Significant effects of tipranavir on platelet aggregation and thromboxane \( \text{B}_2 \) formation \textit{in vitro} and \textit{in vivo}

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**Objectives:** In the past, bleeding events have been described for patients with haemophilia taking HIV-1 protease inhibitors. Recently, the FDA published a warning concerning intracranial haemorrhage in patients taking the HIV-1 protease inhibitor tipranavir co-administered with ritonavir.

**Methods:** We investigated (i) platelet aggregation \textit{in vivo} in HIV-1-infected adult patients \((n=5)\) immediately before and 2 and 4 h after dosing of tipranavir/ritonavir 500/200 mg. To further characterize the effects, we then evaluated (ii) platelet aggregation and (iii) thromboxane \( \text{B}_2 \) (\( \text{TxB}_2 \)) formation (ELISA) with increasing tipranavir concentrations (TPV\text{conc}) \textit{in vitro} of up to 100 000 ng/mL. Platelet aggregation was stimulated either with 2 \( \mu \text{M} \) ADP (ADP) or 10 mg/L collagen (COL). TPV\text{conc} were measured with validated EPI-LC-MS/MS. Intraintividual comparisons of values at time points and TPV\text{conc}, respectively, were carried out with repeated samples ANOVA.

**Results:** Platelet aggregation (mean, maximal light transmission \( A_{\text{max}} \)) was significantly decreased in patients 4 h post-dose in collagen- (from 79.8% to 57.1%; \( P<0.001 \)) and in ADP-stimulated (from 58.5% to 54.0%; not significant) samples at a median (range) TPV\text{conc} of 62 500 ng/mL (22 990–67 500). These results could be reproduced \textit{in vitro} at TPV\text{conc} 50 000 ng/mL (\( A_{\text{max}}/A_{\text{max COL}} = 20.7/36.9\% \); \( P=0.003/0.001 \)) and 100 000 ng/mL (\( A_{\text{max}}/A_{\text{max COL}} = 14.5/17.1\% \); \( P<0.001/0.001 \)). Median (range) \( \text{TxB}_2 \) concentrations were reduced (\( P=0.07 \)) from 327 ng/mL (187–500) at baseline to 265 ng/mL (152–428) at 5000 ng/mL and were significantly reduced (\( P<0.001 \)) to 187 ng/mL (81–362) at a TPV\text{conc} of 50 000 ng/mL, respectively.

**Conclusions:** Five HIV-1-infected patients on tipranavir-containing highly active antiretroviral therapy presented marked decreases in platelet aggregation. \textit{In vitro} these effects were reproduced and decreased \( \text{TxB}_2 \) formation was also demonstrated. Inhibition of platelet aggregation while receiving tipranavir treatment might contribute to increased risk of bleeding.

Keywords: HIV protease inhibitors, HIV therapy, platelet function, thromboxane formation, intracranial haemorrhage

**Introduction**

Although thromboembolic complications and an increased cardiovascular risk have been observed with HIV-1 protease inhibitors,\(^1\) there also have been reports of bleeding events which were attributed to unknown mechanisms\(^2,3\) and severe episodes of haemarthrosis and skin/soft tissue bleeding have been described especially in factor VIII/IX-deficient haemophilic patients. With a lower incidence, non-haemophiliac patients were also affected by haemorrhage, especially in the gastrointestinal tract or mucous membranes.\(^2\) As platelet dysfunction was reported in haemophilic HIV-1-infected patients taking protease inhibitors,\(^3\) this would be a considerable reason for bleeding occurring under protease-inhibitor-containing highly active antiretroviral therapy (HAART).

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Tipranavir affects platelet aggregation

In June 2006, the US Food and Drug Administration and Boehringer Ingelheim alerted healthcare providers and patients about serious and/or fatal intracranial haemorrhage (ICH) events in patients taking the HIV protease inhibitor tipranavir co-administered with ritonavir. A warning and additional information was added to the product description.

