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# Intracellular metabolism of the new antiviral compound 1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine

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ABSTRACT

1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine [HPMP-5-azaC], the 5-azacytosine analogue of cidofovir (HPMPC), represents a new acyclic nucleoside phosphonate with pronounced activity against DNA viruses, and a selectivity index superior to that of cidofovir. Here we investigated the intracellular metabolic pathway of [6-$^{3}$H]-HPMP-5-azaC. By comparing the metabolism in mouse lymphosarcoma S49-wild type (S49-WT) and mutant cells deficient for dCMP deaminase, we identified the mono- and diphosphate metabolites generated from HPMP-5-azaC and its deaminated product HPMP-5-azaU. In human lung carcinoma A549 cells, the relative formation of the deaminated metabolites was only 6%, implying that deamination plays a minor role in the overall metabolism of HPMP-5-azaC. The diphosphorylated metabolite of HPMP-5-azaC accounted for 60% of the total radioactivity, and reached intracellular levels which were 60-fold higher in absolute value than the corresponding diphosphate levels obtained with cidofovir. Consequently to its increased activation, HPMP-5-azaC showed about 45-fold higher incorporation into cellular DNA than cidofovir. Herpes-, pox- or adenovirus infection had no marked influence on the metabolism of HPMP-5-azaC. The HPMP-5-azaC-diphosphate metabolite was shown to have long intracellular stability (half-life: 63 h), suggesting that infrequent administration of HPMP-5-azaC should be possible. HPMP-5-azaC represents a new acyclic nucleoside phosphonate compound with promising anti-DNA virus activity and a favorable metabolic profile that is characterized by low sensitivity to catabolic deamination and a high rate of phosphorylation and DNA incorporation.



## 1. Introduction

Prior to its clinical development as a broad-spectrum anti-DNA-virus agent, the acyclic nucleoside phosphonate compound cidofovir {Vistide®; 1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine; HPMPC} has been the subject of extensive biochemical studies, to elucidate its activation pathway and mode of action. Some of its characteristic features are: slow cellular uptake by endocytosis [1]; conversion to its mono- and diphosphate metabolites by cellular kinases that is, though independent of viral kinases, increased in cytomegalovirus-infected cells [2]; and long intracellular retention of its active diphosphate metabolite and secondary phosphocholine metabolite [3]. In these studies, no catabolic

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conversion of HPMPC (for instance, by dCMP deaminase) to the antivirally inactive uracil derivative was noted, indicating that HPMPC is resistant to enzymatic deamination.

Recently, our ongoing efforts to further investigate the structure–activity relationship of the acyclic nucleoside phosphonate compounds, have pointed to the 5-azacytosine derivative of HPMPC {1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine; HPMP-5-azaC} as a promising antiviral compound with potential clinical relevance. Its antiviral activity spectrum is as wide as that of HPMPC and includes human and animal viruses belonging to the herpesvirus, adenovirus, polyomavirus, papillomavirus and poxvirus families [4–6]. Depending on the virus and the cell line used for the *in vitro* assay, the antiviral potency of HPMP-5-azaC was shown to be comparable or 2- to 7-fold higher than that of HPMPC. Even more importantly, for any of the viruses thus far evaluated, HPMP-5-azaC displayed a superior (i.e. 2- to 12-fold higher) therapeutic index (i.e. ratio of cytotoxic to antivirally effective concentration) compared to HPMPC. Based on these promising data, we recently synthesized a series of lipophilic ester prodrugs of the cyclic form of HPMP-5-azaC [7]. A similar approach has been used to design ester prodrugs of HPMPC, which, *in vitro*, displayed improved cellular penetration and therapeutic index, and, *in vivo*, showed some favorable pharmacokinetic features such as oral bioavailability [8].

The present study was aimed at understanding the metabolism of HPMP-5-azaC in cell culture, with a particular focus on its anabolic (i.e. phosphorylation) as well as its catabolic conversion (i.e. deamination). We found that HPMP-5-azaC differs from HPMPC in generating low yet detectable levels of the deaminated products, and a predominant diphosphate metabolite, which resulted in markedly increased incorporation into DNA.

## 2. Materials and methods

### 2.1. Cells

Human lung carcinoma A549 cells (American Type Culture Collection No. CCL-185) were grown in Minimum Essential Medium, supplemented with 2 mM L-glutamine, 0.075% sodium bicarbonate and 10% heat-inactivated fetal calf serum. The latter concentration was reduced to 2% for the experiments in virus-infected A549 cells. The following mouse T-lymphosarcoma cell lines were a kind gift from Dr. B. Ullman (Oregon Health and Science University, Portland, OR): S49-wild type cells (S49-WT) and an S49-derived mutant deficient for purine nucleoside phosphorylase and dCMP deaminase, that was originally selected under cytarabine (araC) pressure [designated NSU-1/araC-6-1] [9]. These cells were grown in Dulbecco's modified Eagle medium with 10% heat-inactivated horse serum and 1 mM sodium pyruvate. Cell cultures were incubated at 37 °C in a humidified and $CO_2$-controlled incubator.

### 2.2. Compounds

1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine was synthesized as already described [4]. 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine [HPMPC; cidofovir] was a kind gift from Dr. T. Cihlar (Gilead Sciences, Foster City, CA). [6-$^3$H]-HPMP-5-azaC (143 μM; 1 mCi/ml; specific activity: 7 Ci/mmol) and [5-$^3$H]-HPMPC (67 μM; 1 mCi/ml; specific activity: 15 Ci/mmol) were synthesized by Moravek Biochemicals (Brea, CA), and stored at −20 °C in ethanol/water 1:1. The stability of these radiolabeled compounds was verified by reverse-phase HPLC at several time points throughout the course of the study.

