Cancer Chemother Pharmacol (2004) 54: 131–138
DOI 10.1007/s00280-004-0793-2

ORIGINAL ARTICLE

**N. Harry Hendrikse · Folkert Kuipers · Coby Meijer
Rick Havinga · Charles M. A. Bijleveld
Winette T. A. van der Graaf · Willem Vaalburg
Elisabeth G. E. de Vries**

In vivo imaging of hepatobiliary transport function mediated by multidrug resistance associated protein and P-glycoprotein

Received: 25 November 2003 / Accepted: 16 February 2004 / Published online: 30 April 2004

Abstract Multidrug resistance associated proteins (MRPs) and P-glycoprotein (P-gp) are involved in hepatobiliary transport of various compounds. Our aim was (1) to define transporter specificity of the cholescintigraphic agents ^{99m}Tc-HIDA and ^{99m}Tc-MIBI, which are used clinically for myocardial perfusion measurements; and (2) to deduce MRP and P-gp functions in vivo from hepatic ^{99m}Tc kinetics. Accumulation of radioactivity was measured in the human tumor cell lines GLC_4, GLC_4/ADR_{150x} (MRP1-overexpressing/P-gp-negative) and GLC_4/P-gp (P-gp-overexpressing). Bile secretion was quantified in untreated and in glutathione-depleted control and MRP2-deficient (GY/TR^-) rats. Hepatobiliary transport was measured using a gamma camera in both types of rats. ^{99m}Tc-HIDA accumulated 5.8-fold less in GLC_4/ADR_{150x} calls than in GLC_4 or GLC_4/P-gp cells. In GLC_4/ADR_{150x}, the cellular ^{99m}Tc-HIDA content was increased 3.4-fold by the MRP1,2 inhibitor MK571 (50 μM), while MK571 had no measurable effect in GLC_4 and GLC_4/P-gp cells. ^{99m}Tc-MIBI accumulated less in GLC_4/P-gp and GLC_4/ADR_{150x} cells than in GLC_4 cells. Bile secretion of ^{99m}Tc-HIDA was impaired in GY/TR^- compared to control rats and not affected by glutathione depletion in GY/TR^- rats. Hepatic secretion of ^{99m}Tc-HIDA was slower in GY/TR^- ($t_{1/2}$ 40 min) than in control rats ($t_{1/2}$ 7 min). Bile secretion of ^{99m}Tc-MIBI was similar in both rat strains and impaired by glutathione depletion in control rats only, indicating compensatory activity of additional transporter(s) in GY/TR^- rats. ^{99m}Tc-HIDA is transported only by MRP1,2 only, while ^{99m}Tc-MIBI is transported by P-gp and MRP1,2. The results indicate that hepatic P-gp and MRP1,2 function can be assessed in vivo by sequential use of both radiopharmaceuticals.

Keywords GY/TR^- · ^{99m}Technetium · Liver · P-gp · MRP · Functional imaging

Abbreviations DEM: Diethylmaleate · GLC_4: Small-cell lung carcinoma · GSH: Glutathione · P-gp: P-Glycoprotein · MRP: Multidrug resistance associated protein · ^{99m}Tc-HIDA: ^{99m}Technetium-disofenin · ^{99m}Tc-MIBI: ^{99m}Technetium-sestamibi

Introduction

P-glycoprotein (P-gp) and the multidrug resistance associated protein (MRP) efflux pumps are involved in removal of endogenous and exogenous compounds from the body, most likely to protect the body against toxins. Both transporter proteins are members of the ATP-binding cassette superfamily [21]. In the human body, P-gp is expressed in different organs such as endothelial cells of the blood–brain barrier, testes, and liver [7, 40]. Several MRP homologues have been identified [18]. MRP1 is expressed in nearly all tissues, in particular at basolateral membranes of hepatocytes but MRP1 expression is low in quiescent hepatocytes [37]. MRP2 (cMOAT, canalicular multiorganic anion transporter) is mainly expressed at canalicular membranes of hepatocytes, transporting several compounds into bile [18, 25, 28, 34]. Several studies have demonstrated that the substrate specificity of MRP1 is very similar to that of MRP2. Most substrates are conjugated to or cotransported with glutathione (GSH), glucuronide or sulfate [13, 24, 29, 31, 39]. MRP2 is defective in GY/TR^- rats and in patients with the Dubin–Johnson syndrome due to MRP2 mutations [15, 16, 33, 42].