We therefore investigated (i) platelet aggregation and (ii) tipranavir concentrations in HIV-1-infected adult patients (n = 5) immediately before (trough concentration) and 2 and 4 h after dosing (peak concentration) with HAART containing tipranavir/ritonavir 500/200 mg. Following the results of the patient study, we further characterized the effects of tipranavir in an ex vivo–in vitro assay with increasing tipranavir concentrations on (iii) platelet aggregation and (iv) thromboxane B2 (TxB2) formation.

Materials and methods

Sample preparation and patients

Blood samples from patients taking tipranavir plus concomitant antiretrovirals were assessed as part of a study evaluating platelet function parameters in HIV patients receiving protease-inhibitor-containing HAART. The study protocol was approved by the Medical Faculty Ethics Review Board of the Johann Wolfgang Goethe University Frankfurt am Main, Germany, and patients were included in this study after written informed consent. The original study protocol did not include aggregometry, which was amended after the report of the tipranavir ICH events in 2006 had been published. Thus, five additional patients were screened for this study and eligible for the analysis, taking tipranavir 500/200 mg twice daily as part of their HAART with no history of non-steroidal anti-inflammatory drug (NSAID) intake within at least 14 days prior to the study assessments. Exclusion criteria for the study were thrombocytopenia, hepatic impairment, abnormal international normalized ratio or thromboplastin time and the use of NSAIDs, oral anticoagulants or heparin. Patients’ blood samples were assessed at steady state, immediately before sampling and samples were processed as described below. Tipranavir pure substance (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA) was dissolved at 1 mg/mL in methanol, evaporated to dryness and subsequently dissolved in 1 mL of sodium chloride containing 0.25% ammonia and frozen at −80°C until incubation.

Ex vivo–in vitro blood samples were assessed from healthy volunteers (n = 6, aged between 28 and 41 years), not taking any medication 2 weeks prior to the evaluation.

These blood samples were then incubated with tipranavir at final concentrations of 0, 5000, 50 000 and 100 000 ng/mL, respectively. Tipranavir was added to the whole blood immediately after sampling and samples were processed as described below. Tipranavir pure substance (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA) was dissolved at 1 mg/mL in methanol, evaporated to dryness and subsequently dissolved in 1 mL of sodium chloride containing 0.25% ammonia and frozen at −80°C until incubation.

Platelet aggregation

Citrated whole blood (3.18%, Sarstedt, Nümbrecht, Germany) was collected by a 1.2 G needle and transferred to the lab within 30 min. Directly thereafter or after tipranavir-incubation, platelet-rich plasma was prepared by centrifugation at 20°C for 5 min at 409 g. Aggregation was measured with a turbidimetric, light-transmittance aggregometer (APACT 4 S PLUS, LABiTec, Ahrensburg, Germany) within 1 h after blood sampling. Inducers used were collagen (Kollagenreagens Horm®, Nycomed Austria GmbH, Linz, Austria) at a concentration of 10 mg/L and ADP (Boehringer, Mannheim, Germany) at a concentration of 2 μM. The aggregation response is given as percentage of maximal light transmission (Amax) either after ADP stimulation (Amax,ADP) or collagen stimulation (Amax,COL).

TxB2 formation

Blood was collected into 4.9 mL serum separator tubes (Sarstedt, Nümbrecht, Germany) and immediately spiked with tipranavir at concentrations of 5000 and 50 000 ng/mL, respectively, one tube stayed untreated as baseline. Blood was subsequently clotted at 37°C in a water-bath for 1 h, then serum was prepared (10 min, 3200 g, 4°C) for the further analysis of TxB2 concentrations by EIA (assay designs, Ann Arbor, USA). The sensitivity of the TxB2 assay was 10.54 pg/mL with an intra-assay precision ranging from 1.6% to 4.0% and an inter-assay precision of 3.6% to 7.6%; the accuracy ranged between 77.34% and 124.93%.

