Transport mechanisms of a novel antileukemic and antiviral compound 9-norbornyl-6-chloropurine

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Abstract

6-Chloropurines substituted at the position 9 with variously modified bicyclic skeletons represent promising antiviral and anticancer agents. This work aimed to investigate the transport mechanisms of 9-[(1R,2R,4S*)-bicyclo[2.2.1]hept-2-yl]-6-chloro-9H-purine (9-norbornyl-6-chloropurine, NCP) and their relationship to the metabolism and biological activity of the compound. Transport experiments were conducted in CCRF-CEM cells using radiolabeled compound ([3H]NCP). The pattern of the intracellular uptake of [3H]NCP in CCRF-CEM cells pointed to a combination of passive and facilitated diffusion as prevailing transport mechanisms. NCP intracellular metabolism was found to enhance its uptake by modifying NCP concentration gradient. The transport kinetics reached steady state under the conditions of MRP and MDR proteins blockade, indicating that NCP is a substrate for these efflux pumps. Their inhibition also increased the cytotoxicity of NCP. Our findings suggest that the novel nucleoside analog NCP has potential to become a new orally available antileukemic agent due to its rapid membrane permeation.

Introduction

9-Norbornyl-6-chloropurines represent a novel class of antiviral and cytotoxic compounds. 9-[(1R,2R,4S*)-bicyclo[2.2.1]hept-2-yl]-6-chloro-9H-purine (9-norbornyl-6-chloropurine, NCP, Figure 1) represents the prototype structure from a large compound library synthesized at our laboratories. Interestingly, NCP exhibits selective antiviral activity against Coxsackieviruses, causative agents of certain life-threatening conditions (e.g. myocarditis) while safe and efficient antiviral chemotherapy is still lacking. In addition, 9-norbornyl-6-chloropurines were found to possess significant cytostatic activity, particularly toward human leukemia cell lines. We have recently shown that intracellularly, it is rapidly metabolized by glutathione-S-transferase (GST) into the glutathione (GSH) conjugate (NCP-GS). Since the reaction consumes large amounts of cellular GSH, GSH depletion was suggested to play role in its mechanisms of action. Also, NCP was shown to be highly permeable in Caco-2 transepithelial assay indicating a good bioavailability. However, the mode of its intracellular transport (in terms of both uptake and efflux) remains unexplored.

Due to certain conformational resemblance of NCP to natural nucleosides, where bicyclic scaffold mimics sugar moiety, its transport into the cells via nucleoside transporters (NTs) may be expected as it is frequently observed with pharmacologically active nucleoside analogs. NTs belong to solute carrier families 28 and 29 (SLC28 and SLC29), which encode concentrative nucleoside transporters (CNT) and equilibrative nucleoside transporter proteins (ENTs), respectively. The high-affinity translocation of natural nucleosides is mediated by CNTs via the transmembrane sodium gradient, whereas facilitative nucleoside influx and efflux is mediated by ENTs. On the other hand, NCP is considerably more lipophilic compared to physiological nucleosides or traditional synthetic nucleoside analogs, which may favour other transport mechanism such as passive diffusion. Fluid-phase diffusion and receptor-mediated endocytosis have also been reported for 9-(2-phosphonomethoxyethyl)adenine (PMEA) in CCRF-CEM cells and HeLa cells, respectively.

The aim of this study was to investigate the mode of intracellular uptake of the novel pseudonucleoside analog NCP to extend our understanding of the usefulness and the limitations of the therapeutic use of the compound. Attention was paid to the interactions with ABC-class transporters such as P-gp or MRP as well as to the interplay between the drug uptake and its intracellular metabolism. Experiments were conducted in CCRF-CEM cell line, as these leukemia cells represent one of the potential target tissues.