N. H. Hendrikse (✉) · W. Vaalburg
PET Center, University Hospital Groningen,
Groningen, The Netherlands
E-mail: n.h.hendrikse@pet.azg.nl
Tel.: +31-50-3616161
Fax: +31-50-3611687

F. Kuipers · R. Havinga · C. M. A. Bijleveld
Department of Pediatrics, University Hospital
Groningen, Groningen, The Netherlands

C. Meijer · W. T. A. van der Graaf · E. G. E. de Vries
Department of Medical Oncology,
University Hospital Groningen,
Groningen, The Netherlands

In cholescintigraphy studies with ^{99m}Tc-disofenin (^{99m}Tc-HIDA) a decreased hepatobiliary transport in Dubin–Johnson syndrome patients has been observed [1, 2, 35]. ^{99m}Tc-sestamibi (^{99m}Tc-MIBI) is a P-gp and MRP1 substrate in vitro [8, 36]. We hypothesized that ^{99m}Tc-HIDA and ^{99m}Tc-MIBI transport into bile proceeds by MRP2 for ^{99m}Tc-HIDA and by both P-gp and MRP2 for ^{99m}Tc-MIBI. Individual kinetics of ^{99m}Tc-labeled compounds may thus allow functional evaluation of liver transport activity due to differences in drug efflux pump activities. In this study, the substrate specificities of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI for P-gp and MRP1,2 were studied. Thereafter, the possibility to discriminate between P-gp- and MRP1,2-mediated pharmacokinetics in vivo was assessed. Kinetics of both radiopharmaceuticals were compared in normal and MRP2-deficient GY/TR$^-$ rats to evaluate whether comparison would allow conclusions about P-gp and MRP2 function in vivo.

Materials and methods

Chemicals

Ketamine (Ketalar, 50 mg/ml) was obtained from Parke-Davis (Munich, Germany) and xylazine (Rompun, 2% solution) from Bayer (Leverkusen, Germany). Diethylmaleate (DEM) was purchased from Sigma (St. Louis, Mo.) and RPMI 1640 medium and fetal calf serum (FCS) from Gibco (Paisley, UK). ^{99m}Tc-hexakis-2-methoxyisobutylisonitrile (^{99m}Tc-MIBI) was synthesized as described previously [8]. Radiochemical purity was greater than 98% by thin-layer chromatography (Gelman Sciences, Ann Arbor, Mich.) with 0.9% sodium chloride as mobile phase. ^{99m}Tc-*N*-(2,6-dimethylphenylcarbamoylmethyl)-iminodiacetic acid (^{99m}Tc-HIDA) was synthesized by the complexation of ^{99m}Tc with iminodiacetic acid. Briefly, iminodiacetic acid (20 mg, 0.15 mmol) was dissolved in 0.3 ml sodium hydroxide (0.5 *M*). Thereafter, the pH was adjusted to 7 by the addition of approximately 0.2 ml hydrochloric acid (0.1 *M*). Stannous chloride (1 mg/ml $SnCl_2$, 0.26 ml solution) was added and the solution was stirred. A solution of ^{99m}Tc-labeled sodium pertechnetate (1 ml, 300 MBq) was added and the reaction mixture was stirred for 20 min. Saline was added until a final concentration of 100 MBq/ml was reached. The radiochemical purity was greater than 95% as verified by thin-layer chromatography (Gelman Sciences) with methylethylketone (Merck, Darmstadt, Germany) as mobile phase.