Plasma concentrations of tipranavir

Tipranavir concentrations in patient samples were measured in plasma using validated and quality controlled liquid chromatography coupled with electrospray–mass spectrometry (LC-ESI-MS/MS). The calibration curves showed a good linearity (r2 ≥ 0.98) for tipranavir concentrations, ranging from 0.5 to 10 000 ng/mL. Higher concentrations were diluted appropriately. The accuracy of the measurements was 89.6% to 107.0%, the intra-assay precision ranged from 1.8% to 4.0%.

Statistics

We primarily investigated platelet aggregation and tipranavir concentrations in blood samples from five HIV-1-infected adults before and 2 and 4 h after the administration of tipranavir/ritonavir 500/200 mg.

We subsequently tested the differences in platelet aggregation at tipranavir concentrations of 0, 5000, 50 000 and 100 000 ng/mL in vitro. We furthermore assessed TxB2 formation at tipranavir concentrations of 0, 5000 and 50 000 ng/mL.

The effects of tipranavir on platelet aggregation and thromboxane formation as well as tipranavir concentrations were evaluated by intra-individually comparing pre-treatment versus post-treatment values using repeated samples ANOVA (SPSS 12.0.1).

Results

Patients’ demographic and laboratory baseline data

All five patients were male with a mean age of 42.7 years (range 38–62 years). They took tipranavir/ritonavir at therapeutic dosage of 500/200 mg twice daily together with nucleoside reverse transcriptase inhibitors (abacavir, n = 1; didanosine, n = 2; emtricitabine, n = 1; lamivudine, n = 2; tenofovir-DP, n = 3; and zidovudine, n = 1), fosamprenavir (n = 1) and enfuvirtide (n = 5). The median (range) CD4 cell count at study baseline was 318 cells/mm³ (129–444), HIV-RNA-PCR was <49 copies/mL in all five patients, thrombocyte count = 175 x 10⁹ (143–283), international normalized ratio = 1.00 (0.90–1.02), aspartate aminotransferase = 35 U/L (18–71) and alanine aminotransferase = 33 U/L (19–59).
In vivo platelet aggregation and tipranavir concentrations

The median (range) collagen-stimulated platelet aggregation evaluated in patients immediately before dosing was 79.8% (73.7–83.3) and decreased to 73.2% (68.7–79.9) 2 h post-dose and to 57.1% (48.6–66.3) 4 h post-dose (P < 0.001) (Table 1), whereas ADP-stimulated aggregation varied from 58.5% (56.8–74.5) to 59.1% (51.4–72.6) at 2 h to 54.0% (42.3–72.6) at 4 h post-dose.

The median (range) tipranavir concentrations measured in patients were 33 280 ng/mL (18 100–59 950) before dosing, 63 500 ng/mL (39 820–72 500) 2 h post-dose and 62 500 ng/mL (22 990–67 500) 4 h post-dose, and were in agreement with pharmacokinetic data reported from previous trials with tipranavir.5,7,8

In vitro effects in platelet aggregation and TxB2 formation

Compared with the samples without tipranavir, platelet aggregation stimulated with 2 μM ADP or 10 μg/mL collagen was significantly decreased at tipranavir concentrations ranging from 5000–100 000 ng/mL. ADP-induced aggregation was already at 5000 ng/mL strongly and significantly decreased (from 79.2% to 28%, P < 0.009) while the strong stimulus of 10 μg/mL collagen was not significantly decreased at that concentration (from 83.4% to 72.3%, P = 0.78). All other results showed a significant alteration compared with baseline (zero) (Table 1 and Figure 1).

According to the steady-state plasma concentrations of tipranavir at therapeutic dose, we chose a lower (5000 ng/mL) and a higher (50 000 ng/mL) concentration for investigation of TxB2 formation in vitro. At these concentrations, TxB2 formation was markedly reduced: the median (range) TxB2 concentration was 326.5 ng/mL (186.5–499.7) before dosing, compared with 265.4 ng/mL (151.5–428.1) at a concentration of 5000 ng/mL (P = 0.07) and 186.8 ng/mL (80.7–362.5) at a concentration of 50 000 ng/mL (P < 0.001) (Table 1).