Materials and methods

Materials

NCP and the glutathione conjugate of NCP were synthesized as described previously (IOCB, Czech Republic). 9-[(1R,2R,4S*)-bicyclo[2.2.1]hept-2-yl]-6-chloro-9H-purine ([3H]NCP) with specific activity...
47.5 Ci/mmol was prepared by the catalytic tritiation of the corresponding \( \text{\textsuperscript{5}}\text{\textsuperscript{6}}\)-unsaturated precursor at the Laboratory of Radioisotopes (IJC, Czech Republic). Its chemical identity was confirmed by \( ^3\text{H} \) and \( ^1\text{H} \) nuclear magnetic resonance, specific activity was assayed by a combination of radio-high-performance liquid chromatography and liquid scintillation counting. Ethacrynic acid, dipiridamole, buthionine sulfoximine, oligomycin A, antimycin A, N-methyl-D-glucamine, cytochalasin B, cytochalasin D, mineral oil, silicone oil DC 702, streptomycin, penicillin G, PBS, RPMI-1640 media, (deoxy)nucleotides, and glucose were purchased from Sigma-Aldrich (St. Louis, MO), fetal calf serum was obtained from PAA Laboratories GmbH (Pasching, Austria). Dynasore was from Merck Millipore (Darmstadt, Germany). Soluene 350\(^{\text{TM}}\) was provided by Perkin-Elmer (Waltham, MA), salts for buffer preparations were from Serva (Heidelberg, Germany). LY335979 and MK571 (MDR and MRP inhibitors, respectively) were kindly provided by Gilead Sciences (Foster City, CA). Human CCRF-CEM cells were from LGC standard (Teddington, UK).

**Cell culture**

CCRF-CEM cells were cultured under a humidified atmosphere containing 5% \( \text{CO}_2 \) at 37°C. Cultures were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 200 \( \mu \text{g/ml} \) of streptomycin, 200 \( \mu \text{g/ml} \) of penicillin G and 4 \( \mu \text{M} \) glutamine. Cells in the exponential phase of growth were used for the experiments.

**Cytotoxicity evaluation**

The cytotoxicity of the tested compounds was assessed with XTT cell proliferation kit II (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. CCRF-CEM cells were seeded in a 96-well plate at a density of 13 500 cells per well. After 24 h, the tested compounds were added to the culture media and incubated for 72 h before the XTT dye was added. The absorbance at 495 nm was recorded after a 1-h incubation with the dye. Where indicated, CCRF-CEM cells were preincubated with the efflux pump inhibitors (10 \( \mu \text{M} \) MK571 or 2 \( \mu \text{M} \) LY 335979) for 20 min prior to the addition of NCP.

**Intracellular transport of \([3H]\)NCP**

CCRF-CEM cells were washed with 20 ml of PBS, pelleted by centrifugation at 250 \( \times \) g for 5 min and resuspended in RPMI medium. Aliquots containing 1 \( \times \) \( 10^7 \) cells were distributed into the microtubes and \([3H]\)NCP (0.2 \( \mu \text{Ci/ml} \)) was added to the desired concentration. Alternatively, the cell suspension was preincubated with inhibitors (20 \( \mu \text{M} \) MK571, 10 \( \mu \text{M} \) LY 335979, 20 and 100 \( \mu \text{M} \) oligomycin A, 0.2 and 1 \( \mu \text{M} \) antimycin A, 200 \( \mu \text{M} \) dipiridamole, 100 \( \mu \text{M} \) ethacrynic acid, 0–60 \( \mu \text{M} \) cytB and cytD, 0–40 \( \mu \text{M} \) colchicine or 0–200 \( \mu \text{M} \) dynasore) for 20 min prior to the addition of NCP (0.2 \( \mu \text{Ci/ml} \)) \([3H]\)NCP. Analogously, the cell suspension was incubated with a mixture containing 10 \( \mu \text{M} \) (0.2 \( \mu \text{Ci/ml} \)) \([3H]\)NCP and 0–3.2 mM various natural nucleosides or 0–100 mM glucose for 2 min. The incubation was done at 37°C in a CO\(_2\) incubator using a rotary stirrer. At indicated time intervals, the uptake process was terminated by centrifugation at 3500 \( \times \) g for 1 min through an oil layer (a mixture of silicone and mineral oil at final specific density of 1.05 g/ml). The cells were washed by centrifugation (5300 \( \times \) g, 1 min) in PBS, solubilized with Soluene\(^{\text{TM}}\) tissue solubilizer overnight and radioactivity was counted in a toluene-based scintillation cocktail. The intracellular volume of CCRF-CEM for the calculation of the actual cytoplasmic concentration of NCP was 3.38 \( \mu \text{l}/10^7 \) cells\(^{\text{TM}}\). The HPLC analysis of the NCP metabolites was performed as described previously\(^{\text{TM}}\).