^{99m}Tc-HIDA transport in GLC_4 cells

To study the transport characteristics of ^{99m}Tc-HIDA, a small-cell lung carcinoma cell line GLC_4, its MDR1 gene-transfected, P-gp-overexpressing, MRP-negative, GLC_4/P-gp subline and its 150-fold doxorubicin-resistant, MRP1-overexpressing, P-gp-negative subline GLC_4/ADR_{150x} were incubated with ^{99m}Tc-HIDA (64 f *M*) in polystyrene tubes in RPMI/10% FCS for 1 h at 37°C [3, 44, 26, 41]. For modulation studies, a specific modulator of MRP1,2 function, MK571, or a modulator of P-gp function, cyclosporin A (CsA), were used. In addition, it is noted that CsA is also a very weak substrate for MRP [22, 32]. To study modulating effects, the cell lines were incubated with the MRP1,2 blocker MK571 (50 μ*M*) for 1 h. Thereafter, the cells were washed with 5 ml ice-cold phosphate-buffered saline (PBS, 0.14 *M* NaCl, 2.7 m *M* KCl, 6.4 m *M* $Na_2HPO_4{\cdot}2H_2O$, 1.5 m *M* KH_2PO_4, pH 7.4) for 5 min followed by centrifugation (5 min, 300 *g*, 4°C). Cellular ^{99m}Tc-HIDA accumulation was measured in water with a γ-counter (LKB Wallac, Turku, Finland). Corrections were made for extracellular adhesion of ^{99m}Tc-HIDA by subtracting the data obtained after ^{99m}Tc-HIDA incubation for 5 min at 4°C from the 37°C incubation data.

For comparison, accumulation data of ^{99m}Tc-MIBI in GLC_4 and GLC_4/ADR_{150x} cell lines are shown in Fig. 1. The ^{99m}Tc-MIBI accumulation data in GLC_4 and GLC_4/ADR_{150x} cells have been published previously [8].

Animals

Male Wistar rats (280–320 g) and GY/TR$^-$ homozygous transport mutant Wistar rats (280–320 g) were obtained

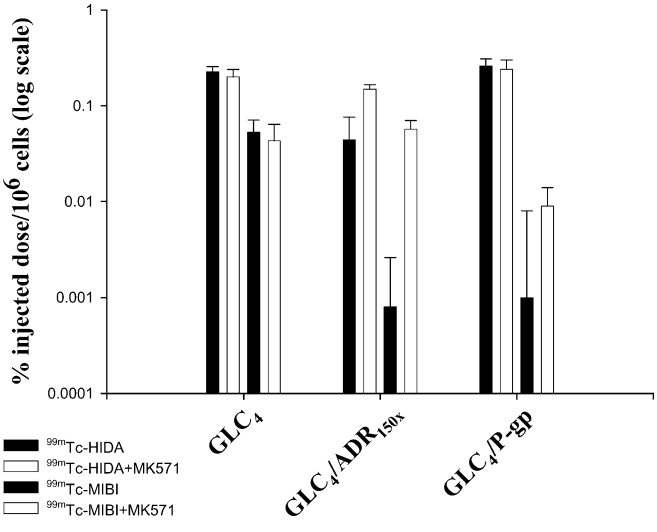

Fig. 1 Cellular uptake of radioactivity in GLC_4, GLC_4/ADR_{150x} and GLC_4/P-gp cells (*x*-axis from *left* to *right*). In every cell line the *first bar* from *left* to *right* represents accumulation of ^{99m}Tc-HIDA, the *second bar* represents cellular ^{99m}Tc-HIDA uptake in the presence of MK571, the *third bar* represents cellular ^{99m}Tc-MIBI uptake and the *fourth bar* represents cellular ^{99m}Tc-MIBI uptake in the presence of MK571. Cellular uptake of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI was measured after 1 h exposure to radioactivity. The data presented are the means ± SD of three independent experiments, each performed in duplicate. Data on the cellular accumulation of ^{99m}Tc-MIBI for GLC_4 and GLC_4/ADR_{150x} cells have been published previously [8]

from the Central Animal Laboratory, University of Groningen, The Netherlands. All experiments were approved by the local animal ethics committee.

Clearance of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI from plasma

To study ^{99m}Tc-HIDA and ^{99m}Tc-MIBI pharmacokinetics in control rats ($n=3$) and GY/TR$^-$ rats ($n=3$), animals were anesthetized with ketamine/xylazine (2:1, 1 ml/kg) and the carotid artery was cannulated. Arterial blood samples (100–200 μl) were drawn at 0, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 40 and 60 min after injection of radioactivity. Plasma and blood cells were separated by centrifugation (3 min, 1000 *g*). Plasma samples (50 μl) were counted in a γ-counter.

Excretion of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI into bile

All experimental studies were performed under anesthesia as described above. A catheter was placed into the bile duct of rats for bile excretion experiments [20, 19]. Subsequently, the animals were injected with ^{99m}Tc-HIDA (37 MBq, 0.3 ml, $n=3$) or ^{99m}Tc-MIBI (37 MBq, 0.3 ml, $n=3$) into the penile vein and the total bile was collected over time intervals for 2 h into tarred test tubes. Bile flow was assessed by weight, assuming a density of 1 g/ml.

