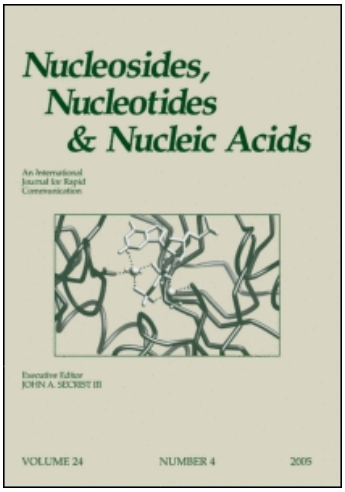

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Parker, William B. , Shaddix, Sue C. , Rose, Lucy M. , Pham, Phuong T. , Hua, Mei and Vince, Robert(2000) 'Metabolism of O⁶-Propyl and N⁶-Propyl-carbovir in CEM Cells', Nucleosides, Nucleotides and Nucleic Acids, 19: 4, 795 — 804
To link to this Article: DOI: 10.1080/15257770008035025
URL: http://dx.doi.org/10.1080/15257770008035025

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(4), 795–804 (2000)

METABOLISM OF O^6-PROPYL AND N^6-PROPYL-CARBOVIR IN CEM CELLS

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ABSTRACT: The metabolism of O^6-propyl-carbovir and N^6-propyl-carbovir, two selective inhibitors of HIV replication, has been evaluated in CEM cells. Both compounds were phosphorylated in intact cells to carbovir-5'-triphosphate. The metabolism of these two agents was inhibited by deoxycoformycin and mycophenolic acid, but not erythro-9-(2-hydroxy-3-nonyl)adenine. No evidence of the 5'-triphosphate of either compound was detected in CEM cells.

CBV (carbovir, carbocyclic analog of 2',3'-dideoxy-2',3'-didehydro-guanosine) has very good *in vitro* anti-HIV activity (1-4). It is metabolized by human enzymes to CBV-5'-triphosphate (CBV-TP) (5-8), which is a potent and selective inhibitor of the HIV reverse transcriptase (9-11). N^6-cyclopropyl-CBV, abacavir, is also metabolized to CBV-TP and has shown good anti-HIV activity in clinical trials as a single agent and in combination with other anti-HIV drugs (12,13). Abacavir (Ziagen™) has recently been approved by the FDA for use in the treatment of people infected with HIV.

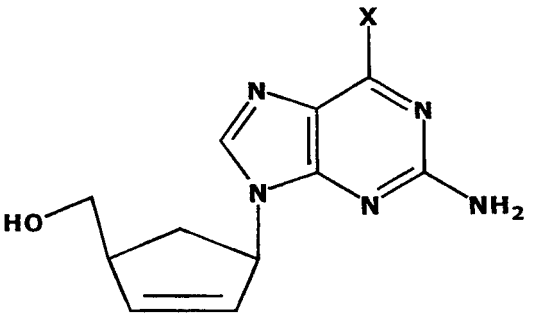
We have also created many 6-substituted CBV analogs and have tested them as inhibitors of HIV replication (14). Two of these compounds, O^6-propyl-CBV and N^6-propyl-CBV (Figure 1), demonstrated anti-HIV activity at concentrations that were similar to that of CBV. Therefore, in the current work the metabolism of these two agents in CEM cells has been evaluated to determine how their metabolism might differ from CBV or N^6-cyclopropyl-CBV.

MATERIALS AND METHODS

Synthesis of O^6-propyl-CBV and N^6-propyl-CBV. **(-)-(1S, 4R)-4-(2-Amino-6-*n*-propoxy-9H-purinyl-9-yl)-2-cyclopentenylcarbinol.** A mixture of (-)-(1S,4R)-4-(2-amino-6-chloro-9H-purinyl-9-yl)-2-cyclopentenylacetylcarbinol (15) (250 mg, 0.810 mmol) and sodium hydride (343 mg, 14.3 mmol) in propanol (25 ml) was heated under reflux for 1 h. The reaction mixture was evaporated in vacuo and the crude product was purified on a silica gel column. Elution with $CHCl_3$ followed by $CHCl_3$/MeOH(20:1) gave the title compound as a white solid (230 mg, 98%): mp 90-92°C; R_f = 0.24 ($CHCl_3$/MeOH, 20:1); ^{1}H NMR (DMSO d6) δ 7.76 (s, 1H, 8-H), 6.34 (s, 2H, NH_2, exchangeable), 6.12 (m 1H, vinyl C-H), 5.88 (m. 1H, vinyl C-H), 5.44 (m, 1H, 4'H), 4.71 (t, 1H, OH, exchangeable), 4.36 (t, 2H, $OCH_2CH_2CH_3$), 3.45 (t, 2H, CH_2OH), 2.88 (m, 1H, 1'H), 2.60 (dt, 1H, CHH), 1.76 (m, 2H, $OCH_2CH_2CH_3$, 0.97 (t, 3H, CH_3); $[\alpha]_D25$ - 88.7° (c, 0.30, MeOH); MS Anal. Calc'd for $C_{14}H_{19}N_5O_2$: C, 58.15; H, 6.62; N, 24.21. Found: C, 58.35; H, 6.79; N, 24.37.

