FEBS Letters 573 (2004) 38–44 FEBS 28704

# Improved antiviral activity of the aryloxymethoxyalaninyl phosphoramidate (APA) prodrug of abacavir (ABC) is due to the formation of markedly increased carbovir 5′-triphosphate metabolite levels

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Received 25 May 2004; revised 15 July 2004; accepted 19 July 2004

Available online 28 July 2004

Edited by Hans-Dieter Klenk

**Abstract The anti-human immunodeficiency virus (HIV) activity of abacavir (ABC; 1-(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol) could be markedly enhanced by administering the aryloxymethoxyalaninyl phosphoramidate prodrug derivative of ABC (pro-ABC-MP) to virus-infected cell cultures. Metabolic studies with radiolabeled ABC and pro-ABC-MP in human T-lymphocyte and primary macrophage cell cultures revealed a significantly increased delivery of the activated (phosphorylated) metabolite of ABC (ABC-MP) by pro-ABC-MP, and the concomittant appearance of markedly higher intracellular levels of carbovir 5′-triphosphate (CBV-TP), which represents the eventual antivirally active metabolite of ABC. The intracellular amounts of ABC-MP and appearance of CBV-TP closely correlated with the extracellular pro-ABC-MP concentrations that were administered to the cell cultures within a concentration range between 0.5 and 100 μM. The highest amounts of CBV-TP were observed within 6–24 h after drug administration. The improved delivery of ABC-MP and metabolic conversion to CBV-TP explain the markedly enhanced antiviral activity of the prodrug of ABC, and warrant further exploration of this prodrug technology on ABC and related compounds to further enhance and optimize their antiviral efficacy.**


*Keywords:* Abacavir; Carbovir; Reverse transcriptase; Human immunodeficiency virus; Aryloxyphosphoramidate; Nucleotide prodrug; Antiviral chemotherapy

## 1. Introduction

Abacavir (ABC) (Fig. 1) represents one of the newer nucleoside reverse transcriptase inhibitors (NRTIs) that are clinically used to treat human immunodeficiency virus (HIV)-infected individuals [1]. Metabolic studies on ABC revealed that it was predominantly anabolised to its 5′-monophosphate (ABC-MP) by an adenosine phosphotransferase and subsequently converted to the deaminated product carbovir 5′-monophosphate (CBV-MP) by a distinct cytosolic deaminase different from adenylate deaminase [2,3]. CBV-MP is then converted in two successive steps to CBV-5′-triphosphate (CBV-TP) by cellular kinases [2]. CBV-TP is the antivirally active metabolite of ABC and inhibits the virus-encoded RT enzyme by acting as DNA chain terminator. The maximum velocity ($V_{max}$) for ABC to be converted by adenosine phosphotransferase was ~55% of the corresponding value for adenosine, but the phosphorylating capacity of this enzyme for ABC ($V_{max}/K_m$) was only 3.6% of the value for adenosine [2]. Also, ABC proved to be a poor substrate for 5′-nucleotidase (inosine phosphotransferase), in contrast with its deamination product CBV which was rather efficiently converted to CBV-MP by 5′-nucleotidase [4,5]. Thus, ABC is not very efficiently converted to its monophosphate derivative by cellular enzymes. In fact, beside ABC, the majority of antiviral nucleoside analogues active against HIV have a relatively poor affinity for their activating (phosphorylating) enzyme [6]. Examples of a relatively poor conversion rate by the activating enzyme other than ABC are stavudine (d4T) by thymidine kinase (TK) [7,8], zalcitabine (ddC) by 2′-deoxycytidine kinase [9,10], and didanosine (ddI) by 5′-nucleotidase [4,5]. Therefore, several attempts have been undertaken to deliver activated (phosphorylated) nucleoside analogues directly into the virus-infected target cells [11–24]. One of these approaches has proven very successful when applied on thymidine analogues such as d4T. Indeed, the aryloxymethoxyalaninyl phosphoramidate (APA) prodrug derivative of d4T proved highly successful in retaining anti-HIV activity in dThd kinase-deficient CEM cells, whereas the parent d4T compound had poor, if any, antiviral activity in this cell line [11,12]. However, when

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*Abbreviations:* CBV, carbovir; ABC, abacavir; CBV-MP, CBV-5′-monophosphate; CBV-DP, CBV-5′-diphosphate; CBV-TP, CBV-5′-triphosphate; ABC-MP, ABC-5′-monophosphate; ABC-DP, ABC-5′-diphosphate; ABC-TP, ABC-5′-triphosphate; Ala-ABC-MP, alaninyl-ABC-MP; pro-ABC-MP, aryloxymethoxyalaninyl phosphoramidate prodrug of ABC