To study the effects of GSH depletion on excretion of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI into bile, control rats and GY/TR$^-$ rats were injected with DEM intraperitoneally (3.9 mmol/kg body weight) as described by Dijkstra et al. [4]. At 45 min after DEM injection, ^{99m}Tc-HIDA or ^{99m}Tc-MIBI was injected as described above, and bile was collected.

Biodistribution studies in control rats and GY/TR$^-$ rats

Male control rats and GY/TR$^-$ rats (300 ± 20 g) were anesthetized with ketamine/xylazine (2:1, 1 ml/kg). Subsequently, 0.3 ml ^{99m}Tc-HIDA or ^{99m}Tc-MIBI (37 MBq) was injected into the penile vein. At 60 min after injection the rats were killed by extirpation of the heart. Several tissues were dissected. Heparin-plasma was obtained from collected blood by centrifugation (3 min, 1000 *g*). Radioactivity was measured with a γ-counter. To study the effects on biodistribution of GSH depletion, the rats were treated with DEM as described above. At 45 min after DEM injection, the rats were injected with ^{99m}Tc-HIDA or ^{99m}Tc-MIBI and 60 min after injection the animals were killed as described above.

Camera experiments

In vivo pharmacokinetics of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI in the liver of control rats and GY/TR$^-$ rats were performed with a MultiSPECT 2 dual headed gamma camera (Siemens, Hoffman Estates, Ill.) equipped with low-energy high-resolution collimators. After anesthesia with ketamine/xylazine (2:1, 1 ml/kg) the rats were positioned on the collimator, to obtain planar images. Radioactivity was injected into the penile vein and then 60 frames of 60 s each were acquired in a 256×256 matrix using a zoom factor of 1.0. Only one gamma camera head (the one with the rats on the collimator) was used to obtain the images. Processing was performed on a dedicated Siemens Icon work-station. Posterior planar images were corrected for radioactive decay. To obtain a time–activity curve representative of clearance of HIDA from the liver, a region-of-interest was positioned on posterior images over the entire liver, including the central gall ducts. From this region-of-interest a time–activity curve was generated, from which liver clearance measurements were obtained.

Statistical analysis

Statistical significance was determined using Student's *t*-test. P values <0.05 were considered significant.

Results

Cellular accumulation of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI in vitro

Cellular accumulation of ^{99m}Tc-HIDA in GLC_4 cells and GLC_4/P-gp cells after a 1-h exposure did not differ between the two cell lines, indicating that ^{99m}Tc-HIDA is not a substrate for P-gp (Fig. 1). In contrast, the accumulation of ^{99m}Tc-HIDA in GLC_4/ADR_{150x} cells overexpressing MRP1 was 5.8-fold lower ($P<0.0025$) than in GLC_4 cells, indicating that ^{99m}Tc-HIDA is a substrate for MRP1. Coincubation with the specific MRP1,2 modulator MK571 increased the cellular ^{99m}Tc-HIDA concentration in GLC_4/ADR_{150x} cells 3.4-fold ($P<0.05$) compared to the concentration in GLC_4/ADR_{150x} cells without MK571, whereas MK571 did not affect the cellular ^{99m}Tc-HIDA accumulation in GLC_4 and GLC_4/P-gp tumor cells, confirming MRP1-mediated transport of ^{99m}Tc-HIDA. In accordance with previously reported data, accumulation of ^{99m}Tc-MIBI was 67-fold lower ($P<0.005$) in GLC_4/ADR_{150x} cells than in GLC_4 cells (Fig. 1) [8].

Plasma clearance of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI in rats

Disappearance of both compounds from plasma was extremely rapid (for ^{99m}Tc-HIDA $t_{1/2}$ was 1.17 ± 0.16 min in GY/TR$^-$ rats and 0.95 ± 0.29 min in control rats; for ^{99m}Tc-MIBI $t_{1/2}$ was 0.51 ± 0.03 min in GY/TR$^-$ rats and 0.29 ± 0.13 min in control rats). No