### 2.3. Chemical stability of HPMP-5-azaC

A solution of HPMP-5-azaC (70 μM) was prepared in water, medium (Minimum Essential Medium), or buffer [50 mM potassium phosphate, pH 6.5; 50 mM Tris–HCl, pH 7.4; or 50 mM Tris–HCl, pH 8.0], and stored at −20 °C, 4 °C, 20 °C or 37 °C during varying periods. The remaining HPMP-5-azaC was quantified by ion-pairing reverse-phase HPLC analysis on an RP-18 column (LiChroCart 4 mm × 250 mm, Superspher 100 RP-18e; from Merck, Darmstadt, Germany) and a Waters (Milford, MA) chromatographic system, equipped with a 717 Plus Autosampler, a 600 Controller pump and a 996 Photodiode Array Detector, and controlled by Millennium software. Elution was done with acetonitrile and buffer (5 mM tetrabutyl-ammonium hydrogen sulphate; 5 mM ammonium dihydrogen phosphate; pH 6.5), using a flow of 1 ml/min, and the following gradient: 100% buffer (5 min); linear gradient to 75% buffer and 25% acetonitrile (5 min); 75% buffer and 25% acetonitrile (2 min); linear gradient to 100% buffer (3 min); reequilibration with 100% buffer (20 min). The peak corresponding to HPMP-5-azaC had a retention time of 14.5 min and a UV spectrum similar to that of 5-azacytidine with a $\lambda_{max}$ of 245 nm.

### 2.4. Metabolism study with [6-$^3$H]-HPMP-5-azaC and [5-$^3$H]-HPMPC

At day 0, [6-$^3$H]-HPMP-5-azaC was added at a final concentration of 10 μM (unless stated otherwise) and 10 μCi per flask, to A549 cells (density: $10^7$ cells per 75-cm$^2$ flask) or mouse lymphosarcoma S49 cells (density: $10^8$ cells per 75-cm$^2$ flask). All conditions were performed in duplicate. After 24 h, 48 h or 72 h incubation at 37 °C, A549 cells were rinsed three times with ice-cold phosphate-buffered saline (PBS), and detached by 15 min trypsinization at 37 °C. Similarly, mouse lymphoma cells were subjected to three steps of PBS rinsing and centrifugation. After collection of the cell pellet by centrifugation, extraction was performed with 300 μL ice-cold methanol 66%. After 10 min standing on ice, followed by 10 min centrifugation at 23,000 × *g*, the clarified extracts and residual methanol-insoluble pellets were frozen at −20 °C. For HPLC analysis, 200 μL extract was injected onto an anion-exchange Partisphere SAX column (dimensions: 4.6 mm × 125 mm) from Whatman (Maidstone, UK), and separated with two phosphate buffers (A: 5 mM and B: 0.3 M ammonium dihydrogen phosphate; both at pH 3.5), and the following gradient (flow: 2 ml/min): 100% A (5 min); linear gradient to 100% B (15 min); 100% B (20 min); linear gradient to 100% A (5 min) and 100% A (15 min). One-min fractions of the eluate were collected, mixed with Hisafe 3 cocktail (Perkin Elmer, Waltham, MA) and analyzed for radioactivity in a scintillation counter.

To determine incorporation of [6-$^3$H]-HPMP-5-azaC into cellular nucleic acid material, the methanol-insoluble pellets were digested in 500 μl 5N sodium hydroxide during 24 h incubation at 37 °C. After neutralization with 500 μl 5N hydrochloride, digested samples were transferred to scintillation vials, mixed with Hisafe 3 cocktail and analyzed for total radioactivity.

In a separate wash-out study, [6-$^3$H]-HPMP-5-azaC was added to A549 cells as described above, and incubated during 24 h. The compound was then removed by three washings with growth medium, and the cells were further incubated during 0 h, 24 h, 48 h or 120 h, after which methanol extraction was performed as above.

### 2.5. Metabolism in virus-infected cells

One day after seeding at a density of $10^7$ cells per 75-cm$^2$ flask, A549 cells were infected with herpes simplex virus type 1 (HSV-1), human adenovirus type 2 (Ad2), or vaccinia virus (VV) [multiplicity of infection: 0.03 (HSV-1); 0.03 (Ad2) and 0.0004 (VV) PFU per cell]. After two hours virus adsorption at 37 °C, the medium was replaced by fresh medium containing 10 μCi [6-$^3$H]-HPMP-5-azaC or [5-$^3$H]-HPMPC (both at a final concentration of 1 μM). After an additional 48 h incubation period, methanolic cell extracts were prepared as described above. Mock- or virus-infected controls receiving unlabeled compound were included to allow microscopic verification that, at 1 μM, the compounds had no inhibitory effect on the virus-induced cytopathic effect.

### 2.6. Incorporation into cellular DNA

Incorporation of [6-$^3$H]-HPMP-5-azaC and [5-$^3$H]-HPMPC was determined in A549 cells grown in six-well plates (plated on day 1 at 700,000 cells per 9.6 cm$^2$). After compound addition at a total concentration of 10 μM and 10 μCi per well, cells were incubated during 24 h, 48 h or 72 h. After three washing steps with cold medium, followed by trypsinization, cells were collected in 200 μl PBS. Total cellular DNA was extracted with the QIAamp DNA Blood Mini kit (Qiagen), following the Manufacturer's instructions. After mixing with 10 ml High-Safe 3 cocktail, the radioactivity in the DNA extract was determined by scintillation counting.