**Na\(^+\)**-dependence of \([3H]\)NCP intracellular transport**

The experiment was conducted in a sodium-free buffer (20 mM Tris/HCl, 3 mM \( K_2\text{HPO}_4 \), 1 mM \( \text{MgCl}_2 \), 6 mM \( \text{H}_2\text{O} \), 2 mM \( \text{CaCl}_2 \), 5 mM glucose and 130 mM N-methyl-D-glucamine, pH 7.4) or sodium-containing buffer (130 mM NaCl instead of N-methyl-D-glucamine). CCRF-CEM cells were washed and suspended in the assay buffer. 1 \( \times \) \( 10^7 \) Cells were distributed into the microtubes, the assay was started by adding 10 \( \mu \text{M} \) \([3H]\)NCP (0.2 \( \mu \text{Ci/ml} \)) and terminated after 2 min as described in section Intracellular transport of \([3H]\)NCP.

**Data analysis and statistical procedures**

Unless otherwise indicated, the data are presented as mean SD from at least three independent experiments. Statistical evaluation, half-maximal saturation (\( K_d \)), maximum rate of uptake (\( V_{\text{max}} \)), and \( IC_{50} \) values were performed using GraphPad Prism v. 5.00 (GraphPad Software, La Jolla, CA). The octanol–water partition coefficient values (\( log P \)) were obtained using ChemDraw v. 8.0 (Perkin Elmer, Waltham, MA).

**Results**

**NCP accumulates within the cells and reaches steady state under the conditions of MRP inhibition**

Initially, the kinetics of NCP accumulation in the cells was studied. When CCRF-CEM cells were incubated with 10 \( \mu \text{M} \) NCP for 0 to 60 min, NCP was accumulated intracellularly to a maximum value of about 600 \( \mu \text{M} \) at 10 min after which its level decreased sharply (Figure 2). To determine the role of major efflux proteins in this decrease, selective MRP and P-gp inhibitors were added. In the presence of the inhibitors, the NCP uptake reached steady state (Figure 2). The effect of MRP inhibition was considerably higher than that of P-gp, indicating that NCP is preferentially (although not exclusively) a substrate for the efflux pumps of MRP class. The efflux of NCP and/or its metabolites is also supported by the analysis of the extracellular medium, which proved the presence of NCP metabolite, NCP-GS, in considerable amount (Figure 3). The uptake of NCP was also dependent on its extracellular concentration (0.1–1000 \( \mu \text{M} \)) and accumulated in cytosol to a level about sixfold higher than in the medium (Figure 4). The concentration of half-maximal saturation (\( K_d \)) and maximum rate of uptake (\( V_{\text{max}} \)) were 0.5 ± 0.1 mM and 4.4 ± 0.4 mmol/l/min, respectively.

**Intracellular transport of NCP is energy independent**

To examine whether NCP might be transported by an energy-dependent carrier, we studied its dependence on ATP and Na\(^+\) level. Two ATP biosynthesis inhibitors (oligomycin and antimycin) were used. Both compounds have been previously shown to inhibit ATP biosynthesis in CCRF-CEM cells at indicated...
concentrations and time with good efficiency. The transport of NCP was found to be independent on both ATP level and the presence of Na⁺ in the medium (Table 1).