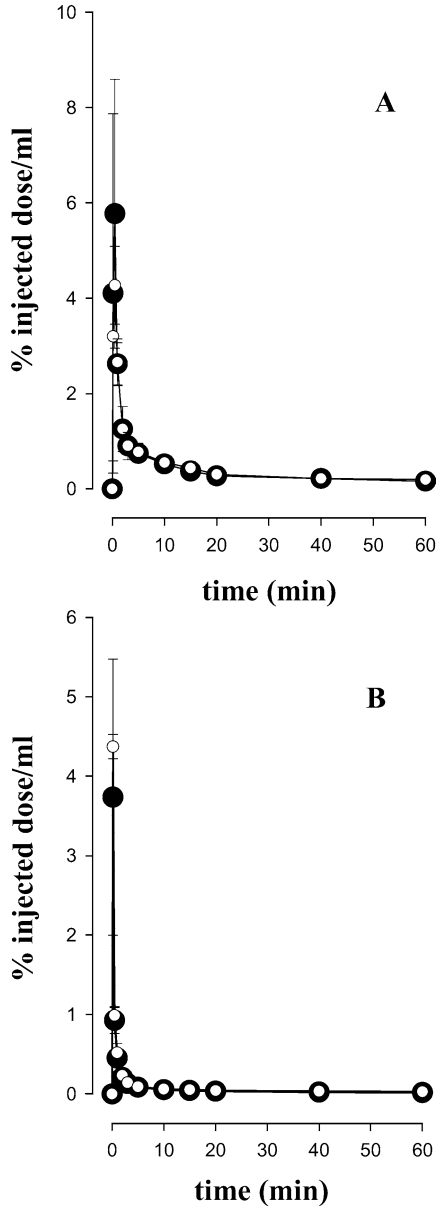

Fig. 2a, b Plasma clearance of (**a**) ^{99m}Tc-HIDA and (**b**) ^{99m}Tc-MIBI in control rats (*filled circles*) and GY/TR$^-$ rats (*open circles*). Each point represents the mean ± SD of three independent experiments

differences in plasma kinetics were observed between control rats and GY/TR$^-$ rats for ^{99m}Tc-HIDA or ^{99m}Tc-MIBI (Fig. 2).

Bile excretion of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI in rats

After intravenous injection of ^{99m}Tc-HIDA, bile secretion of radioactivity was delayed in GY/TR$^-$ rats compared with control rats. Within 20 min, GY/TR$^-$ rats secreted 3.4 ± 0.6% and control rats 7.8 ± 0.7% of the injected dose into the bile (Fig. 3a). After GSH depletion, bile secretion of ^{99m}Tc-HIDA in control rats was decreased to levels similar to that in untreated GY/TR$^-$ rats (Fig. 3b). DEM treatment had no effect on ^{99m}Tc-HIDA secretion in GY/TR$^-$ rats.

After intravenous injection of ^{99m}Tc-MIBI, the bile secretion of radioactivity did not differ between control rats and GY/TR$^-$ rats (Fig. 3c). The cumulative recovery 120 min after injection of radioactivity was 3.1 ± 0.1% in control rats and 3.3 ± 0.1% in GY/TR$^-$ rats. As reported previously, the bile flow was significantly lower in GY/TR$^-$ rats than in control rats [19]. After injection of DEM, the cumulative biliary excretion of ^{99m}Tc-MIBI in control rats was reduced by 73.0% of the bile excretion of untreated control rats (Fig. 3d). In contrast, no effect of DEM treatment was observed in GY/TR$^-$ rats (compare Fig. 3c,d).

Biodistribution studies in rats

At 60 min after injection of ^{99m}Tc-HIDA, the percentage ^{99m}Tc-HIDA in the liver was 0.2 ± 0.1% of the injected dose per gram of tissue in control rats and 0.4 ± 0.3% in GY/TR$^-$ rats. The percentage ^{99m}Tc-MIBI in the liver was 0.3 ± 0.3% of the injected dose per gram in control rats and 0.4 ± 0.2% in GY/TR$^-$ rats. In the liver and in other tissues, the contents of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI in control rats and GY/TR$^-$ rats were similar. DEM treatment had no effect on the biodistribution of ^{99m}Tc-HIDA or ^{99m}Tc-MIBI in control rats or GY/TR$^-$ rats compared to the untreated rats (data not shown).