0014-5793/$22.00 
doi:10.1016/j.febslet.2004.07.049

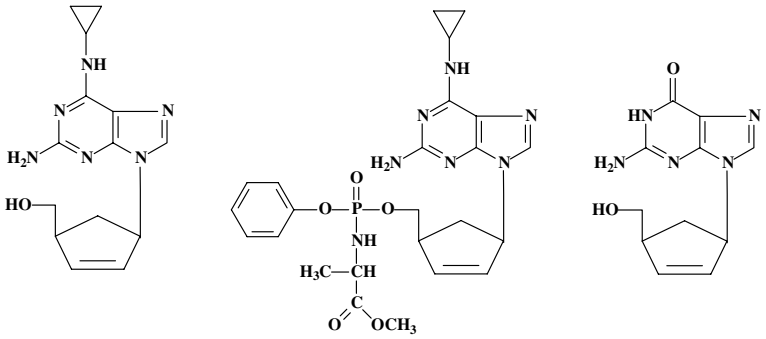


Fig. 1. Structural formulae of test compounds.

the same prodrug technology was applied on zidovudine (AZT), another TK-dependent nucleoside analogue, no significant antiviral activity was retained in TK-deficient CEM cells [12] as also the parent AZT compound failed to do so. However, it is currently still unclear whether this is due to failure of efficient release of AZT-MP from the prodrug molecule or, alternatively, whether it is due to unfavorable kinetics of AZT-MP release allowing other enzymes [i.e., 5′-(deoxy)nucleotidases], to dephosphorylate AZT-MP before the nucleotide kinases (dTMP kinase in the particular case) can further anabolize the monophosphate derivative of AZT. Thus, the aryloxymethoxyalaninyl phosphoramidate prodrug technology is not equally efficient for all types of nucleoside analogues with regard to the eventual conversion of the parent drugs to the triphosphate derivative [25].

It was important to reveal whether the APA approach results in an improvement of antiviral activity when applied on ABC. In particular, a successful direct delivery of ABC-MP into virus-infected target cells may have at least two advantages. (i) Higher amounts of activated ABC-MP can be delivered into the intact cells circumventing the first poor phosphorylation step by the adenosine phosphotransferase and leading to improved antiviral efficacy of ABC. (ii) By directly delivering ABC-MP into the cells, conversion of ABC to its deaminated product CBV at the nucleoside level can be avoided. Although CBV has also proven to be an efficient anti-HIV drug [26], it shows pronounced side-effects (i.e., renal and cardiac toxicity) that are thought to be due, at least in part, to the parent drug itself rather than to one of its phosphorylated derivatives [27,28]. Thus, efficiently delivering the phosphorylated derivative of ABC would not only enhance its antiviral potency by circumventing the relatively poor phosphorylating step by adenosine phosphotransferase, but may also further improve its safety profile in the clinic. Therefore, the antiviral activity and metabolic conversion pathway of ABC and its APA derivative (pro-ABC-MP) have been studied in human T-lymphocyte and primary macrophage cell cultures.

## 2. Materials and methods

### 2.1. Cells

Human lymphocyte CEM cells were obtained from the American Tissue Culture Type Collection (ATCC) (Rockville, MD) and human lymphocyte MT-4 cells were obtained from Prof. N. Yamamoto (at that time at Tokyo University, Japan).

Monocyte/macrophages (M/M) were obtained from the blood of healthy seronegative donors. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque gradient and seeded in plastic 48-well plates (Costar, Cambridge, MA) at $1.8 \times 10^6$ cells/ml in RPMI 1640 (Gibco Labs, Gaithersburg, MD) with the addition of 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 20% heat-inactivated, mycoplasma-free and endotoxin-free fetal calf serum (Hyclone, Logan, UT). On the sixth day of culture, non-adherent cells were removed by repeated gentle washing with warm medium. Adherent cells obtained with this technique consist of over 95% differentiated M/M. Cells were always cultured in a humidified environment containing 5% $CO_2$ at 37 °C.

### 2.2. Viruses

HIV-1 (strain $III_B$ and Ba-L) was obtained from Dr. R.C. Gallo and Dr. M. Popovic (at that time at the National Institutes of Health, Bethesda, MD) and HIV-2 (strain ROD) was obtained from Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France).