### 2.7. In vivo studies

Female NMRI mice (~20 g) (Janvier breeding, France) were given an intravenous bolus injection of HPMP-5-azaC at a dose of 50 mg/kg, by injection into the tail vein of 200 μl HPMP-5-azaC solution in PBS (concentration: 5 mg/ml). At several time points after injection (range: 2 min–8 h), mice were given ether anesthesia and subjected to heart puncture to collect ~1 ml blood into heparinized tubes. Plasma samples were collected by centrifugation and stored at −20 °C until extraction. To this end, 100 μl plasma was mixed with 300 μl methanol, kept on ice during 10 min and clarified by centrifugation at 23,000 × *g*. The extracts were vacuum-evaporated and reconstituted in 250 μl elution buffer A. HPLC-UV analysis was done on a Partisphere SAX column using a flow of 2 ml/min and the same buffers as outlined above for analysis of the cell extracts, yet a different gradient system [100% A (5 min); linear gradient to 85% A and 15% B (5 min); 15% B (5 min); linear gradient to 100% A (10 min); 100% A (10 min)], giving a retention time for HPMP-5azaC of 7 min. A standard curve was drawn from blank mouse plasma samples spiked with known amounts of HPMP-5-azaC, and extracted and analyzed as above.

## 3. Results

### 3.1. Chemical stability of HPMP-5-azaC

The presence of a 5-azacytosine moiety in HPMP-5-azaC raised our concern that the compound might be degraded in solution, as observed for 5-azacytidine and 5-aza-2′-deoxycytidine [10]. The latter two compounds undergo hydrolytic decomposition at alkaline pH, which is characterized by reversible opening of the triazine ring between C-6 and N-1, followed by irreversible deformylation. The mechanism of this ring opening reaction for structurally diverse 5-azacytosine acyclic nucleoside phosphonates was recently studied in more detail, using NMR methodology for identification of reaction intermediates [11].

The chemical stability of HPMP-5-azaC was determined in function of time, temperature and pH. The remaining HPMP-5-azaC was determined by HPLC-UV$_{245nm}$ analysis, and expressed as the percentage of the amount detected in a fresh solution prepared in water. When stored in water or culture medium (MEM; pH 7.4) at −20 °C, HPMP-5-azaC was relatively stable, since ≥86% was left after 6 months. Similarly, our stock solution of [6-$^3$H]-HPMP-5-azaC did not show any sign of degradation after 12 months storage at −20 °C, when analyzed by HPLC coupled with detection of radioactivity. Since the deformylated degradation product lacks the tritium label (Fig. 1), these data show that conversion of HPMP-5-azaC into this antivirally inactive deformylated product is negligible upon storage at −20 °C. In contrast, slow degradation of HPMP-5-azaC was observed upon storage in water at 4 °C (72% and 0% compound remaining after 1 week and 5 months, respectively) or 20 °C (35% HPMP-5-azaC left after 1 week). The stability improved in buffered conditions within a pH range of 6.5–8 (~60% compound left after 1 week storage at 20 °C, in buffer at pH 6.5, 7.4 or 8). This is a comparable result as recently reported by Dračínský et al. [11], who observed a decomposition half-life for HPMP-5-azaC of 5 days, upon incubation in PBS at 20 °C. Likewise, when stored in cell culture medium (containing 10% fetal calf serum) at 37 °C during 7 days, we found HPMP-5-azaC to be 38% intact, indicating that its chemical stability was sufficient to perform biochemical studies at 37 °C.

### 3.2. Characterization of HPMP-5-azaC metabolites

A comparative study was performed on the metabolism of [6-$^3$H]-HPMP-5-azaC in S49-WT and NSU-1/araC-6-1 cells, the latter possessing less than 3% of the dCMP deaminase activity of S49-WT cells [9]. In this way, we were able to identify which metabolites on the radiochromatogram corresponded to HPMP-5-azaU (formed from HPMP-5-azaC by dCMP deaminase) and its phosphorylated metabolites HPMP-5-azaU-monophosphate and HPMP-5-azaU-diphosphate (Fig. 1). As