Camera experiments in rats

After injection of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI, planar images in control rats and GY/TR$^-$ rats were obtained (Fig. 4a,b). Imaging of ^{99m}Tc-HIDA demonstrated a rapid uptake of radioactivity, in particular in the liver of both types of rats. The disappearance of radioactivity from the liver was faster in control rats ($t_{1/2\ (liver)}$ = 7 ± 9 min) than in GY/TR$^-$ rats ($t_{1/2\ (liver)}$ 40 ± 9 min) ($P < 0.05$), indicating a prolonged storage of radioactivity in the liver of GY/TR$^-$ rats (Fig. 5a). ^{99m}Tc-MIBI transport in the liver was not different between control rats and GY/TR$^-$ rats (Fig. 5b).

Discussion

Assessment of functional transport activities by P-gp and MRP1,2 in the liver in vivo by visualization techniques might be of benefit to the diagnosis of transporter deficiency-related diseases. ^{99m}Tc-labeled substrates are potential candidates for this application. In vitro and in vivo studies with ^{99m}Tc-MIBI have already shown that ^{99m}Tc-MIBI is a substrate for P-gp and MRP1 [8, 36]. The current in vitro and in vivo studies showed that ^{99m}Tc-HIDA is a substrate for

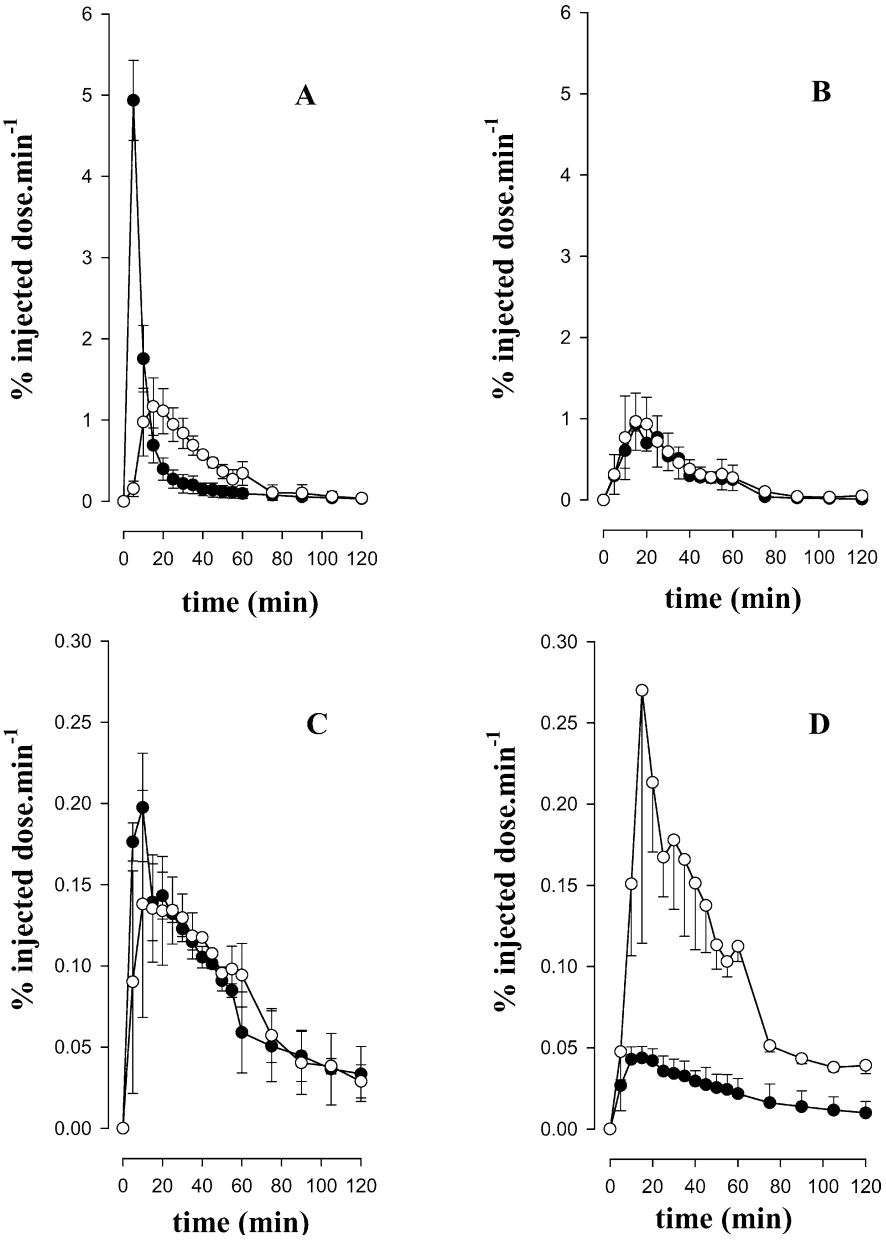

Fig. 3a–d Time-course measurement of the bile excretion of ^{99m}Tc-HIDA (**a**, **b**) and ^{99m}Tc-MIBI (**c**, **d**) after intravenous injection in control rats (*filled circles*) and GY/TR$^-$ rats (*open circles*) (**a**, **c**) and after GSH depletion with DEM (**b**, **d**). DEM (3.9 mmol/kg body weight) was administered intraperitoneally 45 min before injection of radioactivity. Subsequently, radioactivity was injected intravenously. The data presented are the means ± SD from three animals per group

MRP1,2 and not for P-gp. The fact that ^{99m}Tc-HIDA is transported by both MRP1 and MRP2 is in agreement with expectations, given the similar substrate specificity of MRP1 and MRP2 [31, 39, 17]. Therefore, comparison of MRP1 transport in vitro and MRP2 transport in vivo is justified. Bile collection studies in control rats and GY/TR$^-$ rats demonstrated that ^{99m}Tc-HIDA and ^{99m}Tc-MIBI