NCP transport is facilitated by its rapid intracellular metabolism

Since the uptake was found to be energy independent while paradoxically displaying saturation kinetics, a possible link between NCP metabolism (GSH conjugation) and transport was investigated. Indeed, NCP transport was nearly completely blocked following the inhibition of GST by ethacrynic acid (Figure 5A). Similarly, when CCRF-CEM cells were preincubated with GSH-depleting agent buthionine sulfoximine (BSO), the uptake was lower than that of untreated cells (Figure 5B), suggesting that the intracellular metabolism facilitates NCP membrane transport.

NCP does not use NTs to enter the cell

The involvement of NTs in the transport process was studied in the competition experiments using natural nucleosides. The addition of various purine and pyrimidine nucleosides to an eightfold excess had no effect on the NCP uptake (Table 2). Alternatively, the cells were preincubated with an NT inhibitor dipyridamole prior to the addition of 10 or 100 μM NCP. The NCP intracellular concentrations were 93 ± 9% and 112 ± 7%, respectively, compared to the controls treated with NCP only. Importantly, no signs of inhibitor toxicity were detectable under the conditions of the experiment.

Microtubule disruption blocks NCP uptake

To characterize further the nature of NCP transport processes, the effects of the microfilament-disrupting agents, cytochalasin B (cytB) and D (cytD), and a microtubule-disrupting drug, colchicine, were tested. While the influence of cytD on the uptake was insignificant (Figure 6A), when cytB and colchicine were used (Figure 6A and B), the uptake of NCP dropped up to 47% and 11% of control, respectively. Importantly, no signs of inhibitor toxicity were detectable under the conditions of the experiment. Finally, the influence of a dynamin inhibitor, dynasore, on NCP uptake was studied (Figure 7) to find out whether receptor-mediated endocytosis plays a role in NCP transport. Its effect on the NCP uptake was also insignificant.

Efflux pumps inhibition increases cytotoxicity of NCP

Since we have shown that NCP-GS is a potential substrate for drug efflux pumps, we also investigated whether P-gp and MRPs...
Figure 5. The time course of intracellular \[^{[3]H}]9\text{-}\text{norbomyl\text{-}6\text{-}chloropurine (\[^{[3]H}\text{NCP}) levels following the CCRF-CEM cell treatment with ethacrynic acid and buthionine sulfoximine (BSO). CCRF-CEM cells were incubated with 10 \mu M (0.2 \mu Ci/ml) \[^{[3]H}\text{NCP for 0 to 45 min (●)} or preincubated with 50 \mu M ethacrynic acid (glutathione-S-transferase inhibitor, A) for 20 min prior to the addition of 10 \mu M (0.2 \mu Ci/ml) \[^{[3]H}\text{NCP (A). In a parallel experiment, NCP uptake was evaluated after the cell treatment with 50 \mu M BSO (glutathione biosynthesis inhibitor, □) for 24 h before the addition 10 \mu M (0.2 \mu Ci/ml) \[^{[3]H}\text{NCP (B).}

Table 2. The effect of natural nucleosides on the uptake of \[^{[3]H}]9\text{-}\text{norbomyl\text{-}6\text{-}chloropurine (\[^{[3]H}\text{NCP after a 2-min incubation.}

| Competing nucleoside | Intracellular \[^{[3]H}\text{NCP (% of control)} | Intracellular \[^{[3]H}\text{NCP (% of control)} |
|----------------------|----------------------|----------------------|
|                      | \[^{[3]H}\text{NCP = 50 \mu M (0.2 \mu Ci/ml)}; control sample (NCP only) | \[^{[3]H}\text{NCP = 50 \mu M (0.2 \mu Ci/ml)}; control sample (NCP only) |
| Guanosine            | 115 ± 4              | N/A†               |
| Deoxycytidine        | 107 ± 4              | 95 ± 22            |
| Adenosine            | 120 ± 11             | 102 ± 10           |
| Inosine              | 106 ± 5              | 107 ± 14           |
| Uridine              | 80 ± 6               | N/A†               |
| Thymidine            | 116 ± 7              | 133 ± 32           |
| Cytidine             | 105 ± 2              | 138 ± 30           |