### 2.3. Anti-HIV activity of drugs in CEM, MT-4 and monocyte/macrophage cell cultures

The activity of test compounds against HIV-1- and HIV-2-induced cytopathicity was examined in CEM and MT-4 cell cultures at days 4–5 post-infection, and the antiviral activity of the test compounds was estimated by microscopical examination of virus-induced giant cell formation (CEM) or virus-induced destruction of the cell cultures (MT-4). HIV-1 and HIV-2 were added at 100 $CCID_{50}$ to the cell cultures [11,14].

M/M cell cultures were exposed to various concentrations of abacavir or its aryloxymethoxyalaninyl phosphoramidate derivative pro-ABC-MP for 20 min, then M/M were challenged with 300 $TCID_{50}$/ml of HIV-$1_{Ba\text{-}L}$. After 2 h of incubation, M/M were extensively washed with warm medium to remove the excess of virus and then cultured in the presence of the same concentrations of drug under the same conditions as before. M/M were washed and fed every 7 days. Supernatants were collected at day 14 after virus challenge, and virus production was determined by the antigen-capture assay using a commercially available kit. For the assessment of cytotoxicity, mock-infected M/M were treated with various concentrations of the test compounds. Assessment of the cytotoxic effect was performed twice weekly by visual inspection and then by counting cells and by trypan blue dye exclusion at day 14 after the beginning of drug treatment.

### 2.4. Metabolism of [$^3$H]abacavir and its aryloxymethoxyalaninyl phosphoramidate derivative pro-[$^3$H]ABC-MP

The metabolism of radiolabeled [$^3$H]abacavir ([$^3$H]ABC) and its radiolabeled pro-tide aryloxymethoxyalaninyl abacavir monophosphate (pro-[$^3$H]ABC-MP) was studied in human T4 lymphocyte

CEM and monocyte/macrophage cell cultures according to previously established procedures [11,17]. Briefly, CEM cells were seeded at $2–4 \times 10^5$ cells/ml in RPMI-1640 culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.075% $NaHCO_3$. In a first set of experiments, 5-ml cell suspensions in 25 $cm^2$ culture flasks were incubated with 0.5 μM [$^3$H]ABC or 0.5 μM pro-[$^3$H]ABC-MP (5 μCi/5 ml) for different time periods (i.e., 2, 6, 24, 48 and 72 h). At the indicated time points, the drug-treated cell cultures were centrifuged, washed twice with cold RPMI-1640 medium, and precipitated with cold methanol:water (60:40). In a second set of experiments, 5-ml cell suspensions were incubated with a variety of concentrations of [$^3$H]ABC (0.5, 1.5, 10, 100 μM) or pro-[$^3$H]ABC-MP (0.1, 1.1, 10, 100 μM) (5 μCi/5 ml). At 24 h, drug-treated cells were centrifuged, washed and precipitated as described above. After centrifugation, the supernatants of the samples were subjected to HPLC analysis and separated on a Partisphere SAX column (Whatman, Clifton, NJ). A linear gradient of 0.005 M $(NH_4)H_2PO_4$ (pH 5.0) (buffer A) to 0.30 M $(NH_4)H_2PO_4$ (pH 5.0) (buffer B) was used. The retention times of the following metabolites were as follows: ABC, carbovir (CBV) and pro-ABC-MP: 2–3 min; ABC-MP (abacavir-5′-monophosphate): 7–8 min; CBV-MP (carbovir-5′-monophosphate) and alaninyl ABC-MP (Ala-ABC-MP): 11–13 min; CBV-DP (carbovir-5′-diphosphate): 19–21 min; CBV-TP (carbovir-5′-triphosphate): 35–38 min.

Primary M/M were obtained from normal seronegative blood donors. Peripheral blood mononuclear cells separated on a Ficoll-Hypaque gradient were seeded in 5-ml plastic well plates at $\sim 1.8 \times 10^6$ cells/ml in RPMI-1640 medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 20% heat-inactivated mycoplasma/endotoxin-free fetal calf serum (Hyclone, Logan, UT). After 5 days, non-adherent cells were removed by repeated gentle washings with warm medium. Such cultures consist of $\geqslant 95\%$ mature macrophages. [$^3$H]ABC (0.5 μM) and pro-[$^3$H]ABC-MP (0.5 μM) were then added to the M/M cultures for 6 or 24 h. After this incubation time period, the M/M were detached, counted in a haematocytometer chamber, and processed for radiolabeled metabolite determination as described above. The total number of M/M used in the individual experiments varied from $1.53 \times 10^6$ to $2.19 \times 10^6$ cells.