are both excreted into bile. Excretion of ^{99m}Tc-HIDA into bile was rapid in control rats and severely delayed in GY/TR$^-$ rats, indicative of MRP2-mediated transport of ^{99m}Tc-HIDA. These results were also revealed by imaging. Bile collection and measurement of ^{99m}Tc-MIBI kinetics in the liver with the gamma camera showed similar rates of ^{99m}Tc-MIBI transport in both rat strains. Possibly, the lack of ^{99m}Tc-MIBI transport by MRP2 is compensated by upregulation of other drug efflux pumps, as has been reported for MRP2-deficient rats (see below) [9].

Before endogenous and xenobiotic compounds are transported via MRP, they are usually converted into more hydrophilic anionic conjugates with GSH, glucuronate or sulfate [11, 30]. These conjugates can be ATP-dependently transported by MRP [30]. It is suggested that cotransport of MRP substrates and GSH is also possible, but the exact mechanism is still unknown [12, 43]. GSH depletion resulted in decreased biliary excretion of ^{99m}Tc-HIDA and ^{99m}Tc-MIBI in control rats but not in GY/TR$^-$ rats, underscoring MRP2-mediated transport of both radiolabeled compounds in control rats. The fact that GSH depletion had no effect on ^{99m}Tc-HIDA and ^{99m}Tc-MIBI excretion in GY/TR$^-$ rats might be explained by upregulation of other drug

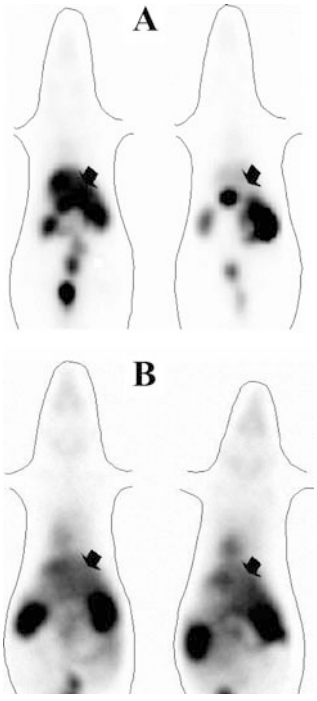

Fig. 4a, b Representative scintigrams of (**a**) ^{99m}Tc-HIDA and (**b**) ^{99m}Tc-MIBI in control rats (*right images*) and in GY/TR$^-$ rats (*left images*). The ^{99m}Tc-HIDA images show that the radioactivity had disappeared from the liver in the control rat and persisted in the liver of the GY/TR$^-$ rat. In contrast, the ^{99m}Tc-MIBI images of the control rat and the GY/TR$^-$ rat show equal radionuclide uptake in the livers. The images were acquired 1 h after injection. The *arrows* in the figure indicate the livers

efflux pumps. For instance, it has been shown that a MRP-like protein (MLP2) is highly expressed in the liver of MRP2-deficient Eisai hyperbilirubinemic rats, while this protein is not expressed in Sprague-Dawley rats [9].

