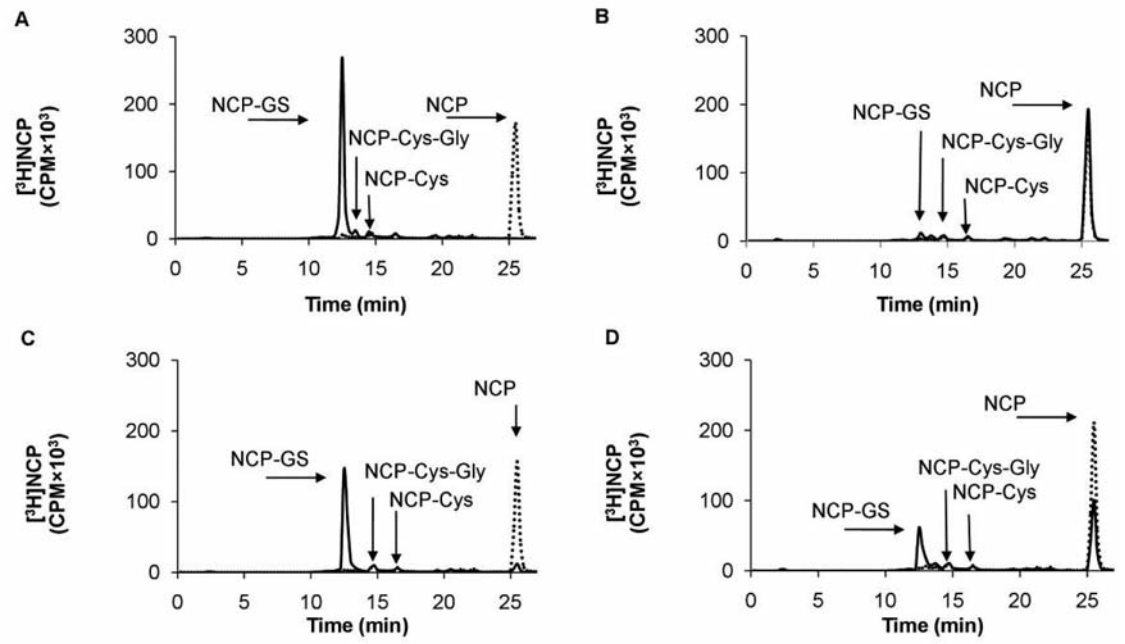

Figure 3. *High-performance liquid chromatography analysis of in vitro [^{3}H]9-norbornyl-6-chloropurine ([^{3}H]NCP) metabolites. CCRF-CEM cell extract (A), rat microsomes (B), cytosol (C) and mitochondria (D) were incubated with 25 μM (1 μCi/ml) [^{3}H]NCP (–) for 30 min. In a parallel experiment, the 1 mM ethacrynic acid (glutathione-S-transferase inhibitor) was added (····). The identity of all peaks was confirmed using the authentic standards. NCP-GS, Glutathione conjugate; NCP-Cys-Gly, NCP-cysteinylglycine; NCP-Cys, NCP-cysteine.*

prevented in BSO-treated, *i.e.* GSH-depleted cells (Figure 2). The identity of all reaction products was verified using the authentic standards. Importantly, the NCP metabolites were detected not only in the cells, but also in the medium. The role of non-enzymatic NCP-GS formation by direct reaction with cellular GSH was found to be negligible.

To specify subcellular localization of NCP-metabolizing GST(s), [^{3}H]NCP conversion in rat liver cytosol, mitochondria and microsomes was also studied and compared to the rate of [^{3}H]NCP conversion using the CCRF-CEM cell extract (Figure 3). Out of all enzyme preparations tested, the CCRF-CEM extract and liver cytosolic GST were the most efficient in catalyzing NCP conversion to NCP-GS. The reaction catalyzed by GST from mitochondria was also robust. No additional metabolic products were identified in any subcellular fraction tested, confirming the validity of the data obtained from CCRF-CEM cells.

NCP depletes tumor cells of GSH. With respect to the excessive GST-mediated biotransformation of NCP, it is of interest what the consequences may be on cellular GSH levels. We found that NCP-treated CCRF-CEM cells indeed exhibited a concentration-dependent drop in the total cellular GSH (3.7-fold decrease *vs.* untreated control at 20 μM NCP (Figure 4). GSH/GSSG ratios remained unchanged due to very low concentrations of GSSG in all samples (data not shown). This suggests that NCP-treated cells were not exposed to significant oxidative stress.

