Influence of Indocyanine Green on Plasma Disappearance and Biliary Excretion of a Synthetic Thrombin Inhibitor of the 3-Amidinophenylalanine Piperazide-type in Rats

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Purpose. The pharmacokinetics of a number of synthetic peptidomimetic thrombin inhibitors is determined by extensive hepatic elimination. The objective was to further characterize the disposition in vivo of Pefa 1023, a novel 3-amidinophenylalanine piperazide-type thrombin inhibitor, by influencing the hepatic handling with indocyanine green (ICG), which is actively taken up by the liver.

Methods. Pefa 1023 was administered intravenously to bile duct-cannulated rats, either alone or in combination with ICG. The concentrations of Pefa 1023 in blood plasma and bile were measured by a bioassay (thrombin clotting time), concentrations of indocyanine green were measured spectrophotometrically.

Results. ICG (10 mg/kg i.v. 15 min prior to or simultaneously with Pefa 1023) markedly influenced the plasma level and biliary excretion rate of the thrombin inhibitor Pefa 1023 given in a dose of 1 mg/kg i.v. The plasma level was more than twice that of the control, the maximum biliary excretion rate about one third and the fraction of dose excreted in bile about two thirds.

Conclusions. The anionic dye ICG is able to interfere with the hepatic handling of a cationic, amidinophenylalanine piperazide-type thrombin inhibitor with the consequence of reduced hepatic clearance leading to higher plasma levels and lower biliary excretion of the latter.

KEY WORDS: thrombin inhibitor; indocyanine green; plasma; bile; rats.

INTRODUCTION

In recent years, the development of small molecule synthetic peptidomimetic inhibitors of coagulation enzymes, especially of thrombin, has led to a number of highly potent and selective inhibitors (1,2). They are effective as anticoagulants and have a potential for use as antithrombotic agents in various clinical settings. Their pharmacokinetic properties, however, present in most cases drawbacks to a clinical application as oral antithrombotics.

Based on the finding that an amidinophenylalanine amide-type thrombin inhibitor reached high concentrations in the bile in rabbits after various routes of administration, we reported on the essential role of the liver for systemic clearance of the 4-amidinophenylalanine amide-type highly potent and selective thrombin inhibitor, NAPAP: "functional hepatectomy" markedly prolonged the plasma half-life of NAPAP (3,4). In a further analysis of the biliary excretion pattern of this thrombin inhibitor in rabbits and rats, it was assumed that the hepatic uptake was not limiting the biliary excretion that showed saturation kinetics (5).

Several other thrombin inhibitors with similar structural characteristics, including tripeptide derivatives, were found to be biliary excreted in various species to a marked extent (6,7,8). The arginine amide-type thrombin inhibitor argatroban, marketed as antithrombotic drug for i.v. use, is excreted via the bile after i.v. administration in rabbits to a somewhat lesser extent than NAPAP and shows slower plasma disappearance (9).

Recently, the active uptake into isolated rat hepatocytes of a thrombin inhibitor of the 4-amidinophenylalanine-type, CRC 220, was described (10). This uptake is mediated via the organic anion transporter polypeptide (OATP), as competition with bile acids and bromosulfophthalein showed (11). NAPAP competed with the uptake of CRC 220 in vitro. The carrier-mediated uptake in the liver brings about a high extent of hepatobiliary first-pass elimination of CRC 220 and a short half-life. In vivo, bile acids were shown to decrease the biliary excretion of CRC 220 (10).

It can be assumed that the structural moieties found in most of the active site-directed synthetic thrombin inhibitors (strongly basic amidino or guanidino groups, other polar groups, several ring structures, considerable molecular size) are the basis for their characteristic pharmacokinetic properties (short half-life, low oral bioavailability). Among the peptidomimetic protease inhibitors, HIV-protease inhibitors, studied extensively in recent years, also present problems with regard to bioavailability resulting from hepatic uptake and biliary excretion (12).

Pefa 1023 is a novel 3-amidinophenylalanine-derived thrombin inhibitor with a longer half-life than NAPAP (13). The present study is aimed at clarifying whether competition for hepatic handling by a compound structurally different is reflected in vivo in the time course of the plasma level and biliary excretion of Pefa 1023. Indocyanine green (ICG), a cholephilic organic anion undergoing extensive hepatic uptake in several species was chosen to study this problem.

MATERIALS AND METHODS

ICG: Indocyanine green sodium iodide (ICG-Pulsion®, 0.838 mg indocyanine green per vial; Pulsion Medizintechnik, München, Germany).

Pefa1023: Nα-(2-naphthylsulfonyl)-L-3-amidinophenylalanine carboxymethylpiperazide hydrochloride (Pentapharm AG, Basel, Switzerland).