For ^{99m}Tc-HIDA, analysis of rat bile has shown that more than 98% of ^{99m}Tc-HIDA is present as parental compound [14]. Reinjection of the ^{99m}Tc-HIDA content of the urinary bladder and the gallbladder in mice produced a biodistribution that was similar to ^{99m}Tc-HIDA itself, indicating that ^{99m}Tc-HIDA is stable in vivo [23]. Administration of ^{99m}Tc-MIBI in guinea pigs has shown that 80–85% of ^{99m}Tc-MIBI is not metabolized, indicating that ^{99m}Tc-MIBI is also rather stable in vivo [6, 27].

In the case of drug treatment, knowledge about drug efflux pump function in the liver and other organs might be of clinical importance. Differences in P-gp and MRP transport function may be able to explain variance in excretion of several drugs from the body. For instance, recently it has been reported that an altered P-gp function may be due to allelic variation in MDR1 gene [10, 38]. This particular polymorphism results in different plasma concentrations of the HIV protease inhibitor nelfinavir. This indicates that concentrations of P-gp substrates in plasma could have a genetic basis for MDR1 [5]. Concerning drugs which are substrates for the drug efflux pumps, variation in function of drug efflux pumps might result in patient-tailored prescription

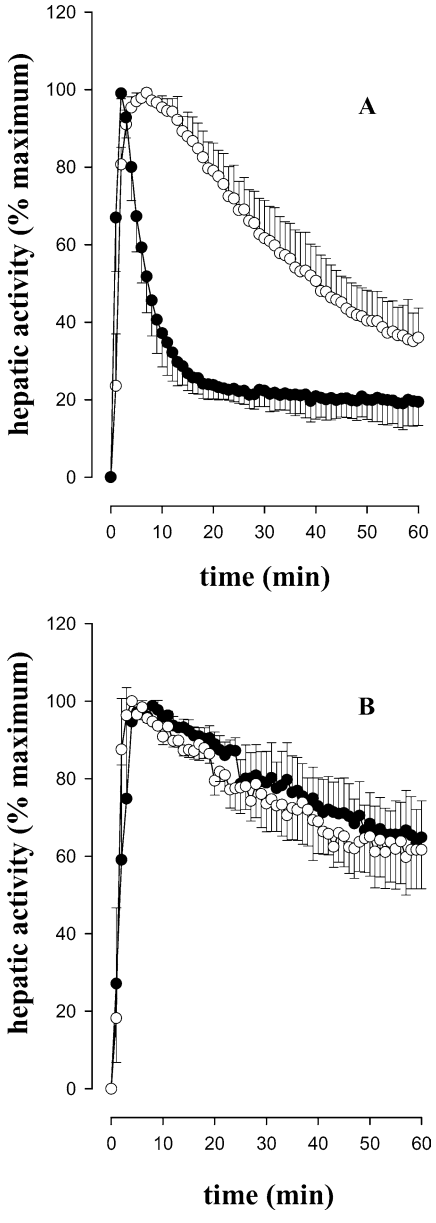

Fig. 5a, b Time course of the hepatic excretion of (**a**) ^{99m}Tc-HIDA and (**b**) ^{99m}Tc-MIBI noninvasively in control rats (*filled circles*) and GY/TR$^-$ rats (*open circles*). The time–activity curves were obtained with a gamma camera. The rats were positioned on the collimator, to obtain planar images. Radioactivity was injected into the penile vein, and 60 frames each of 1 min were acquired. Radioactive decay was corrected automatically. The data presented are the means ± SD from three animals per group

of lower dosages. For this reason, knowledge of drug transport function and polymorphism might allow dosage regimens for drugs which are mainly secreted by P-gp and/or MRP1,2 to be optimized in the individual patient. To avoid toxicity, dosage schedules potentially could be adapted in the future on the basis of measured P-gp/MRP function.

In conclusion, ^{99m}Tc-HIDA is a substrate for MRP1,2 only, while ^{99m}Tc-MIBI is a substrate for both P-gp and MRP1,2 in rats. Transport kinetics of these radiopharmaceuticals in the rat liver can be visualized

using gamma camera imaging in vivo. Comparison of the hepatobiliary kinetics of both ^{99m}Tc-HIDA and ^{99m}Tc-MIBI may give clinical information about the transport capacity of P-gp and/or MRP1,2 drug transporters in the human liver.

Acknowledgement This study was supported by grant GUKC 94-783 from the Dutch Cancer Society.

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