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Abacavir increases platelet reactivity via competitive inhibition of soluble guanylyl cyclase

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Abstract

Objective—To provide a molecular mechanism that explains the association of the antiretroviral guanosine analogue, abacavir, with an increased risk of myocardial infarction.

Design—Drug effects were studied with biochemical and cellular assays.

Methods—Human platelets were incubated with nucleoside analogue drugs *ex vivo*. Platelet activation stimulated by ADP was studied by measuring surface P-selectin with flow cytometry. Inhibition of purified soluble guanylyl cyclase was quantified using an ELISA to measure cGMP production.

Results—Pre-incubation of platelets in abacavir significantly increased activation in response to ADP in a time and dose-dependent manner. The active anabolite of abacavir, carbovir triphosphate, competitively inhibited soluble guanylyl cyclase activity with a K_i of 55 $\mu\text{mol/l}$.

Conclusion—Abacavir competitively inhibits guanylyl cyclase, leading to platelet hyper-reactivity. This may explain the observed increased risk of myocardial infarction in HIV patients taking abacavir.

Keywords

abacavir; blood platelets; guanylate cyclase; myocardial infarction; P-selectin

Introduction

Abacavir, a guanosine analogue reverse transcriptase inhibitor, has been linked in five large observational studies with an approximately two-fold increase in relative risk of myocardial

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Conflicts of interest

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infarction [1–5]. This observation is concerning because it may translate into a significant absolute risk in aging HIV-infected patients who are expected to remain on lifelong antiretroviral therapy. In randomized studies, however, this finding has not been uniformly replicated. Although the Simplification of antiretroviral therapy with Tenofovir-Emtricitabine or Abacavir-Lamivudine trial found a significant increase in myocardial infarction risk in individuals randomized to abacavir [6], the preponderance of trial data has not shown a significant association [7–10]. However, when compared with the observational data, the randomized trial dataset represents smaller numbers of healthier patients who may not be at sufficient risk to develop a myocardial infarction, and in many of these studies myocardial infarction was not a predefined endpoint. The resulting controversy over the potential cardiovascular safety of abacavir has been particularly difficult to resolve because there is no established mechanism to account for this side-effect [11]. Several surveys of the effect of abacavir on biomarkers associated with cardiovascular disease have not revealed consistent findings [12–18]. Should a possible mechanism be identified *in vitro*, then epidemiologic and randomized trial studies might better ascertain whether and to what extent it plays a significant role in patients.

Although the myocardial infarction risks attributed to other HIV drugs (i.e. protease inhibitors and efavirenz) are thought to be mediated through derangements in lipid metabolism, two epidemiologic findings for abacavir suggested that it might act through a different mechanism. First, the risk of myocardial infarction was only seen in patients currently taking the drug. Second, the risk was not affected by statistical adjustment for lipids [1]. These findings suggested to us that abacavir might instead increase myocardial infarction through effects on platelet activation. Thrombus formation is a key event in acute myocardial infarction, and platelets are a well validated target for treatment of acute coronary syndromes [19]. Further, purine nucleotides are key regulators of platelet function [20]. Indeed, several small observational studies have found that patients taking abacavir have increased platelet aggregation compared with patients on other antiretroviral regimens [21,22].

Here, we present evidence that platelet activation is increased after ex-vivo incubation with abacavir, but not other nucleoside reverse transcriptase inhibitor drugs. We provide a molecular mechanism for this platelet effect by showing that the active anabolite of abacavir, carbovir triphosphate, can competitively inhibit the enzyme, soluble guanylyl cyclase, by mimicking the natural substrate guanosine triphosphate (GTP). Nitric oxide signaling normally downregulates platelet function through induction of soluble guanylyl cyclase [23,24]. Soluble guanylyl cyclase increases platelet cGMP levels, which in turn leads to the activation of multiple targets (cyclic nucleotide-gated ion channels, phosphodiesterases, and protein kinases) that inhibit platelet function [25]. In the presence of abacavir, platelet soluble guanylyl cyclase activity is low and platelet activation increases.

Methods

Phlebotomy

Five individuals, recruited from laboratory personnel, participated under a protocol approved by the University of California San Francisco (UCSF) Committee on Human Research. Individuals were healthy nonsmokers who had not taken any medications, including aspirin and NSAIDs, for 2 weeks before phlebotomy. Blood was mixed 9 : 1 with 3.2% sodium citrate anticoagulant. All drugs and reagents were added to blood in a PBS vehicle and incubated in tightly capped polypropylene tubes at 25 °C for 1 h unless otherwise noted.

Reagents

Carbovir triphosphate and entecavir were from Moravек Biochemicals (Brea, California, USA). All other nucleoside analogue drugs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. All other chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Flow cytometry

After incubation with drug or vehicle, ADP was added for 2 min at 25°C to stimulate platelet activation. Blood or platelets were stained with fluorescently labeled anti-P-selectin (CD62P) and anti-CD61 antibodies (Becton Dickinson, San Jose, California, USA) for 15 min at 25°C and then fixed in 1% paraformaldehyde before analysis on an LSR-II flow cytometer (Becton Dickinson). A minimum of 10 000 platelet events was acquired per sample. Data were analyzed with FlowJo software (Treestar Software, Ashland, Oregon, USA). Platelets were identified as CD61⁺ events, refined by forward and side scatter gating to eliminate red blood cell-associated or coincident events. Population P-selectin expression was characterized using median fluorescence, rather than mean, to minimize any artifacts due to poisoned platelets from possible aspirin use more than 2 weeks before phlebotomy.