Rats: Female Wistar rats, 260–300 g body weight (Charles River-Wiga, Sulzfeld, Germany) were used. The animals were kept under conventional conditions with free access to standard diet and tap water. The animal experiments were in accordance with the "Principles of Laboratory Animal Care".

Anaesthesia was performed with ethylurethane (1.4 g/kg intraperitoneally). The body temperature was kept constant by means of a thermostated infrared lamp. The right carotid artery was exposed and cannulated for drawing blood samples; the left femoral vein was exposed for intravenous injection. The bile duct was cannulated in typical manner. Blood was taken into sodium citrate (1/10, v/v), citrated plasma was obtained by centrifugation at 1200g for 10 min. Bile was collected for the first 30 min of the experiment in 5 min-fractions, thereafter

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in 15 min-fractions (up to 1 hr) and in 30 min-fractions later on. Bile volume was determined gravimetrically. The duration of the experiments was limited to 4 hours.

ICG in plasma and bile was measured spectrophotometrically at 800 nm directly in appropriate dilutions (with distilled water) of these media and concentrations were read from a calibration curve.

In the HPLC-determination, developed in our laboratory for Pefa 1023 (and other thrombin inhibitors) (13), the high concentrations of ICG in the plasma and bile samples from animals dosed with ICG interfered with the chromatography of Pefa 1023 under the respective conditions. Therefore, a bioassay (thrombin clotting time of plasma) based on the antithrombin-activity of Pefa 1023 was used and the concentrations in plasma and bile were quantified in the following way: 50 µl samples each (or appropriate dilutions in saline) of rat plasma and bile were added to 100 µl of human plasma (pool plasma from healthy blood donors); coagulation was started by addition of 50 µl of bovine thrombin (5 NIH-U/ml) and the clotting time was measured in a coagulometer. Prolongations of clotting times in comparison to the control samples taken before administration of Pefa 1023 were converted to concentrations of Pefa 1023 by means of a calibration curve obtained with graded concentrations of the thrombin inhibitor added to blank rat plasma and bile. The detection limit of the assay was about 70 ng/ml for rat plasma. ICG did not interfere with this assay.

Systemic clearance was calculated from AUC. Biliary clearance was calculated as $Cl_b = v \times c_b / c_p$, where v is bile flow (ml/min) and c_b and c_p are the concentrations (µg/ml) in bile and plasma, respectively, for the period from 5 to 60 min after administration. Concentrations in plasma corresponding to the middle of the respective bile sampling period were interpolated for calculation of biliary clearance. Mean biliary clearance was calculated from the individual time point data.

The data presented are means with standard deviations. Statistical significance ($p \leq 0.05$) was calculated by two-sided t-test or ANOVA.

RESULTS

In control groups of 4 animals each, ICG and Pefa 1023 were administered i.v. in doses of 10 and 1 mg/kg, resp. Bile flow and the concentrations in plasma and bile of the compounds administered were measured.

The time course of the plasma level of ICG in the control group (data not shown) was in principle in accordance with literature data, the plasma level was somewhat higher whereas the biliary excretion rates were lower than those reported for doses of 6 and 12 mg/kg (14). Mean biliary clearance was 1.5 ml/min×kg.

In the control group, Pefa 1023 showed a time course of plasma levels (Fig. 1) measured with the bioassay very close to those measured via HPLC in a separate group of animals (13), the absolute concentrations were, however, lower by a factor of about 1.2. There was an excellent correlation of the plasma levels determined by both methods in an individual control with Pefa 1023. Also the mean values of the plasma levels (7 time points for the period 2 min to 90 min after administration) and of the biliary excretion rates (10 time points for the period 5 min to 120 min) of Pefa 1023 measured via bioassay correlated well ($r = 0.9982$ and $r = 0.9897$, respectively) with those measured via HPLC. From the group using HPLC-measurements a systemic clearance of 31.8 ± 9.6 ml/min × kg and an elimination half-life of the β-phase of 43 min were calculated. The distribution volume V_{Dss}, calculated from a further group of 3 animals (5 mg/kg infusion over 60 min), was 1.32 ± 0.25 l/kg.

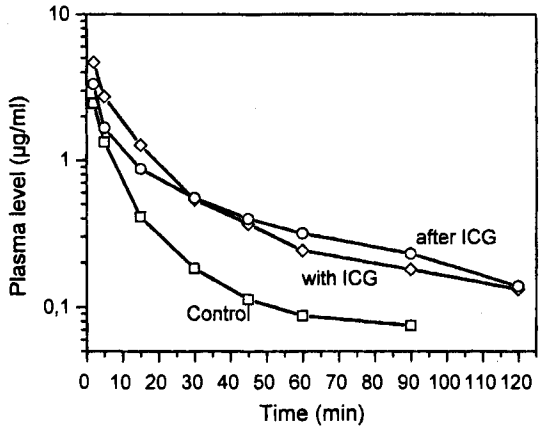

Fig. 1. Time course in rats of plasma levels of the thrombin inhibitor Pefa 1023 (1 mg/kg i.v.) given alone, 15 min after ICG (10 mg/kg i.v.) or simultaneously with ICG (10 mg/kg i.v.) (Mean values from 4–6 animals; standard deviation omitted because of clarity of graph).