*: \[^{[3]H}\text{NCP = 50 \mu M (0.2 \mu Ci/ml); control sample (NCP only) = 9.9 ± 0.9 nmol.}†|\[^{[3]H}\text{NCP = 400 \mu M (0.2 \mu Ci/ml); control sample (NCP only) = 3.4 ± 0.6 nmol.}†Nucleoside not soluble at the designated concentration.

Discussion

NCP represents a class of original pseudonucleoside compounds inspired by carbocyclic nucleosides. NCP was demonstrated to inhibit replication of the Cox sackie B3 virus with considerable efficiency (EC\textsubscript{50} = 0.8 ± 0.2 \mu M). Studies in our laboratory also suggested an antitumor potential.

In this transport study, NCP accumulated in the cells and exhibited saturation kinetics. As it was clearly expelled from the cells when longer incubation times were used, the mechanism of this efflux was investigated. Blocking the multidrug-resistance proteins efficiently prevented the excretion of NCP and increased the cytotoxicity of NCP to CCRF-CEM cells indicating that NCP is likely a substrate for these transporters.

Although the intracellular concentration of NCP was clearly higher compared to this in the medium, the transport was surprisingly both ATP and Na\textsuperscript{+} independent. It should be noted that the radiometric method used to detect intracellular NCP does not discriminate between the parent compound and its metabolites and the presented intracellular uptake data always represent the sum of both. Therefore, we proposed that the intracellular metabolism of NCP (i.e. GSH conjugation) represents a major driving force of its cellular uptake by modifying the concentration gradient across the plasma membrane. Indeed, both GST inhibition by ethacrynic acid\textsuperscript{13} and GSH depletion by BSO\textsuperscript{14} resulted in a significant impairment of the uptake rate. The effect was somewhat less pronounced in BSO-treated cells compared to GST inhibition, which can be explained by the fact that the BSO-induced decrease of GSH in the cells was incomplete (more than 40% of residual GSH following an overnight incubation with BSO)\textsuperscript{15}. The conjugation reaction is therefore clearly determining the rate of NCP transport. Our results suggested that the NCP transport mechanism is likely a process not utilizing chemical energy, likely diffusion (passive and/or facilitated) or endocytosis. Since the plots of NCP uptake versus NCP extracellular concentration were nonlinear, simple nonfacilitated diffusion and fluid-phase endocytosis should be excluded, because neither of them show saturation kinetics with respect to the extracellular concentration. This assumption is further supported by the fact that the inhibitor of fluid-phase endocytosis cytD\textsuperscript{16,17} had no effect on NCP uptake. On the other hand, reaching the apparent steady-state could also be the effect of the intracellular driving force (i.e. GSH or GST) depletion.

Interestingly, NCP transport was also found to be independent of NTs. According to Damaraju et al.\textsuperscript{18}, the wild-type CEM cells possess only hENT1 activity. hENT1 is inhibited by NBTI or dipyridamole\textsuperscript{7}. However, dipyridamole did not have any effect on the NCP uptake. In addition, not even high levels of natural NTs substrates did not compete with NCP transport. As for the role of passive diffusion, its contribution to the NCP uptake is heavily supported by its lipophilic nature. NCP is considerably more lipophilic (log \textit{P} = 2.12) compared to its physiological counterpart adenosine (log \textit{P} = −1.05) and although the concentration-dependent plot of NCP uptake rather favours the protein-mediated mode of transport, we suggest that passive diffusion also takes place.