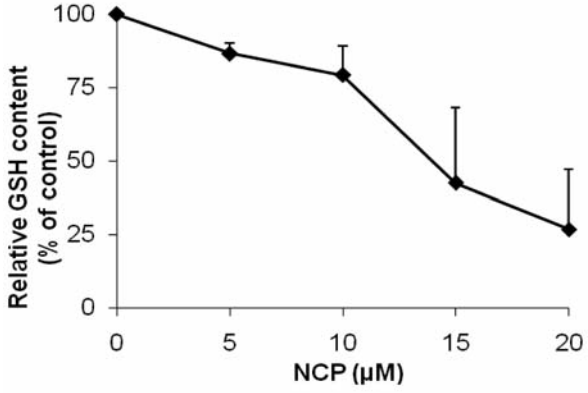

Figure 4. *Effect of 9-norbornyl-6-chloropurine (NCP) on total glutathione (GSH) level in CCRF-CEM cells after 72 h incubation. *p<0.05 vs. untreated cells (ANOVA with Dunnett's post-hoc test).*

NCP readily crosses the intestinal epithelial barrier. The monolayers of differentiated Caco-2 epithelial cells were used to simulate NCP absorption in the intestine. The apparent permeability coefficient (P_{app}) of NCP was

Table II. *The cytotoxicity of 9-norbornyl-6-chloropurine (NCP) in cancer and normal cells and the effect of glutathione depletion on NCP cytotoxicity towards CCRF-CEM cells.*

Cell line	CCRF-CEM		HL-60	HeLa	HepG2	HUVEC-2	NHDF-Ad
	w/o BSO	with BSO					
IC_{50} (μM)	18±2	7±2	18±1	124±5	176±6	476±18	≥500

IC_{50}, Concentration of NCP causing 50% decrease in cells viability using XTT cytotoxicity test (calculated using the non-linear regression method by GraphPad Prism). BSO, L-buthionine sulfoximine concentration used was shown not to cause any intrinsic toxicity to the cells (IC_{50} value for CCRF-CEM=250±19 μM). Data are means from six independent experiments±SD.

$12.6\pm0.3\times10^{-5}$ cm/s which corresponded to 42±1% of the drug permeated from apical to basolateral compartment after the 3 h incubation with NCP. These data indicate a very good transepithelial permeability of NCP. The P_{app} of the paracellular flux marker, [^{14}C]mannitol, was $21.2\pm0.4\times10^{-7}$ cm/s indicating good monolayer integrity and standard conditions of the permeation experiments.

Differential cytotoxicity of NCP to tumor vs. normal cells. The selectivity of the cytotoxic effects of NCP was tested in human tumor and normal cells. Two leukemia cell lines (CCRF-CEM, HL-60), two solid tumor-derived cell lines, (HeLa-S3, HepG2) and two normal cell lines (HUVEC-2, NHDF-Ad) were employed. NCP was found to be particularly cytotoxic to leukemia cells with much lower effects on carcinoma cell lines and negligible toxicity towards normal cells (Table II).

Conjugation with GSH is generally a detoxifying step. It was, therefore, of interest whether the impairment of GST-mediated NCP metabolism could potentiate NCP cytotoxicity. Indeed, under BSO-induced GSH depletion, NCP exerted enhanced cytotoxicity towards CCRF-CEM cells (Table II).

Discussion

NCP represents a class of original pseudonucleoside compounds inspired by carbocyclic nucleosides. Apart from its well-demonstrated antiviral effects, preliminary studies in our laboratory also suggested its antitumor potential. This study confirms that it may be considered a novel antileukemic agent with significantly reduced toxicity against solid tumors and very low toxicity against normal cells. To our knowledge, the metabolic fate of NCP has not been reported.

In the present study, NCP was shown to be a substrate for GST to form glutathione conjugate (NCP-GS) as a major metabolite. Its degradation products, NCP-cysteinylglycine and NCP-cysteine, were also detected, albeit in much lower amounts. NCP metabolism by GST is therefore identical to that of 6-chloropurine base (6). On the other hand, another metabolic pathway previously described for 6-CP, oxidation by xanthine oxidase (5), was not found in the case of NCP. No signs of norbornane hydroxylation, oxidative dehalogenation or cleavage of the C-N bond between norbornane and the purine ring were observed. Taken together, conjugation with GSH (and the subsequent reactions) is the sole metabolic pathway of NCP detected in CCRF-CEM cells. These results were consistent in both CCRF-CEM cells (target cells) and subcellular fractions from rat liver, routinely used as a standard model system to study drug metabolism. No additional metabolic products were identified in any subcellular fraction, not even after the inhibition of the main metabolic pathway.