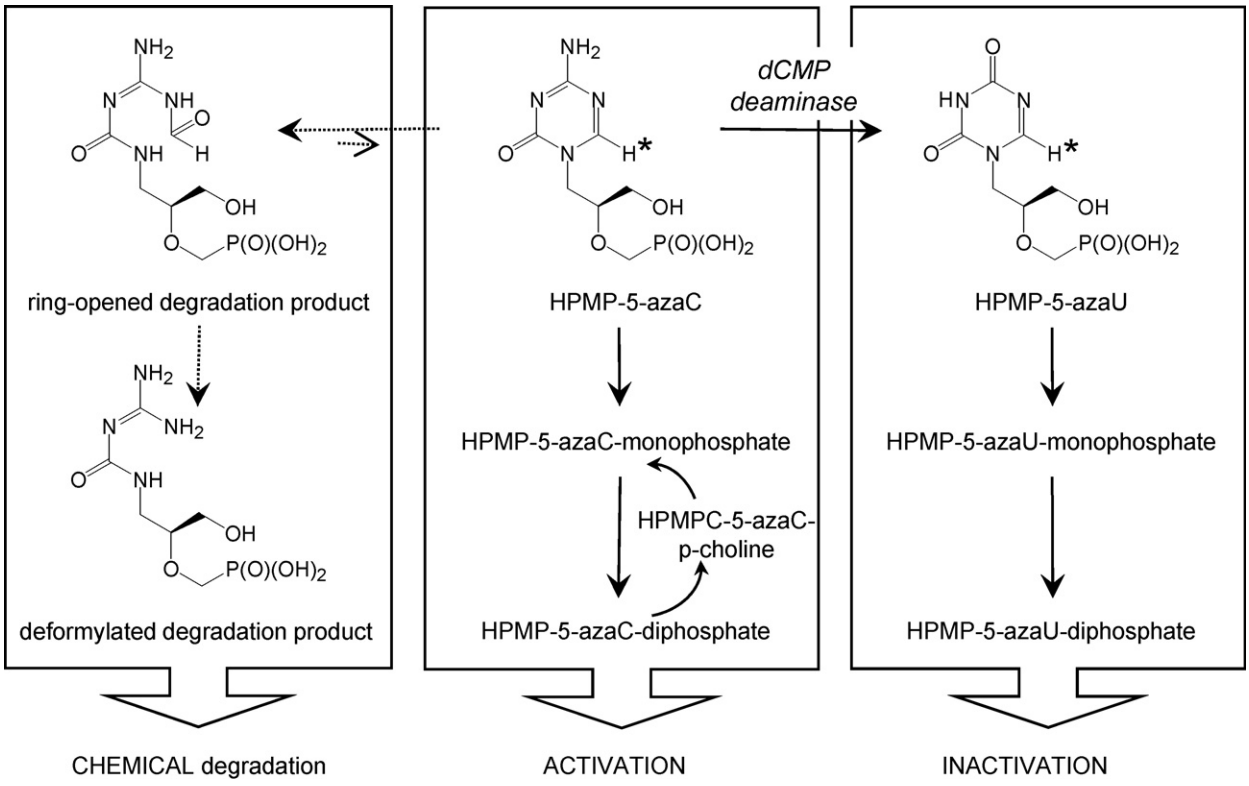
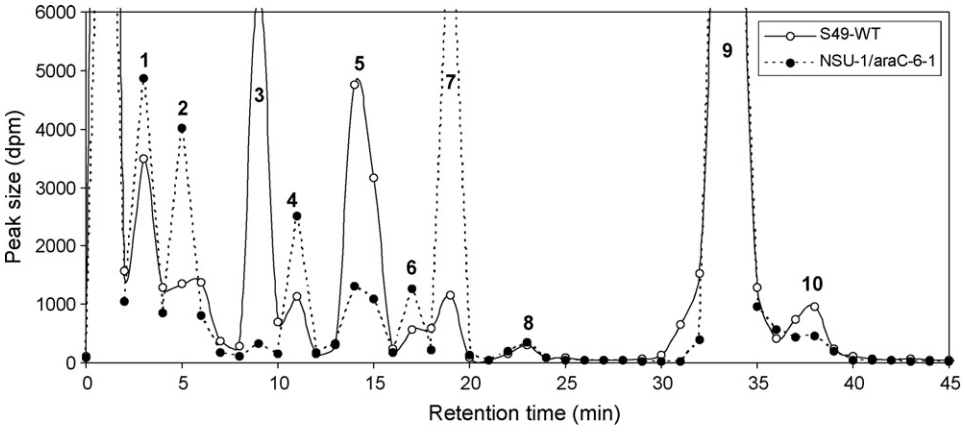



**Fig. 1 – Proposed scheme for metabolic activation, metabolic inactivation and chemical degradation of HPMP-5-azaC. The (*) symbol indicates the position of the radiolabel in the [6-$^3$H]-HPMP-5-azaC compound used in this study.**

shown in Fig. 2, the levels of these three metabolites were significantly smaller in NSU-1/araC-6-1 compared to S49-WT cells, the ratio being 0.1, 0.3 and 0.4, for HPMP-5-azaU, its monophosphate and its diphosphate, respectively. Conversely, two metabolites having significantly higher levels in the dCMP deaminase-deficient cell line were identified as parent HPMP-5-azaC and its monophosphate, the latter being 7-fold increased in NSU-1/araC-6-1 cells compared to S49-WT cells. Remarkably, the predominant metabolite identified as HPMP-5-azaC-diphosphate represented 46% of total radioactivity. The observation that this metabolite reached equal levels in the dCMP deaminase-deficient and WT cells suggests that, in order to compensate for their deranged nucleotide pools, the araC-resistant cell line may have acquired alterations in other enzymes than dCMP deaminase [12]. The mutant and wild-type cells also showed similar formation of the HPMP-5-azaC-phosphocholine adduct, for which the peak was identified from its similar elution profile as a CDP-choline standard. Finally, three minor metabolites (encoded X, Y and Z; Fig. 2) were observed, each representing 1–2% or less of the total radioactivity in S49-WT cells, with about 2-fold higher levels in the dCMP deaminase-deficient cells.

### 3.3. *Metabolism of HPMP-5-azaC in A549 cells*

In a preliminary experiment, we investigated the metabolism of [6-$^3$H]-HPMP-5-azaC in human embryonic lung (HEL) fibroblasts, to allow direct comparison with literature data on the metabolism of cidofovir (HPMPC) in the similar MRC-5 cell line [2,3,13]. After 24 h incubation with 10 μM [6-$^3$H]-HPMP-5-azaC, one predominant peak was observed which eluted at 35 min, represented 28% of total radioactivity and obtained an intracellular concentration of 2 pmol/$10^6$ HEL cells (data not shown). However, the other peaks on the radiochromatogram were too small to allow accurate analysis, and, hence, we performed all subsequent metabolism studies in human lung carcinoma A549 cells. A compound concentration of 10 μM was chosen, since it is well above the $EC_{50}$ value of HPMP-5-azaC for several DNA viruses [4]. This concentration of 10 μM was also used by Srinivas et al. to compare the metabolism of cidofovir in lymphoblast *versus* HeLa cells [14]. As shown in Table 1, ten metabolites of HPMP-5-azaC were detected in A549 cells, with identical retention times as seen in S49-WT cells, allowing their identification as HPMP-5-azaC, HPMP-5-azaU and the corresponding phosphorylated forms, or the unknown metabolites X, Y and Z. In analogy to what was observed in S49-WT and HEL cells, the major metabolite formed in A549 cells corresponded to HPMP-5-azaC-diphosphate, which represented about 60% of the total radioactivity. Using a single cell volume of 1200 μm$^3$, the intracellular concentration of HPMP-5-azaC-diphosphate (35 pmol/$10^6$ cells) was estimated to be 29 μM, after 72 h incubation with 10 μM HPMP-5-azaC. In parallel with the increasing levels of HPMP-5-azaC-monophosphate and -diphosphate as a function of incubation time (Table 1), there was a reduction in the relative contribution of the choline adduct, which represents an intracellular reservoir. Interestingly, after 72 h incubation, the sum of HPMP-5-azaC and its phosphorylated metabolites was 90% in A549 cells (Table 1), compared to 62% in S49-WT cells (Fig. 2), reflecting cell-type dependent dCMP