Discussion

The key finding of our study was a significant decrease in platelet aggregation in the presence of therapeutic tipranavir concentrations in HIV patients under HAART. This effect could be validated in an ex vivo–in vitro assay using ADP and collagen to induce platelet aggregation in a dose-dependent fashion.

There is evidence that HAART with tipranavir might be connected to an increased risk of associated major bleeds: the incidence and the median time to onset of fatal ICH events were 0.2% and 525 days, respectively, evaluated in a cohort of 6840 HIV-1-infected patients.9

However, recently presented data from a retrospective cohort analysis including over 33 000 patients on non-tipranavir-based HAART in the USA estimated an ICH incidence rate of 0.23 cases per 100 person-years. This compared with 0.26 cases per 100 person-years estimated for tipranavir based on clinical development data.10

For those cases reported in the context of tipranavir therapy, all subjects had additional risks that may have contributed to bleeding, such as CNS lesions, head trauma, recent neurosurgery, coagulopathy, hypertension, alcohol abuse or the concomitant intake of anticoagulants and antiplatelet agents.9

If these effects are compared with those caused by genuine antiplatelet agents, the strength of platelet inhibition and its risks for ICH are comparable to those under chronic intake of aspirin at doses of 75–325 mg, which was 0.19% per 5 years of aspirin intake11 in a meta-analysis of four studies evaluating secondary prophylaxis of cardiovascular events. Another analysis indicates that aspirin increases the risk of haemorrhagic stroke with a summary odds ratio (95% CI) of 1.4 (0.9–2.0). This translates to one to two excess events per 1000 persons given aspirin for 5 years.12

Thus, the clinical relevance of the detected effects should be appraised carefully, particularly as the effects seen with tipranavir intake in vivo were somewhat lower than with aspirin intake. Previously published data from our own group showed an ADP- and collagen-stimulated platelet aggregation that was decreased by 12% and 57%, respectively, when aspirin was taken at a dose of 300 mg once daily.13 This impact on platelet aggregation was similar to that in nine healthy volunteers who took 300 mg of aspirin once daily in a further evaluation, showing a mean (SD) collagen-stimulated thromboocyte aggregation of 84 ± 5% at baseline and a reduction to 50 ± 20% at day 3 and 43 ± 20% at day 5 of aspirin intake.14

If compared with the five patients in our study, the impact on platelet function at therapeutic tipranavir concentrations (Cmax of ~60 000 ng/mL) occurred at a lower level: ADP-induced platelet aggregation was altered by +1% and −7.7%, whereas collagen-induced platelet aggregation was reduced by 6% and 27% at 2 and 4 h post-dose, respectively. Our results indicated that the impact of tipranavir on platelet aggregation in vivo appeared with a certain latency, 2 h after reaching maximum tipranavir plasma concentrations, and was undetectable at tipranavir plasma trough concentrations.

Regarding the mechanisms of platelet aggregation, a reduced TxB2 formation is one surrogate marker for decreased platelet function. TxB2 is the stable metabolite of the short-lived functional thromboxane A2 (TxA2) which stimulates the activation of new platelets and increases platelet aggregation.15 It appeared that TxB2 formation was decreased by more than 40% in ex vivo–in vitro analysis at therapeutic tipranavir concentrations. However, the capacity of platelets to form TxA2 and platelet function is non-linear,16 10% of the residual capacity to generate TxA2 is sufficient to sustain TxA2-dependent aggregation.17

Hence, a significant reduction of TxA2 formation and the decreased ADP-induced aggregation of purigenic P2Y12 receptor and collagen-induced aggregation predominantly over the GPIII/FC gamma chain18 lead to the conclusion of a rather non-specific platelet inhibition that affects several activation pathways.