(-)-(1S, 4R)-4-(2-Amino-6-*n*-propylamino-9H-purinyl-9-yl)-2-cyclopentenylcarbinol. A solution of (-)-(1S,4R)-4-(2-amino-6-chloro-9H-purinyl-9-yl)-2-cyclopentenyl-acetylcarbinol (50 mg, 0.16 mmol) in EtOH (2 ml) and *n*-propylamine (6 ml) was heated under reflux for 8 h. The solution was cooled and 1N NaOH (0.2 ml) was added. The volatile materials were removed in vacuo and the crude product was purified on a silica gel column. Elution with $CHCl_3$/MeOH(50:1). The crude product was crystallized from EtOAc and gave the title compound as a white solid (39 mg, 84%): mp 144-146°C; 1H NMR ($CDCl_3$) δ: 7.52 (s, 1H, H-8), 5.82 and 6.14 (dd, 2H, CH=CH), 5.42 (m, 1H, 4'H), 5.00-5.30 (broad 2H, NH_2), 3.92 (m, 2H, OCH_2), 3.15 (m, 1H, 1'H), 2.80 (m, 1H, 5'H a), 2.18 (m, 1H, 5'H b), 1.70 (m, 2H, CH_2CH_3), 1.00 (t, 3H CH_3); $[\alpha]_D25$ = -59.7° (c = 0.30, MeOH); Anal. Calc'd for $C_{14}H_{20}N_6O$: C, 58.31; H, 6.99; N, 29.15. Found: C, 58.10; H, 6.89; N, 28.96.

Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Melting points were determined on a Mel-Temp apparatus and are corrected. Nuclear magnetic resonance spectra were obtained on a General Electric, 300. Thin layer chromatography was performed on 0.25 mm layers of EM Science silica gel and column chromatography was done on Whatman 60 (230-400 mesh).



X = OH (CBV)
X = $OCH_2CH_2CH_3$ (O-PROPYL-CBV)
X = $NHCH_2CH_2CH_3$ (N-PROPYL-CBV)
X = NH- (N-CYCLOPROPYL-CBV, ABACAVIR)

Figure 1. Structure of CBV, N⁶-propyl-CBV, O⁶-propyl, and N⁶-cyclopropyl-CBV.

Materials. The [-] enantiomers of [^{3}H]O⁶-propyl-CBV (600 Ci/mole), [^{3}H]N⁶-propyl-CBV (300 Ci/mole), and [^{3}H]CBV (2500 Ci/mole) were obtained from Moravek Biochemicals (Brea, CA). These radiolabeled compounds were greater than 95% pure based on HPLC analysis. The impurities associated with O⁶-propyl-CBV or N⁶-propyl-CBV did not elute with CBV. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was obtained from Sigma Chemical Company (St. Louis, MO). 2'-Deoxycoformycin (dCF) was obtained from the National Cancer Institute (Bethesda, MD). CCRF-CEM cells, obtained from the American Type Culture Collection (Rockville, MD), were grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 100 μg/ml penicillin, 100 units/ml streptomycin, 20 μg/ml gentamycin, and 25 mM HEPES buffer. Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. All experiments were conducted with cells that were proliferating at maximal rates. The anti-HIV activity of these compounds was performed by Dr. R.W. Buckheit Jr. as described (16). All other chemicals used were of standard analytical grade.