| Peak number: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| RT (min): | 3 | 5 | 9 | 11 | 14 | 17 | 19 | 23 | 34 | 38 |
| % of total radioactivity in S49-WT: | 7.4 | 5.8 | 13.7 | 2.4 | 16.9 | 1.2 | 2.5 | 1.0 | 45.6 | 3.6 |
| % of total radioactivity in NSU-1/araC-6-1: | 10.2 | 10.0 | 0.7 | 5.2 | 5.7 | 2.6 | 16.5 | 1.2 | 46.5 | 1.4 |
| ratio NSU-1/araC-6-1 to S49-WT: | 1.4 | 1.7 | 0.1 | 2.2 | 0.3 | 2.2 | 6.7 | 1.2 | 1.0 | 0.4 |
| | HPMP-5-azaC-p-choline | HPMP-5-azaC | HPMP-5-azaU | X | HPMP-5-azaU-p | Y | HPMP-5-azaC-p | Z | HPMP-5-azaC-pp | HPMP-5-azaU-pp |

**Fig. 2 – Metabolism of [6-$^3$H]-HPMP-5-azaC in S49-WT cells and dCMP deaminase-deficient (NSU-1/araC-6-1) cells, after 72 h incubation with 10 μM radiolabeled compound. For each cell line, a representative chromatogram is shown, with the numbers in bold referring to the different metabolites, for which the identity and relative contribution to the total radioactivity are shown in the bottom panel.**

deaminase activity [15]. Like S49-WT cells, A549 contained three minor, as yet unidentified, HPMP-5-azaC metabolites, which represented 1–5% of total radioactivity (Table 1; metabolites X, Y and Z).

The efficiency for formation of the active diphosphate metabolite was compared for HPMP-5-azaC and its congener HPMPC. In A549 cells incubated with 10 μM of [5-$^3$H]-HPMPC during 72 h, HPMPC-diphosphate represented 10% of total

**Table 1 – Metabolism of HPMP-5-azaC in human lung carcinoma A549 cells**

| Metabolite[a] | HPLC retention time (min) | Intracellular concentration (pmol/$10^6$ cells)[b] | | |
|---|---|---|---|---|
| | | Incubation time with [6-$^3$H]-HPMP-5-azaC[c] (h) | | |
| | | 24 | 48 | 72 |
| HPMP-5-azaC-p-choline | 3 | 2.2 ± 0.7 (23) | 2.6 ± 0.3 (12) | 4.5 ± 0.7 (8.8) |
| HPMP-5-azaC | 5 | 0.25 ± 0.06 (2.7) | 0.45 ± 0.26 (2.1) | 1.6 ± 0.6 (3.1) |
| HPMP-5-azaU | 9 | 0.12 ± 0.05 (1.3) | 0.11 ± 0.04 (0.5) | 0.17 ± 0.05 (0.3) |
| X | 11 | 0.47 ± 0.39 (4.9) | 0.19 ± 0.03 (0.9) | 0.34 ± 0.20 (0.7) |
| HPMP-5-azaU-p | 14 | 0.41 ± 0.08 (4.4) | 1.6 ± 0.0 (7.3) | 1.8 ± 0.4 (3.6) |
| Y | 17 | 0.27 ± 0.15 (2.9) | 0.91 ± 0.50 (4.2) | 1.9 ± 0.2 (3.8) |
| HPMP-5-azaC-p | 19 | 0.43 ± 0.18 (4.6) | 1.7 ± 1.6 (8.0) | 5.3 ± 1.5 (10) |
| Z | 23 | 0.15 ± 0.06 (1.6) | 0.10 ± 0.02 (0.5) | 0.19 ± 0.01 (0.4) |
| HPMP-5-azaC-pp | 34 | 5.0 ± 0.8 (53) | 14 ± 11 (66) | 35 ± 5 (68) |
| HPMP-5-azaU-pp | 38 | 0.22 ± 0.10 (2.3) | 0.44 ± 0.25 (2.0) | 1.1 ± 0.0 (2.1) |
| Total | | 9.5 ± 0.3 (100) | 22 ± 14 (100) | 51 ± 3 (100) |

[a] Abbreviations: HPMP-5-azaC-p-choline, phosphocholine metabolite; HPMP-5-azaC-p and HPMP-5-azaU-p, monophosphate metabolites; and HPMP-5-azaC-pp and HPMP-5-azaU-pp, diphosphate metabolites.
[b] Results shown are the mean ± S.E.M. of two independent experiments, each performed in duplicate. Data between brackets represent the relative formation (i.e. percentage *versus* sum of all metabolites).
[c] Compound concentration: 10 μM.