Interestingly, the detected effects were more distinct in the ex vivo–in vitro analysis of blood samples drawn from healthy volunteers than in the HIV-infected patients, which leads to the assumption that HIV-related cofactors may have attenuated the detected effects. HIV infection has been shown to correspond with endothelial dysfunction, elevated levels of von Willebrand factor antigen19 and enhanced activation of platelets.20 These effects, however, diminish at HAART with HIV protease inhibitors,19,21 which itself causes in return atherogenic lipoprotein changes.22 In light of these discrepant reports, we cannot eliminate the possibility that the platelet-inhibiting effects of tipranavir may be attenuated by simultaneously active pro-thrombotic mechanisms in HIV-1-infected patients.
Table 1. Thrombocyte aggregation and tipranavir concentrations in blood samples ($n = 5$ from 5 patients) spiked with three different concentrations of tipranavir before and 2 and 4 h after dosing with tipranavir/ritonavir 500/200 mg, respectively.

| Tipranavir (ng/mL), median (range) | In vitro thrombocyte aggregation in ex vivo blood samples ($n = 5$) spiked with 5000 ($n = 5$), 50 000 ($n = 5$) and 100 000 ($n = 5$) ng of tipranavir, respectively | In vivo thrombocyte aggregation of five patients before and 2 and 4 h after dosing of tipranavir/ritonavir 500/200 mg | C1 | C2 | C3 | C4 | C1 versus C2 | C1 versus C3 | C1 versus C4 | 0 h | 2 h | 4 h | 0 h versus 2 h | 0 h versus 4 h |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 5000 | $P < 0.001$ | 50 000 | $P < 0.001$ | 100 000 | $P < 0.001$ | 33 280 | (18 100–59 950) | 63 500 | $P = 0.001$ | 62 500 | (22 990–67 500) | $P = 0.006$ |
| 79.2 (40.9–86.4) | 28.0 (24.3–47.7) | $P = 0.009$ | 20.7 (13.0–45.7) | $P = 0.003$ | 14.5 (7.3–21.4) | $P < 0.001$ | 58.5 (56.8–74.5) | 59.1 (51.4–72.6) | NS | 54.0 (42.3–72.6) | NS |
| 83.4 (66.6–90.2) | 72.3 (46.9–79.3) | $P = 0.078$ | 36.9 (26.1–52.3) | $P < 0.001$ | 17.1 (11.6–28.6) | $P < 0.001$ | 79.8 (73.7–83.3) | 73.2 (68.7–79.9) | NS | 57.1 (48.6–66.3) | $P = 0.001$ |

C, concentration; ADP-stim, aggregometry stimulated with 2 µM ADP; COL-stim, aggregometry stimulated with 10 mg/L collagen; NS, statistically not significant.
In conclusion, our results suggest a degree of decreased platelet function under the influence of therapeutic concentrations of tipranavir, which appears to be less distinct in vivo than in vitro. At this stage of our investigations, the exact mechanism of the platelet inhibition remains unclear.

However, an indication for tipranavir therapy should consider a possibly higher risk for ICH in patients with additional risk factors or receiving concomitant oral anticoagulation, NSAIDs or thrombin inhibitors as long as pharmacodynamic interactions have not been fully evaluated.

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Transparency declaration

None to declare.

References


Figure 1. (a) Correlation between platelet aggregation (%) and tipranavir concentration (ng/mL) in ex vivo blood samples, without tipranavir and spiked with 5000, 50,000 and 100,000 ng/mL of tipranavir, respectively. (b) Platelet aggregation in five patients before (0 h) and 2 and 4 h after dosing with tipranavir/ritonavir 500/200 mg. Upper and lower boundaries of box-plots indicate 75th and 25th percentiles, and whiskers indicate upper and lower range of values. Platelet aggregation was induced with either 2.0 μM ADP (grey box-plots) or 10 ng/L collagen (white box-plots). *Statistically significant at a level of P ≤ 0.01. **Statistically significant at a level of P ≤ 0.001.
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