Due to its increased polarity, NCP-GS is readily excreted from the cells. Based on the fact that NCP was more cytotoxic when GST was inhibited, its conjugation can be considered a detoxifying step. The observed binding of NCP to cytosolic proteins (unpublished data) could result from its general affinity to protein thiols and may selectively affect certain target structures (*e.g.* enzymes and receptors) determining the mechanism of antileukemic or antiviral activity of NCP. However, the specificity/selectivity for certain proteins over others is still hypothetical and will be the subject of further research. Since NCP clearly depletes the cells of GSH, which is frequently found in elevated amounts in cancerous cells (11), we propose that GSH depletion may represent an important mechanism of the antileukemic action of NCP. It can also sensitize the cells to the action of other chemotherapeutics and the immune system (12).

Adequate transepithelial permeability is a necessary prerequisite for possible oral administration of the drug. The Caco-2 cell transwell assay represents one of the most reliable *in vitro* models of drug absorption in the intestine (13). Completely absorbed drugs display high permeability coefficients ($P_{app}\geq1\times10^{-6}$ cm/s) whereas P_{app} values $\leq1\times10^{-7}$ cm/s in the Caco-2 monolayers indicate poor absorption (14). As NCP was found to be highly permeable in this assay, good bioavailability of the compound is predicted.

In summary, this study identified GSH conjugation as the sole metabolic pathway of NCP, leading to the formation of less cytotoxic metabolites. As a consequence, NCP induces significant reduction in a major cellular reductant – GSH. This likely presents the underlying mechanism of action of NCP in terms of its anticancer activity. Transepithelial transport of NCP is fast and suggests good bioavailability of this novel potential drug.

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic [grant #P303/11/1297] and the Research Project of the IOCB #RVO:61388963.

References

1 Šála M, De Palma AM, Hřebabecký H, Dejmek M, Dračínský M, Leyssen P, Neyts J, Mertlíková-Kaiserová H and Nencka R: SAR studies of 9-norbornylpurines as Coxsackievirus B3 inhibitors. Bioorg Med Chem Lett *21*: 4271-4275, 2011.

2 Šála M, Hřebabecký H, Leyssen P, Dejmek M, Dračínský M, De Palma AM, Neyts J and Nencka R: Novel substituted 9-norbornylpurines and their activities against RNA viruses. Bioorg Med Chem Lett *22*: 1963-1968, 2012.

3 Verma NA, Zheng XT, Harris MU, Cadichon SB, Melin-Aldana H, Khetsuriani N, Oberste MS and Shulman ST: Outbreak of life-threatening coxsackievirus B1 myocarditis in neonates. Clin Infec Dis *49*: 759-763, 2009.

4 DeLong DC and Reed SE: Inhibition of rhinovirus replication in organ culture by a potential antiviral drug. J Infect Dis *141*: 87-91, 1980.

5 Duggan DE and Titus E: 6-Chloropurine and 6-chlorouric acid as substrates and inhibitors of purine-oxidizing enzymes. J Biol Chem *234*: 2100-2104, 1959.

6 Hwang YI and Elfarra AA: Detection and mechanisms of formation of *S*-(6-purinyl)glutathione and 6-mercaptopurine in rats given 6-chloropurine. J Pharm Exp Ther *264*: 41-46, 1993.

7 Groves JT and McClusky GA: Aliphatic hydroxylation by highly purified liver microsomal cytochrome-P450. Evidence for a carbon radical intermediate. Biochem Biophys Res Commun *81*: 154-160, 1978.

8 Šála, M, De Palma AM, Hřebabecký H, Nencka R, Dračínský M, Leyssen P, Neyts J and Holý A: Design, synthesis, and biological evaluation of novel coxsackievirus B3 inhibitors. Bioorg Med Chem *18*: 4374-4384, 2010.

9 Okamura T, Kikuchi T, Fukushi K, Arano Y and Irie T: A novel noninvasive method for assessing glutathione-conjugate efflux systems in the brain. Bioorg Med Chem *15*: 3127-3133, 2007.

10 Procházková E, Jansa P, Dračínský M, Holý A and Mertlíková-Kaiserová H: Determination of the antioxidative activity of substituted 5-aminopyrimidines. Free Radic Res *46*: 61-67, 2012.

11 Singh S, Khan AR and Gupta AK: Role of glutathione in cancer pathophysiology and therapeutic interventions. J Exp Ther Oncol *9*: 303-316, 2012.

12 Morales MC, Pérez-Yarza G, Nieto-Rementeria N, Boyano MD, Jangi M, Atencia R and Asumendi A: Intracellular glutathione levels determine cell sensitivity to apoptosis induced by the antineoplasic agent *N*-(4-hydroxyphenyl) retinamide. Anticancer Res *25*: 1945-1951, 2005.

13 Press B and Di Grandi D: Permeability for intestinal absorption: Caco-2 assay and related issues. Curr Drug Metab *9*: 893-900, 2008.

14 Artursson P, Palm K and Luthman K: Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev *46*: 27-43, 2001.

Received May 24, 2013
Revised June 14, 2013
Accepted June 17, 2013