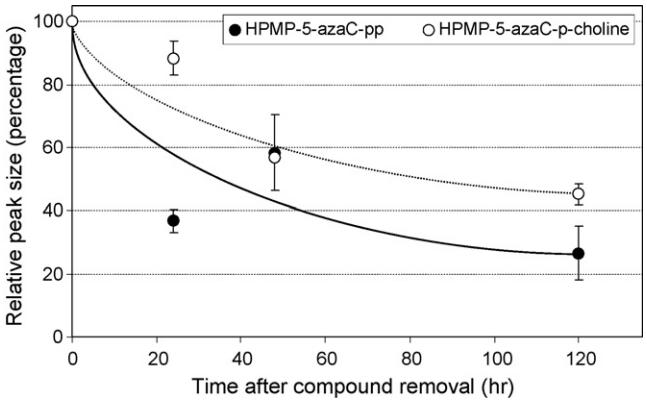



**Fig. 3 – Intracellular stability of the HPMP-5-azaC-diphosphate (●) and HPMP-5-azaC-phosphocholine (○) metabolites in A549 cells. After 24 h incubation with 10 μM [6-$^3$H]-HPMP-5-azaC, the compound was washed away and cells were further incubated during 0 h, 24 h, 48 h or 120 h. The amount of metabolite remaining is presented as the percentage of the amount measured at time zero. Data shown are the mean ± S.E.M. for three (●) or two (○) separate experiments.**

radioactivity and attained a concentration of 0.6 pmol/$10^6$ cells (data not shown), which is ~60-fold lower than the diphosphate level obtained after incubation with HPMP-5-azaC. In the case of HPMPC, about 66% of total radioactivity was associated with the first peak on the radiochromatogram (representing HPMPC and its phosphocholine adduct which, unfortunately, could not be separated by the HPLC method used). The total sum of HPMPC and its metabolites was 6 pmol/$10^6$ cells (data not shown), which is 8-fold lower than the total sum of HPMP-5-azaC metabolites (51 pmol/$10^6$ cells; Table 1), suggesting that the cellular uptake of HPMP-5-azaC may be more efficient than that of HPMPC.

Next, the intracellular stability of the phosphorylated metabolites of HPMP-5-azaC was determined in a wash-out experiment in which the compound was removed after 24 h incubation, and all metabolites were measured after 0 h, 24 h, 48 h or 120 h incubation in compound-free medium. The diphosphate of HPMP-5-azaC showed a slow and mono-exponential decline, with an estimated half-life of 63 h (Fig. 3). An even higher intracellular stability was noted for the phosphocholine adduct (calculated half-life: 103 h).

### 3.4. *Incorporation in cellular DNA*

To compare the efficiency for DNA incorporation of HPMP-5-azaC or HPMPC, we first performed a rough analysis of the methanol-insoluble pellets obtained from A549 cells incubated with 10 μM radiolabeled compound during 24 h, 48 h or 72 h. As shown in Table 2, incorporation of HPMP-5-azaC was 36- to 146-fold higher for HPMP-5-azaC than for HPMPC. However, the analytical variation of these experiments was high, probably due to a variable efficiency for alkaline digestion of the DNA pellet and/or contamination of the pellets with soluble metabolites. We therefore conducted another study in which DNA incorporation was more accurately determined in cellular genomic DNA extracts prepared by silica-based affinity chromatography (Table 2). Consistent with the above data, the level of DNA incorporation was 28- to 59-fold higher for HPMP-5-azaC than for HPMPC.

### 3.5. *Metabolism of HPMP-5-azaC in virus-infected cells*

Virus infection may influence the activity of cellular kinases or deaminases involved in nucleotide metabolism [16]. We therefore investigated the metabolism of HPMP-5-azaC and

**Table 2 – Incorporation of HPMP-5-azaC and HPMPC into cellular DNA of human lung carcinoma A549 cells**

| Compound[a] | DNA incorporation (pmol/$10^6$ cells) | | |
|---|---|---|---|
| | Incubation time with radiolabeled compound (h) | | |
| | 24 | 48 | 72 |
| By digestion of methanol-insoluble material[b] | | | |
| HPMP-5-azaC | 43 ± 9 | 120 ± 42 | 611 ± 214 |
| HPMPC | 1.2[#] | 0.82[#] | 7.0 ± 0.0 |
| Ratio (HPMP-5-azaC to HPMPC) | 36 | 146 | 88 |
| By DNA extraction[c] | | | |
| HPMP-5-azaC | 17 ± 1 | 38 ± 8 | 40 ± 2 |
| HPMPC | 0.61 ± 0.08 | 0.65 ± 0.05 | 0.84 ± 0.11 |
| Ratio (HPMP-5-azaC to HPMPC) | 28 | 59 | 48 |

[a] Compound concentration: 10 μM.
[b] Compounds were added to A549 cells, grown in 75-cm$^2$ flasks. After 24 h, 48 h or 72 h, methanol extracts were prepared to quantify the individual metabolites (see Table 1). The methanol-insoluble pellets were subjected to overnight digestion with NaOH, followed by neutralization and scintillation counting. Data are the mean ± S.E.M. of two to four independent experiments, each performed in duplicate (except #, which is from one experiment).
[c] Compounds were added to A549 cells, grown in six-well plates. After 24 h, 48 h or 72 h, total cellular DNA extracts were prepared on silica-based spin columns, and subjected to scintillation counting. Data are the mean ± S.E.M. of two independent experiments, each performed in duplicate.

HPMPC in A549 cells infected with herpes simplex virus type 1, adenovirus type 2 or vaccinia virus. Microscopical evaluation confirmed that, at the multiplicity of infection used, full-blown cytopathic effect was obtained within 3 days post infection. The compound concentration was reduced from 10 μM (as used in all previous studies) to 1 μM, at which concentration the compounds had no inhibitory effect on viral replication. In the case of HPMPC, the overall metabolism was not affected by HSV-1, Ad2 or VV infection (Table 3), although the relative formation of the diphosphate metabolite was slightly increased in Ad2-infected A549 cells. With regard to HPMP-5-azaC, the total sum of all deamination products (i.e. HPMP-5-azaU and its phosphorylated forms) was not altered by either of the three viruses. Whereas HSV-1 and VV appeared to have no effect on the formation of HPMP-5-azaC-diphosphate, the amount of this metabolite was 2.5-fold greater in Ad2-infected cells, and the total sum of all HPMP-5-azaC metabolites was increased accordingly.