Measurement of acid-soluble metabolites. Cells incubated with radiolabeled compounds were collected by centrifugation and resuspended in ice-cold 0.5 M perchloric

acid. The samples were centrifuged at 12,000 x g for 5 minutes to remove acid-insoluble material, and the supernatant fluid was neutralized with 1 M potassium phosphate (pH 7.4) and 4 M KOH as described (7). Precipitated $KClO_4$ was removed by centrifugation, and a portion of the supernatant fluid was injected onto a strong anion exchange column (Partisil-10 SAX, Keystone Scientific Inc, State College, PA). Elution of the nucleotides was accomplished with a 50-minute linear gradient from 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.7) buffer with a flow rate of 2 ml/min. At the end of the 50-minute gradient the column was washed with 750 mM $NH_4H_2PO_4$ (pH 3.7) for another 20 minutes. This extended wash was necessary to elute CBV-TP from the column. The natural nucleotides were detected by measurement of the UV absorbance at 260 nm, and the radioactive acid-soluble metabolites were detected by counting 1 minute fractions that eluted from the column.

To determine the identity of the nucleoside portion of the nucleotide metabolites found in cells treated with CBV, O^6-propyl-CBV, or N^6-propyl-CBV, the peak fractions eluting from the SAX HPLC column were collected separately and diluted with an equal volume of water. The pH was adjusted to approximately 8.5 with NaOH, and 0.3 units of phosphodiesterase (Pharmacia, Piscataway, NJ) and 100 units of alkaline phosphatase (Sigma Chemical Company, St. Louis, MO) were added to each sample. After incubation at 37°C for 16 to 20 hours the reaction was stopped by boiling for 5 minutes, and the precipitated proteins were removed by centrifugation. The samples were then injected onto a 250 x 4.6 mm Spherisorb ODS reverse phase column (Keystone Scientific, Bellefonte, PA) equilibrated with a 50 mM $NH_4H_2PO_4$ buffer mixed with acetonitrile (95 to 5: V/V). CBV, O^6-propyl-CBV, and N^6-propyl-CBV were separated from each other and the natural nucleosides using a 20 minute gradient from 5% to 50% percent acetonitrile in 50 mM $NH_4H_2PO_4$. Retention times for cytidine, deoxycytidine, uridine, thymidine, adenosine, deoxyadenosine, CBV, N^6-propyl-CBV, and O^6-propyl-CBV in this system were 2.0, 2.5, 2.5, 4.8, 5.6, 6.4, 11.3, 21.2, and 22.2 minutes, respectively.

RESULTS AND DISCUSSION

In previous studies we have determined that CBV, O^6-propyl-CBV, and N^6-propyl-CBV have activity against HIV replication in MT-2 cells (14). Because the anti-HIV activity of N^6-propyl-CBV was determined with the racemic mixture (14), we have

repeated the evaluation of the [-] enantiomer of these three compounds against HIV in CEM cells. The concentration of compound (CBV, O^6-propyl-CBV, and N^6-propyl-CBV) that resulted in 50% reduction of HIV replication was 2.0, 2.2, and 6.0 μM, respectively (average of two determinations). In MT-2 cells, the concentration of compound that inhibited HIV replication by 50% were: 0.4 μM for [-]CBV; 2.5 μM for [-]O^6-propyl CBV; and 16 μM for [±]N^6-propyl CBV (14). The IC_{50} for inhibition of MT-2 cell growth was 98 μM for O^6-propyl CBV, but was greater than 100 μM for CBV and N^6-propyl CBV, which indicated that these compounds had good selectivity for inhibition of HIV replication. In the current work we have characterized the metabolism of the [-] enantiomer of these three compounds in CEM cells.

Because of the similarities in structure between O^6-propyl-CBV, N^6-propyl-CBV, and N^6-cyclopropyl-CBV, it was of interest to determine if O^6-propyl-CBV and N^6-propyl-CBV were converted to CBV-TP as is seen with N^6-cyclopropyl-CBV (13). The nucleotide metabolites that were formed in CEM cells treated with [^{3}H]O^6-propyl-CBV and [^{3}H]N^6-propyl-CBV were separated using SAX HPLC, and a representative profile is shown in Figure 2. The nucleotide metabolites that were formed in CEM cells treated with [^{3}H]CBV were also determined in each experiment to identify the retention times of the CBV nucleotides, but these data are not shown. As can be seen in Figure 2, CBV-TP was detected in CEM cell extracts treated with either O^6-propyl-CBV or N^6-propyl-CBV. Carbovir was the only nucleoside detected when the fractions eluting between 45 and 50 minutes were collected, degraded to their respective nucleosides, and analyzed by reverse phase HPLC, which confirmed that this peak was CBV-TP. In addition, authentic CBV-TP eluted from the SAX HPLC column in this same position, and the peak of radioactivity eluting between fraction 45 and 50 coincided with the CBV-TP peak seen in cells treated with CBV. The amount of CBV-TP formed after incubation with 10 μM of either N^6-propyl-CBV or O^6-propyl-CBV was 6 ± 3 or 32 ± 10 percent of that seen after incubation with 10 μM of CBV (Figure 3). Assuming that 1 pmole/10^6 cells is equivalent to 1 μM intracellular concentration, the concentrations of triphosphate formed in 24 hours in CEM cells from 10 μM of each compound was 25 nM for N^6-propyl CBV, 115 nM for O^6-propyl CBV, and 345 nM for CBV, which were in the appropriate range to inhibit HIV-reverse transcriptase (K_i for CBV-TP is 30 to 50 nM, see references 8, 10, and 11).