## 3. Results

### 3.1. Antiviral activity of ABC and pro-ABC-MP in HIV-infected cell cultures

The anti-HIV-1 and -HIV-2 activity of ABC and its aryloxymethoxyalaninyl phosphoramidate prodrug derivative (pro-ABC-MP) were evaluated in CEM, MT-4 and M/M cell cultures (Table 1). Carbovir (CBV) and its prodrug pro-CBV-MP were included for comparative purpose. The antiviral activity of the prodrug of abacavir against HIV-1 and HIV-2 was substantially increased when compared with the parent compound ABC both in CEM (50–70-fold) and MT-4 (~30-fold) cell cultures. In contrast, the antiviral activity of carbovir (the deamination product of ABC) could not significantly be boosted when its prodrug derivative was administered to the HIV-infected cell cultures. The antiviral activity of pro-ABC-MP was not only improved, but its antiviral selectivity (ratio $CC_{50}/EC_{50}$) in the CEM cell cultures had also increased by 6- to 8-fold when compared

Table 1
Inhibitory activity of test compounds against HIV in cell culture

| Compound | $EC_{50}$[a] (μM) | | | | | $CC_{50}$[b] (μM) | | |
|---|---|---|---|---|---|---|---|---|
| | CEM | | MT-4 | | M/M | CEM | MT-4 | M/M |
| | HIV-1 | HIV-2 | HIV-1 | HIV-2 | Ba-L | | | |
| ABC | 2.3 ± 1.0 | 4.2 ± 1.7 | 5.9 ± 0.03 | 7.3 ± 1.2 | 0.1 ± 0.05 | 97 ± 34 | 192 ± 13 | 150 ± 30 |
| Pro-ABC-MP | 0.05 ± 0.01 | 0.07 ± 0.02 | 0.20 ± 0.06 | 0.26 ± 0.04 | 0.01 ± 0.002 | 13 ± 1.7 | 4.6 ± 0.04 | 110 ± 10 |
| CBV | 2.0 ± 0.0 | 2.3 ± 2.4 | – | – | – | 157 ± 12 | – | – |
| Pro-CBV-MP | 1.3 ± 0.66 | 0.85 ± 0.21 | – | – | – | 123 ± 13 | – | – |

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity in MT-4 or syncytium formation in CEM cell cultures.
[b] 50% Cytostatic concentration, or compound concentration required to inhibit CEM or MT-4 cell proliferation by 50%.

Table 2
Metabolism of [$^3$H]ABC and pro-[$^3$H]ABC-MP in CEM cell cultures in function of incubation time

| Time (hours) | 0.5 μM [$^3$H]ABC (pmol/$10^9$CEM cells) | | | | |
|---|---|---|---|---|---|
| | ABC + CBV fr. 2–3 | ABC-MP fr. 7–8 | CBV-MP fr. 10–12 | CBV-DP fr. 20–21 | CBV-TP fr. 37–38 |
| 2 | 239 ± 112 | 0.99 ± 1.4 | 2.2 ± 1.2 | 0.92 ± 1.3 | 2.5 ± 3.5 |
| 6 | 137 ± 30 | ND | 2.5 ± 3.5 | 1.7 ± 0.69 | 5.0 ± 1.9 |
| 24 | 62 ± 11 | 0.73 ± 1.0 | 4.5 ± 2.3 | 0.76 ± 1.0 | 4.3 ± 2.5 |
| 48 | 100 ± 33 | 1.7 ± 0.21 | 3.1 ± 0.57 | 1.9 ± 0.43 | 1.1 ± 0.45 |
| 72 | 74 ± 24 | 1.2 ± 0.01 | 3.9 ± 0.70 | 2.4 ± 0.06 | 0.57 ± 0.60 |
| | 0.5 μM pro-[$^3$H]ABC-MP (pmol/$10^9$ CEM cells) | | | | |
| | Pro-ABC-MP + ABC + CBV fr. 2–3 | ABC-MP fr. 6–7 | CBV-MP + Ala-ABC-MP fr. 11–13 | CBV-DP fr. 19–21 | CBV-TP fr. 35–38 |
| 2 | 136 ± 1.41 | 4.7 ± 0.8 | 92 ± 1.9 | 6.0 ± 1.8 | 7.5 ± 3.7 |
| 6 | 111 ± 37.4 | 3.8 ± 0.53 | 94 ± 1.7 | 15 ± 2.2 | 47 ± 8.5 |
| 24 | 77 ± 68 | 3.4 ± 1.0 | 54 ± 30 | 25 ± 12 | 30 ± 14 |
| 48 | 24 ± 13 | 1.4 ± 0.57 | 26 ± 7.4 | 8.9 ± 1.7 | 9.2 ± 2.7 |
| 72 | 13 ± 1.6 | 0.64 ± 0.46 | 8.0 ± 0.24 | 4.3 ± 0.28 | 3.2 ± 0.88 |