Enzyme inhibition assay

Two preparations of soluble guanylyl cyclase (purified from bovine lung and recombinant human enzyme expressed in baculovirus) were obtained from Alexis Biochemicals (Enzo Life Sciences; Farmingdale, New York, USA). Enzyme activity was assayed during a 15-min incubation at 37°C in 50 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.4 buffer with 0.05% BSA, 4 mmol/l MgCl₂, and 10 mmol/l 1-methyl 3-isobutyl xanthine (a phosphodiesterase inhibitor). cGMP production was assayed with a competitive ELISA [Assay Designs (Enzo Life Sciences)]. Abacavir and carbovir did not cross-react with the ELISA.

Results

Guanosine analogues increase platelet activation

When nucleoside analogue drugs were added alone to platelets at concentrations up to 20 µg/ml, initial experiments using optical aggregometry found that abacavir, zidovudine, zalcitabine, didanosine, stavudine, lamivudine, emtricitabine, ribavirin, and tenofovir did not induce platelet activation (not shown). In addition, these drugs did not potentiate the effect of low doses (2–5 µmol/l) of the platelet agonist, ADP, when added simultaneously to platelets (not shown). We considered that abacavir might need time to be absorbed and/or metabolized by platelets and we, therefore, changed our experimental approach to assess time-dependent effects by flow cytometry. Studies of radiolabeled nucleosides indicated that they are rapidly taken up inside platelets [26]. We incubated samples of whole blood or platelet-rich plasma with a panel of nucleoside drugs and stimulated the platelets with the agonist ADP. The blood was then stained with fluorescent monoclonal antibodies to detect the upregulation of the activation marker P-selectin on the platelet surface [27]. As shown in Fig. 1a, abacavir exposure was significantly associated with an increase in platelet P-selectin expression, as compared with PBS. This effect was seen in five separate experiments using a different individual platelet donor for each experiment. Of the two other guanosine analogues in clinical use, ribavirin also demonstrated a moderate effect on platelet activation, and entecavir did not.

The abacavir effect increased with incubation time (Fig. 1b). Platelets had to be present for the incubation to have an effect; preincubation of abacavir in platelet-poor plasma did not

increase its effects on platelets (not shown). These experiments suggested that abacavir needed to be internalized and presumably metabolized within platelets before an effect could be observed. We also found that the abacavir treatment exhibited a dose–response relationship down to 10 $\mu\text{g/ml}$ (Fig. 1c). By comparison, the average plasma C_{max} (maximum drug concentration) in patients receiving once-daily dosing of 600 mg of abacavir is 4.3 $\mu\text{g/ml}$ [28]. Although platelets could be incubated *in vitro* for a few hours at most before loss of activity, platelets in a patient taking abacavir are exposed to drug over many days. Therefore, although we do not know the intraplatelet drug concentrations achieved with the in-vitro incubation doses we used, they are likely to approximate those of patients taking abacavir.

Carbovir triphosphate is a competitive inhibitor of soluble guanylyl cyclase

To explain the observed effects of abacavir on platelet activation, we investigated whether the active metabolite of abacavir, carbovir triphosphate, could inhibit soluble guanylyl cyclase, a negative regulator of platelet function. As shown in Fig. 2a, the structure of carbovir triphosphate mimics that of the natural enzyme substrate, GTP, but lacks a 3' hydroxyl group for cyclic nucleotide formation. Fig. 2b shows that carbovir triphosphate, but not abacavir nucleoside, was able to inhibit the activity of purified soluble guanylyl cyclase (both purified native bovine enzyme and recombinant human enzyme) *ex vivo*. The known strong inhibitor dialdehyde-GTP was included as a positive control [29]. To characterize the type of inhibition and to quantify its potency, we measured the amount of inhibition while varying the concentrations of GTP and carbovir triphosphate, and then plotted the data as Dixon (Fig. 2c) and Lineweaver–Burk (Fig. 2d) plots. These plots suggest that carbovir acts as a competitive inhibitor (which does not reduce V_{max} , the maximum reaction velocity) and that the K_i (dissociation constant for the enzyme-inhibitor complex) is approximately 80 $\mu\text{mol/l}$. Assuming that carbovir triphosphate concentrations in platelets are similar to the 50–500 $\mu\text{mol/l}$ measured in lymphocytes [30–32] and given that uninduced soluble guanylyl cyclase has a K_m (concentration of substrate that leads to half-maximal velocity) of approximately 100 $\mu\text{mol/l}$ for GTP [33,34], it is likely that the enzyme is partially inhibited in patients taking abacavir.