Compared to the effect of cytD, cytB and colchicine decreased NCP uptake. Several studies have reported that cytB inhibits the facilitated diffusion of hexoses and nucleosides\textsuperscript{19,20}. In contrast, an increase in fluid-phase endocytosis in various cell types treated with cytB was also observed\textsuperscript{21,22}. Colchicine has been reported to inhibit the receptor-mediated and nucleoside transport in several mammalian cell lines\textsuperscript{16,23}. NCP was not found to be transported by nucleoside or glucose transporters, yet its transport was sensitive to cytB. This may be caused by the binding of cytB to the substrate binding site or to membrane sites in close proximity to the transporters\textsuperscript{24}. Apparently, GTP hydrolysis-driven clathrin-mediated endocytosis\textsuperscript{25} is not involved in the mechanism of NCP transport based on its insensitivity to dynamin inhibitor dynasore\textsuperscript{26}. Nevertheless, with respect to the effect of the microtubule network-disrupting agents on NCP transport, a contribution of facilitated diffusion is likely.

Conclusions

Overall, our data point to a combination of passive and facilitated diffusion as general transport mechanisms of NCP uptake.
Figure 6. The effect of the microtubule inhibitors on \([^{3}H]9\)-norbornyl-6-chloropurine (\([^{3}H]\)NCP) uptake. CCRF-CEM cells were incubated with 0 to 30 \(\mu\)M cytochalasin B (■) and cytochalasin D (□, A) or with 0 to 20 \(\mu\)M colchicine (■, B) for 20 min before the addition of 10 \(\mu\)M (0.2 \(\mu\)Ci/ml) \([^{3}H]\)NCP. The intracellular NCP content was measured at \(t = 2\) min. The absolute value of control sample (NCP only) \(= 1.4 \pm 0.2\) nmol.

\(*p < 0.05\) (one-way ANOVA with Tukey’s post hoc test).

Figure 7. The dependence of \([^{3}H]\)9-norbornyl-6-chloropurine (\([^{3}H]\)NCP) uptake on the clathrine-dependent endocytosis inhibitor, dynasore. CCRF-CEM cells were preincubated with 0 to 200 \(\mu\)M dynasore (□) for 20 min at \(37^\circ\)C after which 10 \(\mu\)M (0.2 \(\mu\)Ci/ml) \([^{3}H]\)NCP was added and the intracellular level was measured following 2-min incubation. The absolute value of control sample (NCP only) \(= 0.9 \pm 0.1\) nmol.

Table 3. The cytotoxicity of 9-norbornyl-6-chloropurine (NCP) in CCRF-CEM cells in the presence of the inhibitors of drug efflux proteins.

| Inhibitor       | None | LY335979* (P-gp) | MK571* (MRP) |
|-----------------|------|------------------|--------------|
| IC50 (\(\mu\)M) | 17.7 ± 1.8 | 14.1 ± 0.9       | 9.9 ± 1.0    |

*2 \(\mu\)M LY 335979 and 10 \(\mu\)M MK571, these concentrations did not cause any intrinsic toxicity to the cells.

†The concentration causing 50% decrease in cells viability using XTT cytotoxicity test (calculated using the non-linear regression method by GraphPad Prism).

These are supported by the saturability and ATP-independent character and the sensitivity to the inhibition by cytB and colchicine. Intracellular transport is fast and together with the previously reported high permeability in Caco-2 assay suggests good bioavailability of the compound. Once NCP reaches intracellular compartment, it immediately undergoes a GST-catalyzed conjugation with GSH. The rapid metabolism further facilitates NCP uptake in the direction of concentration gradient. NCP excretion is mediated by efflux proteins of MDR/MRP type, largely via its polar metabolite NCP-GS.

Declarations of interest

The authors report no conflicts of interest. 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.

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Supplementary material available online