ND: not detectable. Data are means (±S.D.) of two independent experiments.

with parental ABC (Table 1). In the MT-4 cell model, antiviral activity was markedly improved, but the antiviral selectivity was not substantially changed. As observed for HIV-1-infected lymphocytes, the antiviral activity of Pro-ABC-MP in M/M was also markedly greater than that of ABC ($EC_{50}$s, 0.01 and 0.1 μM, respectively) (Table 1). The antiviral selectivity of pro-ABC-MP in M/M increased more than 6-fold when compared with the parent ABC drug (Table 1).

### 3.2. Metabolism of [$^3$H]ABC and pro-[$^3$H]ABC in CEM cell cultures as a function of different incubation times

After exposure of CEM cell cultures to 0.5 μM [$^3$H]ABC and 0.5 μM pro-[$^3$H]ABC-MP, the intracellular levels of the parent compounds and their metabolites were measured at different time points during drug exposure (Table 2). In the [$^3$H]ABC-exposed cell cultures, ABC-MP was detected as well as the 5′-mono-, 5′-di- and 5′-triphosphates of carbovir. The highest levels of CBV-TP, the antivirally active metabolite of ABC, were formed within 6–24 h post-exposure of the drug to the cell cultures (4.3–5.0 pmol/$10^9$ cells). At later time points (i.e., 48 and 72 h), CBV-TP levels progressively decreased to ∼one-tenth of their highest levels recorded after 24 h. Instead, the ABC-MP and CBV-MP levels kept fairly constant during the whole time incubation period (0.73–1.7 pmol/$10^9$ cells for ABC-MP and 2.5–4.5 pmol/$10^9$ cells for CBV-MP).

When the CEM lymphocyte cell cultures were incubated with the aryloxymethoxyalaninyl phosphoramidate prodrug of ABC, ∼10-fold higher levels of CBV-TP (30–70 pmol/$10^9$ cells) were recorded within 6–24 h compared with ABC-incubated cells. All other metabolites (i.e., ABC-MP, CBV-MP and CBV-DP) were also more abundantly formed in the pro-ABC-MP than in the ABC-treated cell cultures, especially during the first 24 h of incubation. Also the alaninyl derivative of ABC-MP (Ala-ABC-MP) was released from pro-ABC-MP but could