In the combination experiments, ICG was administered i.v. in a dose of 10 mg/kg either simultaneously with (n = 6) or 15 min prior to(n = 5) Pefa 1023 which was given in a dose of 1 mg/kg i.v. Mean bile flow in the 30 min control period was 54.0 ± 10.4 µl/min × kg in the control group, 51.8 ± 5.7 µl/min × kg in the group given ICG simultaneously, and 52.8 ± 5.5 µl/min × kg in the group pretreated with ICG. In the three groups that received ICG, bile flow decreased considerably after ICG injection for about 45 min and normalized thereafter to values similar to controls. In contrast, Pefa 1023 alone did not lead to marked depression of bile flow. Transient variations in bile flow also translated into variations of the biliary excretion rates of ICG and Pefa 1023.

ICG plasma levels were not significantly different from the control group in the group administered Pefa 1023 simultaneously. The plasma levels of Pefa 1023 administered simultaneously with ICG, however, showed a pattern different from the control group (Fig. 1): they were significantly higher at every time point after administration. The AUC (calculated up to 90 min after administration) was increased about 2.5-fold by simultaneous administration of ICG. For the ICG-pretreated group, the time course of plasma levels of Pefa 1023 was similar to that after simultaneous administration, however, the values were significantly lower at 2 and 5 min than those after simultaneous administration of ICG. Table I shows the values calculated for systemic and biliary clearance. There was no significant difference between the two groups coadministered ICG.

The pattern of the biliary excretion rates is shown in Figure 2: Compared to Pefa 1023 alone, ICG delayed the maximum of biliary excretion of Pefa 1023 and decreased the maximum rates about 3-fold. After 30 min, the excretion rates did no longer differ significantly from the control; there was, however, a tendency to relatively higher rates in the group given ICG

Table I. Clearance Parameters of the Thrombin Inhibitor Pefa 1023 (1 mg/kg i.v.) and Influence of ICG-coadministration (10 mg/kg i.v.) in Rats

Clearance (ml/min × kg)	Pefa 1023 control (n = 4)	Pefa 1023 with ICG (n = 6)	Pefa 1023 15 min after ICG (n = 5)
Systemic clearance	39.3 ± 3.6	17.3 ± 2.7*	23.0 ± 9.4*
Biliary clearance	16.8 ± 2.6	5.4 ± 2.6*	5.5 ± 3.5*

* Significantly different from control ($p < 0.05$).

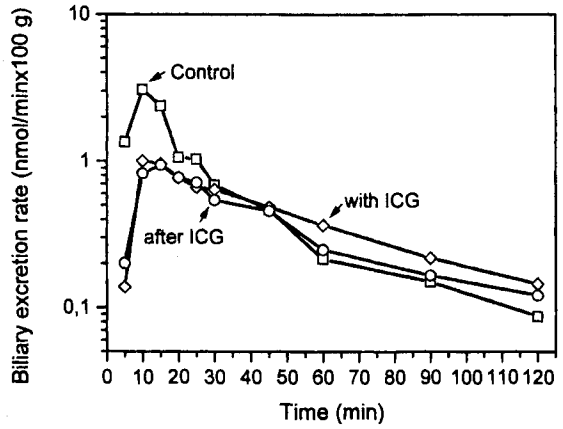

Fig. 2. Time course in rats of biliary excretion rates of the thrombin inhibitor Pefa 1023 (1 mg/kg i.v.) given alone, 15 min after ICG (10 mg/kg i.v.) or simultaneously with ICG (10 mg/kg i.v.) (Mean values from 4–6 animals; standard deviation omitted because of clarity of graph).

simultaneously. The fraction of dose of the thrombin inhibitor excreted with bile during the whole course of the experiment (240 min) was reduced by about one third (Fig. 3). The values for biliary clearance, calculated for individual bile fractions (data not shown), declined over 60 min by about 40 percent in the control group, whereas in the groups given ICG additionally, the biliary clearance was very low during the first 5 min after