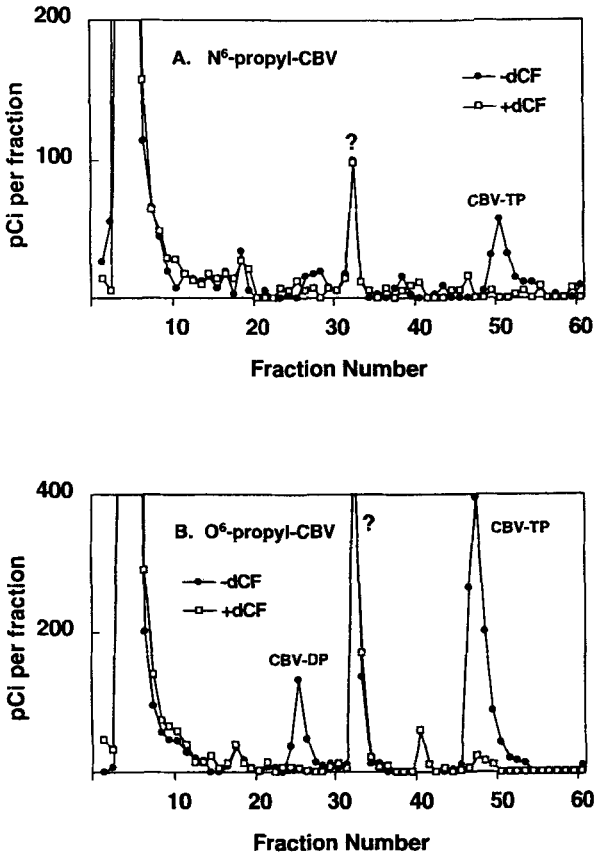


Figure 2. Effect of deoxycoformycin on the metabolism of N^6-propyl-CBV and O^6-propyl CBV. CEM cells (approximately 750,000 cells/ml) were incubated for 8 hours with 6.7 μM of [^{3}H]N^6-propyl-CBV (300 Ci/mole) (Panel A) or 8.3 μM of [^{3}H]O^6-propyl-CBV (600 Ci/mole) (Panel B) in the presence and absence of 10 μM deoxycoformycin. The acid-soluble intracellular metabolites were collected and analyzed by SAX HPLC as described in the Materials and Methods. One minute fractions were counted for radioactivity. This experiment was repeated one time with similar results.

Addition of dCF decreased the amount of CBV metabolites that were formed from both O^6-propyl-CBV and N^6-propyl-CBV (Figure 2). However, EHNA did not affect the metabolism of either compound (data not shown). Because EHNA inhibits adenosine deaminase activity, whereas deoxycoformycin inhibits adenosine deaminase, AMP deaminase, and a newly discovered enzyme which is also capable of deaminating AMP and its analogs (13), these results indicated that both compounds were phosphorylated to their

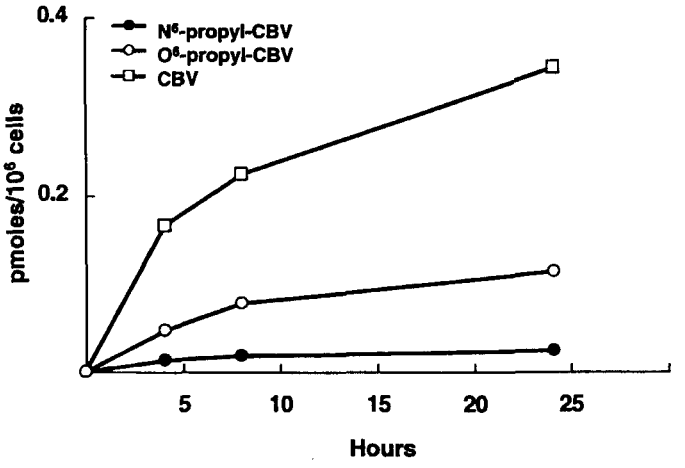


Figure 3. Phosphorylation of N^6-propyl-CBV, O^6-propyl CBV, and CBV. CEM cells (840,000 cells/ml) were incubated with 10 μM of N^6-propyl-CBV (300 Ci/mole), O^6-propyl CBV (600 Ci/mole), or CBV (50 Ci/mole). An aliquot was taken from each incubation 4, 8, or 24 hours after the addition of compounds, and the amount of label in the triphosphate peak of each cell sample was determined using SAX HPLC as described in the Materials and Methods. This particular experiment is representative of 4 or 6 experiments that measured the quantitative differences in the phosphorylation of these three compounds (N^6-propyl-CBV or O^6-propyl CBV, respectively).