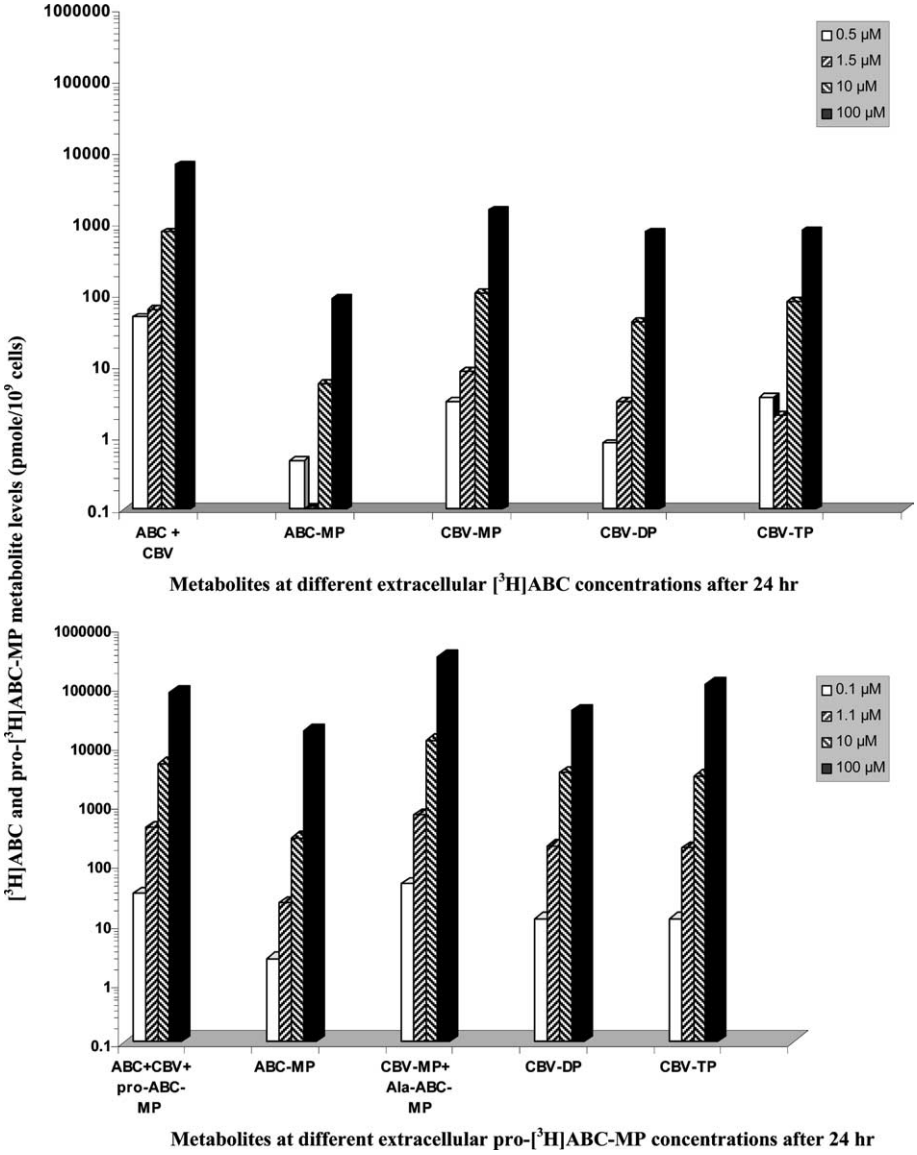


Fig. 2. Metabolic conversion of ABC and pro-ABC-MP in CEM cell cultures at different initial drug concentrations.

not be adequately separated from CBV-MP in the HPLC chromatograms. However, this metabolite seems to markedly accumulate within the first 2–6 h of incubation, whereafter its levels gradually decreased.

Minor traces of radiolabeled products were also found at HPLC elution times between 4–5, 15–17, 26–27 and 30–32 min (data not shown). Although the nature of these metabolites could not be identified, they were comparable in both the ABC- and pro-ABC-MP-treated cell cultures in terms of quantity, and therefore, are unlikely to play a contributing role in the markedly increased antiviral activity of the ABC prodrug.

### 3.3. Metabolic conversion of different concentrations of [$^3$H]ABC and pro-[$^3$H]ABC-MP in CEM cell cultures after 24 h of incubation

Since the highest metabolite levels of ABC and pro-ABC-MP accumulate within 6–24 h after the start of incubation, a variety of ABC and pro-ABC-MP concentrations, ranging between 0.5 and 100 μM, were now exposed to CEM cell cultures for 24 h (Fig. 2). The higher the initial extracellular drug concentration, the higher the intracellular drug metabolite levels recorded. This phenomenon was observed for all metabolites (i.e., ABC-MP, CBV-MP, CBV-DP and CBV-TP) formed. In fact, in contrast with the ABC-derived metabolites, the intracellular formation of the phosphorylated ABC and CBV metabolites derived from pro-ABC-MP increased even more abundantly at higher extracellular prodrug concentrations than expected from a linearly increased dose dependency (Fig. 2). In the ABC-exposed cell cultures the highest drug metabolite levels were recorded for CBV-MP and CBV-TP irrespective of the initial extracellular drug concentrations. In the pro-ABC-MP treated cell cultures, the highest drug levels were recorded for CBV-MP + alaninyl-ABC-MP (Ala-ABC-MP), followed by CBV-TP. At all extracellular drug concentrations tested (up to 100 μM) invariably markedly higher CBV-TP levels appeared in the pro-ABC-MP-exposed cells than in the ABC-exposed cells. The observation that the amounts of metabolite formation progressively increased at higher extracellular drug concentrations indicate that none of the enzymes responsible for the formation of the different metabolites of ABC and pro-ABC-MP seemed to be saturated with regard to their substrate conversion capacity at drug concentrations up to 100 μM.