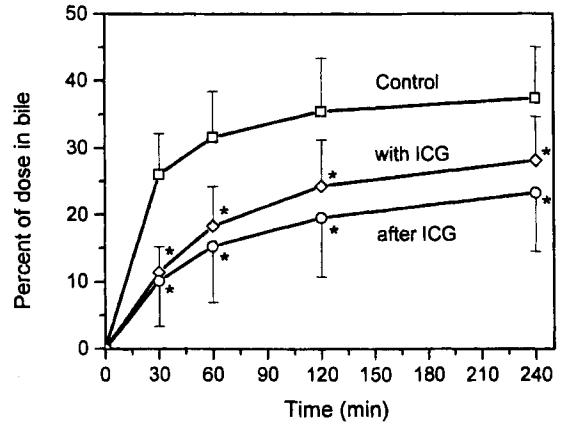

Fig. 3. Cumulative biliary excretion (per cent of dose administered) in rats of the thrombin inhibitor Pefa 1023 (1 mg/kg i.v.) given alone, 15 min after ICG (10 mg/kg i.v.) or simultaneously with ICG (10 mg/kg i.v.).

administration and increased with time, reaching nearly control values after 120 min.

DISCUSSION

The determination of the concentrations of the amidinophenylalanine-type thrombin inhibitor, Pefa 1023, in plasma and bile of rats by a bioassay based on inhibition of thrombin yielded results in principal agreement with those of the HPLC-assay that did not reveal significant appearance of metabolites (13,15). In contrast to our results with the bioassay showing some underestimation, in the case of a tripeptide thrombin inhibitor, efegatran, comparison of a bioassay and HPLC used for measuring concentrations in dog plasma showed a trend to slight overestimation by the bioassay with good correlation of both assays (7).

The coadministration in rats of the anionic dye ICG, a typical OATP substrate, had a siginificant influence on the plasma level and the biliary excretion of the cationic amidinophenylalanine piperazide Pefa 1023. The effect of ICG on the plasma disappearance of Pefa 1023 in rats was in principle the same as the effect of "in situ hepatectomy" in rabbits on the pharmacokinetics of NAPAP (4). The higher plasma levels, compared to controls, of Pefa 1023, administered intravenously in combination with ICG indicate competition with the hepatic uptake of Pefa 1023, which represents the most important route of elimination of this compound. Pefa 1023 was shown to be taken up into isolated rat hepatocytes like the amidinophenylalanine-derived thrombin inhibitor CRC 220 (15). Clearance by the liver (expressed in terms of biliary clearance, comprising hepatic uptake and subsequent biliary excretion processes) accounts for a high percentage of systemic clearance of Pefa 1023 in rats (15).

The inhibitory effect of ICG on the hepatic uptake of Pefa 1023 is obviously not critically dependent on the plasma level of ICG, since the effects of ICG after pretreatment are similar to those after simultaneous administration: In the first case, the ICG plasma levels at the time of administration of the thrombin inhibitor have already declined to less than one tenth of those on simultaneous administration.

Total plasma clearance of ICG in rats is about 7 ml/min × kg (14); the comparatively low biliary clearance of ICG (1.5 ml/min × kg in this study) is apparently the result of restricted canalicular secretion in this species (16,17).

The kinetics of the hepatic uptake is of prime importance for the disappearance of Pefa 1023 from plasma. This kinetics, however, does not directly translate into corresponding biliary excretion data. In the controls, about 75% of the total amount excreted in bile over 240 min appears already in the first 30 min in bile. The ratio of the biliary excretion rates, on a molar basis, of ICG and Pefa 1023 increased with time after administration reaching figures of 30–40, whereas the molar ratio of the administered doses was about 7. This obviously indicates different modes and kinetics of the interaction of ICG with the uptake and the excretion processes of Pefa 1023, respectively, which were not further characterized in this study.

As regards the structural specificity of the hepatic uptake process, it is interesting to note that the most potent inhibitor of uptake into isolated rat hepatocytes of the cationic amidinophenylalanine-type thrombin inhibitor CRC 200 was a structural analogue lacking the basic amidino group, the lowest inhibitory

effect showed an analogue with an amidino group, but having a less hydrophobic Nα-substituent (11). Obviously, the multispecific organic anion transporter, which has been cloned from rat and human liver, shown to be responsible for the uptake of CRC 220, tolerates a (strongly) basic group in a sufficiently hydrophobic molecule (18). The amidinophenylalanine moiety is not an absolute requirement for hepatic uptake of peptidomimetic thrombin inhibitors as the findings with arginine derivatives show (6,7,9).

The characteristics demonstrated for Pefa 1023, i.e. marked hepatic first-pass effect, result in low oral bioavailability and possible pharmacokinetic drug interactions. Recently, in patients with impaired liver function decreased clearance and increased half-life of the arginine-derived thrombin inhibitor, argatroban, were described (19). There is a challenge for further search for therapeutically useful orally bioavailable synthetic thrombin inhibitors; at present, the majority of structures found suited for high-affinity binding to the target enzyme, thrombin, obviously also fulfill the not yet fully characterized structural requirements for molecules to be transported by OATP in the liver.

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