Discussion

The epidemiologic association between abacavir and myocardial infarctions has been an unexpected and controversial finding, and no mechanism has been shown by which abacavir could lead to myocardial infarctions. We hypothesized that abacavir, as a guanosine analogue, might inhibit soluble guanylyl cyclase, leading to platelet hyperreactivity and, ultimately, to increased risk of myocardial infarction.

Our data show that ex-vivo incubation with abacavir results in increased platelet activation as assessed by P-selectin expression. Platelet measurements are notoriously difficult because they vary significantly among healthy individuals and are subject to many individual confounders (including NSAID use, smoking, platelet number, and inflammatory state) [35], and details of the phlebotomy procedure and timing of the testing all introduce additional error. By adding drug to blood samples directly after phlebotomy, assay variability was reduced, and it was possible to detect the small changes in platelet activation that likely correspond to the increase in relative risk for myocardial infarction. One possible limitation of our method is that ex-vivo loading of platelets with abacavir may not achieve the concentrations of active metabolites found in platelets of patients receiving continuous oral dosing. However, our dose–response analysis of platelet activation found detectable effects on platelets, even after brief incubations at concentrations of abacavir similar to those measured in the plasma of patients taking the drug at standard dosages. Additionally, our enzyme inhibition measurements suggest that soluble guanylyl cyclase inhibition could be

physiologically significant at intracellular carbovir triphosphate concentrations measured in patients taking abacavir [30–32]. Endothelial function is also mediated by cGMP and it is possible that guanylyl cyclase inhibition may contribute to the defects in endothelial dysfunction observed in patients receiving abacavir [36].

Although the mechanisms underlying drug toxicities are rarely known, three separate molecular mechanisms have been elucidated for abacavir. First, like many nucleoside reverse transcriptase inhibitors, abacavir can cause mitochondrial toxicity by inhibiting DNA polymerase- γ resulting in a range of syndromes from lipoatrophy to neuropathy to lactic acidosis [37]. Second, a hypersensitivity syndrome specific for abacavir has been linked to the human leukocyte antigen B*5701 haplotype [38]. Finally, we provide evidence here for a third mechanism for abacavir toxicity: inhibition of cGMP synthesis resulting in platelet and, by extension, myocardial infarctions.

These experimental results have several clinical implications. First, they provide a plausible mechanism that would allow us to infer that the correlation between abacavir use and myocardial infarction observed in nonrandomized studies may be a causal one (they do not, however, provide any information about the magnitude of this association). Second, they provide a means for screening for cardiovascular safety of different purine analogues (and, potentially, a means to screen patients for differences in relative risk) at low cost and without exposing people to the drug. Third, the platelet mechanism suggests that patients at risk of cardiovascular disease who take abacavir might benefit from antiplatelet therapy with, for example, aspirin or clopidogrel. As a corollary, if epidemiologic analyses of abacavir risk were adjusted for antiplatelet agent use, it might be possible to resolve the inconsistent findings that have been reported to date. Finally, these results are a reminder that many of the surrogate markers used to predict cardiovascular risk in HIV-infected patients (such as lipid concentrations and intimal medial thickness measurements) may not capture all medication-related cardiovascular toxicity.

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Fig. 1. Increased platelet activation after ex-vivo incubation with abacavir

Activation was measured with flow cytometry and is reported as the median fluorescence intensity (MFI) of platelets stained with conjugated monoclonal antibodies to P-selectin (CD62P). Platelets were defined using CD61 as well as forward and side scatter gating. (a) Whole blood was incubated with nucleoside analogues at 50 μg/ml concentration for 1 h followed by stimulation with 5 μmol/l ADP. Stimulation and staining was done in quintuplicate; error bars represent SDs. Abacavir and ribavirin were associated with significant increases in P-selectin staining compared with the PBS control (Dunnett's test, $P < 0.01$). 3TC, lamivudine; ABC, abacavir; ZDV, zidovudine; d4T, stavudine; ddC, zalcitabine; ddI, didanosine; ETV, entecavir; FTC, emtricitabine; RBV, ribavirin; TDF, tenofovir. (b) Time course of the effect of abacavir incubation on P-selectin expression. Abacavir (80 μg/ml) was added for the indicated time before stimulation with 40 μmol/l ADP (or PBS). No abacavir was added to the 0 min incubation samples. The median fluorescence of P-selectin antibody stained platelets is reported. (c) Dose-response of the effect of abacavir on P-selectin expression. Platelets were incubated with the indicated concentrations of abacavir for 1 h before ADP stimulation.

Fig. 2. Abacavir is a competitive inhibitor of soluble guanylyl cyclase

(a) Structures of carbovir triphosphate and guanosine triphosphate. (b) Comparison of inhibitory effects of abacavir, carbovir triphosphate, and dialdehyde guanosine triphosphate on bovine and recombinant human soluble guanylyl cyclase. (c) Dixon plot of guanosine triphosphate inhibition of purified bovine soluble guanylyl cyclase. Intersection of the plots points to a K_i of 55 $\mu\text{mol/l}$. (d) Data from C replotted in Lineweaver–Burk format suggests a competitive inhibition mechanism.