### 3.4. Metabolic conversion of [$^3$H]ABC and pro-[$^3$H]ABC-MP in monocyte/macrophage cell cultures

The metabolic fate of [$^3$H]ABC and pro-[$^3$H]ABC-MP was studied in primary monocyte/macrophage cell cultures after incubation of the cells with the radiolabeled drugs for 6 and 24 h. For ABC, no metabolites above the detection limit could be detected in the cell extracts (detection limit: ~0.5 pmol/$10^9$ cells). In contrast, ABC-MP, CBV-MP, CBV-DP and CBV-TP as well as alaninyl-ABC-MP appeared to a measurable extent in the pro-ABC-MP-treated M/M cell cultures. The CBV-TP levels were $6.0 \pm 1.65$ pmol/$10^9$ cells after 6 h and $2.5 \pm 0.66$ pmol/$10^6$ cells after 24 h of drug exposure. The alaninyl-ABC-MP levels were as high as $53.7 \pm 8.9$ pmol/$10^9$ cells after 6 h, but dropped to $8.8 \pm 2.3$ pmol/$10^9$ cells after 24 h. Also, ABC-MP levels were very pronounced at the 6-h-evaluation point ($2.4 \pm 0.8$ pmol/$10^9$ cells) but were already markedly lower after 24 h ($2.0 \pm 0.90$ pmol/$10^9$ cells). Thus, all pro-ABC-MP metabolite levels were highest at the 6-h-incubation period, but markedly declined between 6 and 24 h. Also, the CBV-TP levels formed after 24 h were at least 5- to 10-fold higher in pro-ABC-MP-treated than in ABC-treated M/M cell cultures where they were under the detection limit.

## 4. Discussion

In contrast with the APA prodrug of CBV which did not afford a superior antiviral activity against HIV in cell culture compared with the parent CBV, the APA prodrug technology, applied to ABC, resulted in an at least 30- to 70-fold potentiation of the anti-HIV activity of this drug in human T-lymphocyte cell cultures and ~10-fold in primary monocyte/macrophage cell cultures. These findings suggest that efficient circumvention of the first phosphorylation step of ABC by adenosine phosphotransferase and thus direct intracellular delivery of ABC-MP had most likely been very efficient to boost the antiviral activity of the ABC prodrug. We could indeed demonstrate that the biochemical basis of the increased antiviral efficiency of the ABC prodrug is due to an increased intracellular release of ABC-MP and, consequently, an increased metabolic conversion of ABC-MP to CBV-TP, the eventual antivirally active metabolite of ABC. This could be shown in both T-lymphocyte and macrophage cell cultures. In fact, when the intracellular CBV-TP levels formed at equally antiviral ABC and pro-ABC-MP concentrations were compared in T-lymphocyte CEM cell cultures (i.e., ~3 μM ABC versus 0.05–0.07 μM pro-ABC-MP), comparable intracellular CBV-TP concentrations were detected after 24 h [5–10 pmol/$10^9$ CEM cells (extrapolated from levels obtained at 1.5 and 10 μM extracellular ABC concentrations) (Fig. 2) versus ~5 pmol/$10^9$ CEM cells (extrapolated from levels obtained at 0.1 μM extracellular pro-ABC-MP concentrations (Fig. 2)]. These findings also point to CBV-TP as the antivirally active metabolite of both ABC and pro-ABC-MP.

Interestingly, although pro-ABC-MP was markedly more inhibitory against HIV-1 in M/M than in T-lymphocyte cell cultures, the eventual CBV-TP levels were clearly lower in M/M than in T-lymphocytes. This observation seems contradictory at the first glance, but one should take into account that M/M cultures have markedly lower dNTP pool levels than T-lymphocytes [29], and thus, CBV-TP levels can much better compete with the lower cellular dNTP pools to act as a (DNA chain-terminating) substrate for HIV reverse transcriptase in M/M than in T-lymphocytes. A similar phenomenon has been observed earlier for other nucleoside analogues such as AZT and ddI [29]. Our metabolic studies also revealed that all cellular enzymes involved in the conversion of pro-ABC-MP to CBV-TP (i.e., carboxyesterase(s), phosphoramidase, ABC-MP deaminase, and the nucleotide kinases such as GMP kinase and NDP kinase) are neither rate-limiting nor saturated to metabolically convert ABC-MP to CBV-TP when extracellular drug concentrations as high as 100 μM were administered to the T-lymphocyte cell cultures. This means that in vivo intracellular delivery of higher ABC-MP concentrations through the APA prodrug technology will concomittantly result in higher eventual concentrations of the antiviral metabolite CBV-TP. Fig. 3 depicts the overall metabolic pathway of CBV,