Likewise, we found that in human T-lymphoblast HSB-2 cells infected with HHV-6A, or MOLT-3 cells infected with HHV-6B, virus infection did not influence the absolute levels or relative contribution of the different metabolites of HPMP-5-azaC (data not shown). In analogy to the data collected in A549 cells, the HPMP-5-azaC-diphosphate metabolite accounted for ~65% of total radioactivity, attaining similar intracellular levels (i.e., 2 and 6 pmol/$10^6$ HSB-2 and MOLT-3 cells, respectively, after 48 h incubation with 1 μM HPMP-5-azaC).

### 3.6. *Plasma elimination of HPMP-5-azaC in mice*

In a small pharmacokinetic study, the plasma concentrations of HPMP-5-azaC were determined at several time points (range: 0–8 h) after intravenous bolus injection in mice at a dose of 50 mg/kg. At the time of bleeding, all blood samples were immediately placed at 4 °C (to halt any ongoing deamination reactions) for a maximum of 45 min, until collection of plasma. HPLC analysis on plasma extracts revealed no signs of the deaminated metabolite HPMP-5-azaU. The plasma concentration of HPMP-5-azaC showed a mono-exponential decline, with an estimated elimination half-life of 9 min. Using an analytical detection limit of 0.5 μg/ml, HPMP-5-azaC was detected until 60 min after injection, at which time point the plasma concentration was 1.3 μg/ml.

## 4. Discussion

This biochemical study is focussed on the activation pathway of a new acyclic nucleoside phosphonate, the 5-azacytosine analogue of cidofovir (HPMPC). HPMP-5-azaC and HPMPC have a comparable broad antiviral activity spectrum which includes several DNA viruses, i.e. herpesviruses, poxviruses, polyomaviruses, papillomaviruses and adenoviruses [4–6,17,18]. However, depending on the virus species and host cell used for *in vitro* evaluation, the selectivity index of HPMP-5-azaC is 2- to 12-fold higher compared to that of HPMPC.

Our present data reveal some marked differences in the metabolic activation pathway of HPMP-5-azaC and HPMPC. First, whereas HPMPC is resistant to enzymatic deamination, we observed that HPMP-5-azaC is sensitive to deamination by dCMP deaminase, yielding the HPMP-5-azaU metabolite, which is subsequently phosphorylated to its mono- and diphosphate forms. The relative contribution of these deamination products in the overall metabolism of HPMP-5-azaC depended on the cell line, since the sum of HPMP-5-azaU and its mono- and diphosphates represented only 6% of all metabolites in human A549 fibroblast cells, compared to 35% in murine S49 lymphosarcoma cells. One explanation could be that HPMP-5-azaC is a better substrate for murine dCMP deaminase compared to the human enzyme. However, this is contradicted by our observations in human lymphoblast MOLT-3 cells incubated with HPMP-5-azaC, in which the total level of HPMP-5-azaU and its corresponding phosphates represented 27% of the total radioactivity (data not shown), which is comparable to the data in mouse S49 cells. We therefore presume that the increased deamination of HPMP-5-azaC in S49 and MOLT-3 cells (compared to A549 cells), can be explained by their higher proliferative capacity, resulting in a higher activity of dCMP deaminase, which has a role in the *de novo* synthesis of thymidine nucleotides and is

**Table 3 – Effect of virus infection on metabolism of HPMP-5-azaC and HPMPC**

| Compound[a] | Intracellular concentration (pmol/$10^6$ cells) after infection with[b] | | | |
|---|---|---|---|---|
| | Mock | HSV-1 | Ad2 | VV |
| HPMP-5-azaC | | | | |
| Sum of all C metabolites[c] [of which HPMP-5-azaC-pp][d] | 5.7 [4.1] | 8.0 [5.8] | 13.7 [10.0] | 4.6 [3.3] |
| Sum of all U metabolites[e] | 0.31 | 0.53 | 0.48 | 0.34 |
| Sum of X + Y + Z | 0.29 | 0.31 | 0.41 | 0.31 |
| Total sum of all metabolites | 6.4 | 9.0 | 14.8 | 5.2 |
| HPMPC | | | | |
| Total sum of all metabolites [of which HPMPC-pp][d] | 0.30 [0.04] | 0.25 [0.04] | 0.34 [0.07] | 0.29 [0.03] |

[a] Compound concentration: 1 μM.
[b] A549 cells were infected with HSV-1, adenovirus type 2 (Ad2) or vaccinia virus (VV), and incubated with radiolabeled compound during 48 h. After methanol extraction, the metabolites were quantified by HPLC analysis and scintillation counting.
[c] Combined concentration of HPMP-5-azaC, and its phosphocholine, monophosphate and diphosphate metabolites.
[d] Data between square brackets represent the intracellular concentration of the diphosphate metabolite of HPMP-5-azaC or HPMPC.
[e] Combined concentration of HPMP-5-azaU, and its monophosphate and diphosphate metabolites.

thus associated with cell proliferation [15]. Nevertheless, we conclude that deamination has no key role in the overall metabolism of HPMP-5-azaC, in contrast to other cytosine-containing agents such as gemcitabine [19], cytarabine [20], and 5-azacytidine or 5-aza-2′-deoxycytidine (decitabine) [21].

Instead, we found strikingly high intracellular levels for the diphosphate metabolite of HPMP-5-azaC, since this metabolite represented about 60% of all metabolites. In comparison, in cells incubated with HPMPC, the relative formation of HPMPC-diphosphate was only about 10%, whereas parent HPMPC together with its phosphocholine metabolite represented 66% of the total. Our data agree with published data on the metabolism of HPMPC in different cell lines, in which the relative formation was about 17%, 28% and 50%, for HPMPC-diphosphate, the phosphocholine metabolite and parent HPMPC, respectively [2,3,14].