respective monophosphates prior to deamination. Treatment with deoxycoformycin decreased both CBV-TP formation and anti-HIV activity (14), which supports the conclusion that CBV-TP is the active metabolite of both N^6-propyl-CBV and O^6-propyl-CBV.

Treatment with mycophenolic acid, an inhibitor of IMP dehydrogenase, did not increase the metabolism of either O^6-propyl-CBV or N^6-propyl-CBV. Incubation in the presence of 5 μM mycophenolic acid resulted in a decrease in the formation of CBV-TP from both O^6-propyl CBV and N^6-propyl CBV (51% and 17% of control, respectively, average of two experiments). In experiments conducted at the same time, mycophenolic acid increased the metabolism of CBV by 30-fold (average of 2 determinations). Inhibition of IMP dehydrogenase activity by mycophenolic acid results in an increase in the intracellular concentration of IMP, which is the phosphate donor for 5'-nucleotidase. Because the 5'-nucleotidase reaction in cells is limited by the amount of IMP that is

ıvailable, increasing its concentration increases the activity of 5'-nucleotidase activity and increases the phosphorylation of nucleosides, such as CBV (6,7). Therefore, these results indicated that O^6-propyl-CBV or N^6-propyl-CBV were phosphorylated by an enzyme other than 5'-nucleotidase.

A significant peak of radioactivity eluting around fraction 30 was consistently detected in the SAX HPLC chromatograms of acid-soluble extracts prepared from cells treated with either O^6-propyl-CBV or N^6-propyl-CBV. A similar peak of radioactivity was detected in CEM cells that were treated with N^6-cyclopropyl-CBV (13). The retention time of this peak was very similar to that of ATP, dATP, CTP, dCTP, UTP, and TTP. Because it was possible that this peak could represent the 5'-triphosphate of the parent compound, this peak fraction was collected as it eluted from the HPLC column and degraded with phosphodiesterase and alkaline phosphatase. However, the radioactivity did not elute with authentic standards of either O^6-propyl-CBV or N^6-propyl-CBV, which indicated that this peak of radioactivity was not a phosphorylated metabolite of either compound. The HPLC method that was used resolved both O^6-propyl-CBV and N^6-propyl-CBV from CBV and natural nucleosides, such as adenosine, deoxyadenosine, uridine, thymidine, cytosine, and deoxycytidine, whose triphosphates eluted close to the unknown peak. Guanosine nucleosides were not considered, because the unknown peak was clearly separated from GTP and dGTP in the SAX HPLC system. The peak of radioactivity also did not elute with any of the natural nucleosides. The identity of this peak is still not known, but it is likely due to a contaminant of the radiolabeled compounds used in these experiments (7,13). The radioactive peak eluting at fraction 40 in Figure 2B had a retention time similar to that of GTP, and is also likely due to a contaminant in the radiolabeled O^6-propyl-CBV.

Falletto et al. (13) have shown that the inhibition of HIV reverse transcriptase by CBV-TP is responsible for the anti-HIV activity of N^6-cyclopropyl-CBV. These investigators showed that this compound is phosphorylated in CEM cells by adenosine phosphotransferase to N^6-cyclopropyl-CBV-5'-monophosphate, which is not further phosphorylated, but is instead deaminated by an enzyme other than AMP deaminase to CBV-5'-monophosphate. GMP kinase and nucleoside diphosphate kinase then convert CBV-5'-monophosphate to CBV-TP. In these studies (13) mycophenolic acid and EHNA did not affect the metabolism of N^6-cyclopropyl-CBV, and dCF prevented the conversion

of N^6-cyclopropyl-CBV to CBV-TP. The results reported in the current work indicated that the metabolism of both O^6-propyl-CBV and N^6-propyl-CBV in CEM cells was very similar to that of N^6-cyclopropyl-CBV in CEM cells.

Acknowledgments. This work was supported by NIH grant numbers AI29157 and CA23263.

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