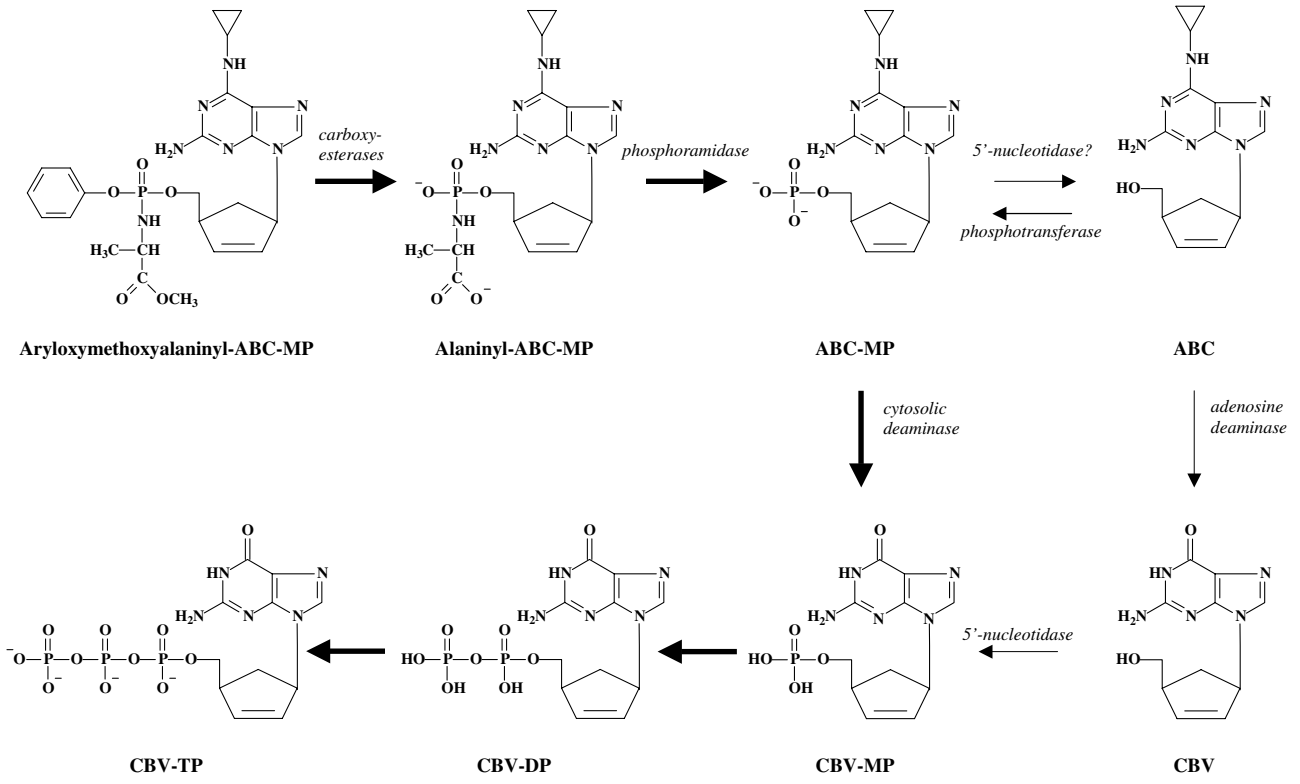


Fig. 3. Metabolic conversion scheme of the aryloxymethoxyalaninyl ABC-MP prodrug to its antivirally active carbovir 5′-triphosphate derivative CBV-TP.

ABC and pro-ABC-MP. Bold arrows represent the predominant metabolic flow when pro-ABC-MP is administered to the cell cultures.

The favorable properties of the aryloxymethoxyalaninyl ABC-MP prodrug in HIV-infected cell cultures may have several important advantages. First, the antiviral potency of ABC is markedly enhanced (~30- to 70-fold) through the prodrug technology. Second, the antiviral selectivity (ratio $CC_{50}/EC_{50}$) is also increased in CEM cell cultures (~5-fold) as well as in monocyte/macrophage cell cultures (~7-fold) (but not in MT-4 cell cultures). It would be interesting to reveal whether in vivo formation of free CBV nucleoside may become less prominent due to direct conversion of pro-ABC-MP to CBV-MP through alaninyl ABC-MP and ABC-MP. Since the toxic side effects observed for CBV in drug-treated patients has been at least partly ascribed to free CBV [26,27], appearance of CBV nucleoside in drug-exposed individuals should be kept as minimal as possible.

In conclusion, the aryloxymethoxyalaninyl phosphoramidate prodrug approach has been proven very successful to be applied to abacavir to increase its anti-HIV potency and selectivity in cell culture. These findings were supported and rationalized by our metabolic studies showing a markedly improved intracellular formation of CBV-TP, the antivirally active metabolite of ABC.

*Acknowledgements:* This work was supported by the European Commission (HPAW-2002-90001), the Geconcerteerde Onderzoeksacties – Vlaanderen (GOA 00/12) and a grant from GlaxoSmithKline to J.B. and C.McG. We thank Ann Absillis and Ria Van Berwaer for excellent technical assistance and Christiane Callebaut for dedicated editorial help.

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