HPMP-5-azaC-diphosphate is presumed to be the active metabolite, in analogy to HPMPC-diphosphate, that was shown to be recognized by cytomegalovirus DNA polymerase causing termination of viral DNA synthesis after incorporation of two consecutive HPMPC molecules [22]. The diphosphate levels obtained with HPMP-5-azaC were about 60-fold higher in absolute value than those measured for HPMPC. In parallel, we observed that incorporation of HPMP-5-azaC into cellular DNA is about 45-fold higher than that of HPMPC. These data implicate that the diphosphate metabolites of HPMP-5-azaC and HPMPC may be equally good substrates for cellular DNA polymerases. Whether this is also the case for viral DNA polymerases is currently unknown, since the HPMP-5-azaC-diphosphate needed for enzymatic (polymerase) assays has not yet been synthesized. However, the fact that the antiviral potency of HPMP-5-azaC exceeds that of HPMPC by no more than a factor 7 [4], despite a 60-fold higher phosphorylation rate for HPMP-5-azaC, suggests that HPMP-5-azaC-diphosphate is less effective than HPMPC-diphosphate in terms of substrate recognition by viral DNA polymerases and/or inhibition of viral DNA synthesis. In the absence of enzymatic data, this still remains a hypothesis. Likewise, the consequences of DNA incorporation of HPMP-5-azaC are still unclear. The acyclic side chain of HPMP-5-azaC is identical to that of HPMPC, for which incorporation leads to chain termination and inhibition of viral DNA synthesis, yielding prematurely ended and dysfunctional DNA fragments [22,23]. However, Magee et al. [24] recently showed that HPMPA, the adenine analogue of HPMPC, displays efficient incorporation into vaccinia virus DNA without causing much chain termination. Rather, the block in viral DNA synthesis appears to occur in the second replication round, due to inefficient primer extension from the viral DNA template containing incorporated HPMPA. Given that HPMP-5-azaC is more efficiently incorporated into cellular DNA than HPMPC, HPMP-5-azaC could have a similar indirect effect on cellular and/or viral DNA synthesis as HPMPA. Also, we cannot fully exclude that incorporated HPMP-5-azaC could bind DNA methyltransferase and induce a DNA-hypomethylating effect (due to the presence of the nitrogen atom at position 5 of the cytosine ring). DNA hypomethylation accounts for the antineoplastic activity of the anticancer drug 5-aza-2′-deoxycytidine [21]. Recent data reported by Hájek et al. [25] showed that the cytostatic activity of HPMP-5-azaC (observed at concentrations above 1000 μM) is due to an S-phase arrest (resulting from inhibition of cellular DNA synthesis), rather than to hypomethylation. Likewise, the high antiviral selectivity index of HPMP-5-azaC, which is in fact higher than that of HPMPC, indirectly argues against such hypomethylating activity.

Although enzymatic deamination appears to have no major impact on the metabolism of HPMP-5-azaC in uninfected cells, we wondered whether this may be different in virus-infected cells. Increased expression of dCMP deaminase in cells infected with cytomegalovirus has been reported [16], and attributed to the dependence of this virus on dNTP-synthesizing enzymes within host cells. We observed that the levels of HPMP-5-azaU and its phosphate metabolites were not increased after infection with HSV-1, adenovirus or vaccinia virus. For both HPMP-5-azaC and HPMPC, adenovirus infection resulted in a slight (2.5-fold) increase in the diphosphate level. Similarly, Cihlar and Chen reported that CMV-infection increases the HPMPC-diphosphate levels by 8-fold [2]. Our observation that, at least for adenovirus, infection appears to promote activation of HPMP-5-azaC rather than enhancing its catabolic deamination, is of course relevant to its potential use as an antiviral agent for the treatment of adenovirus infections.

Another clinically relevant finding is related to the long intracellular stability of HPMP-5-azaC-diphosphate and HPMP-5-azaC-phosphocholine, with an estimated half-life of 63 h and 103 h, respectively. This is very similar to the data reported for HPMPC (intracellular retention of HPMPC-disphosphate and HPMPC-phosphocholine: 65 h and 87 h, respectively) [26], for which the long intracellular stability is at the basis of its infrequent administration schedules in patients. Our preliminary *in vivo* data indicate that infrequent use of HPMP-5-azaC is indeed achievable. Adult mice were fully protected against vaccinia virus or HSV-1 infection when treated with a single dose of 50 mg/kg of either HPMP-5-azaC or HPMPC at the day of virus infection (Andrei, unpublished data). In this regard, the rapid plasma elimination of HPMP-5-azaC after intravenous bolus injection in mice, with an elimination half-life of 9 min, is of poor predictive value in the design of administration schedules. On the other hand, the lack of any signs of HPMP-5-azaU formation in these mice supports our *in vitro* biochemical data that catabolic deamination plays a minor role in the overall metabolism of HPMP-5-azaC.

The antiviral activity spectrum of HPMP-5-azaC is similar to that of its congener HPMPC, and, hence, its therapeutic use should be similar. Compared to HPMPC, the metabolism of HPMP-5-azaC displays some noticeable differences, i.e., markedly higher efficiency for conversion to the active diphosphate metabolite and consecutive incorporation into DNA, and slight sensitivity to catabolism by dCMP deaminase. On the other hand, HPMP-5-azaC shows a similarly long intracellular retention as HPMPC. The significance of these biochemical data should become clear from our ongoing *in vivo* studies in relevant animal models, in which we will perform a direct comparison between HPMP-5-azaC and HPMPC for their antiviral efficacy and safety profile (including their short- and long-term toxicity).

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