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ORIGINAL PAPER

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Ex vivo stimulation of renal transport of the cytostatic drugs methotrexate, cisplatin, topotecan (Hycamtin) and raltitrexed (Tomudex) by dexamethasone, T3 and EGF in intact human and rat kidney tissue and in human renal cell carcinoma

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Abstract Previous experiments have shown that both in vivo and in vitro pre-treatment with various hormones increases the renal transport capacity for weak organic acids, such as PAH, in rats. The aim of the present study was to test whether or not accumulation of the anticancer drugs methotrexate (MTX), cisplatin (CP), raltitrexed (Tomudex) and topotecan (Hycamtin) can be increased in intact, healthy rat and human renal cortical slices and in human renal cell carcinoma (RCC). Intact, healthy human tissue was obtained from tumour bearing kidneys of patients suffering from RCC. Experiments were intended as a new approach to overcome so-called multidrug resistance. Kidney tissue slices were incubated for 24 h in William's medium E containing various concentrations of dexamethasone, T_3, or EGF. Thereafter slices were placed in anticancer drug containing Cross-Taggart medium and the drug uptake into kidney tissue was measured for 2 h. In intact rat and human renal tissue slices, the uptake of p-aminohippurate (PAH = reference substance) increased significantly after incubation in dexamethasone containing medium (134% and 156%, respectively). There were no stimulating effects of either T_3 or EGF on PAH accumulation. On the other hand, only the accumulation of MTX, but not of CP, raltitrexed or topotecan, was significantly enhanced after hormone pre-treatment both in intact renal tissue and in RCC. A stimulation of renal PAH accumulation can be performed ex vivo, as reported previously, both in intact rat and human renal cortical slices and in RCC. Discrepancies between the effects of dexamethasone and T_3 or EGF indicate different modes of action of these substances at the cellular level. Unfortunately, with the exception of MTX, the uptake of anticancer drugs can not be stimulated effectively ex vivo in human RCC tissue by the substances used. Evidently the transport of these anticancer drugs out of the kidney cells is more effective than their uptake.

Keywords Renal tubular transport · Stimulation · Multidrug resistance · Renal cell carcinoma · Anticancer drugs · Methotrexate

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Introduction

The present study is the fourth in a series of investigations dealing with the ex vivo characterisation of drug transport in the human kidney. Previously, it was shown that p-aminohippurate (PAH) accumulation in intact rat and human kidneys and in renal cell carcinoma (RCC) can be stimulated after the ex vivo incubation of renal cortical slices in dexamethasone containing medium [9, 10]. Organic anions are secreted into the urine via organic anion transporters across the renal basolateral membrane. However, no apical membrane transporter for organic anions such as PAH has yet been identified [38]. Therefore, PAH was investigated as a substrate for the apical multidrug resistance (MDR) protein MRP2 [20]. This ATP-dependent export pump for anionic conjugates and amphiphilic anions was cloned recently and localized to the apical membrane of the proximal tubules in human and rat kidney. PAH is a good substrate for the ATP-dependent export pump MRP2 which has a similar substrate specificity to the multidrug

resistance protein MRP1, and may contribute to the multidrug resistance of renal clear-cell carcinoma [32].

Without doubt, PAH transport in renal cells is quite different from that of anticancer drugs. It is well-known that RCC and its metastases are nearly insensitive to any kind of cancer chemotherapy [2]. This MDR is characterised by a broad spectrum cross resistance to many anticancer drugs [41]. MDR is explained first of all by an increase in the transport of anticancer drugs out of RCC-cells [19]. Therefore, a stimulation of the uptake of cytostatic drugs could be beneficial in the treatment of RCC and its metastases. However, the drug resistance phenotype of primary RCC may not reflect that of their metastases [11].

The final goal of the present experiments was to stimulate the uptake of cytostatics into the human kidney, and especially RCC, in order to overcome MDR in RCC because MDR remains an obstacle to the chemotherapy of this cancer [43]. We have previously shown that the accumulation capacity of RCC tissue is significantly reduced [8]. Therefore, a stimulation of the uptake of cytostatic drugs would be beneficial in the treatment of RCC. For this reason, the accumulation of methotrexate (MTX), cisplatin (CP), topotecan and raltitrexed (Tomudex) was characterized in intact rat and human kidneys and in RCC with and without pre-treatment with dexamethasone, triiodothyronine (T_3), or epidermal growth factor (EGF). The different anticancer drugs were chosen for the following reasons: the human MRP gene family contains at least six members; MRP3 is an organic anion and multidrug transporter responsible for high-level resistance to MTX [17]. Recently a MTX carrier was reported in the kidney [15]. These results support the hypothesis that both OAT-K1 and OAT-K2, two apical membrane bidirectional organic anion transporters, participate in the epithelial transport of lipophilic organic anions, such as anticancer drugs, in the kidney. The OAT-K1-mediated MTX transport was significantly inhibited in the presence of several organic anions [24], therefore MTX seems to be transported by OAT1 [40].

Despite nephrotoxicity being one of the major dose-limiting side-effects of CP [18], this drug is an interesting substance because it induces the renal expression of P-glycoprotein and canalicular multispecific organic anion transporter (cMOAT or Mrp2) [4]. Drug resistance to CP appears to be modulated by human cMOAT through transport of CP out of the cells [16]. Furthermore, in the kidney, endothelins (ETs) are important regulators of blood flow, glomerular hemodynamics, and sodium and water homeostasis. Masereeuw et al. have reported for the first time results linking ET to the control of xenobiotic (e.g. CP) transport and demonstrated the control of renal multidrug resistance by a hormone [23].

Numerous new antifolate drugs have been developed to overcome the potential mechanisms of tumour cell resistance to MTX [35]. Promising antifolate compounds undergoing clinical testing as anticancer agents include the thymidilate synthase inhibitor raltitrexed [37].

RCC exhibits only a marginal response rate against cytostatics [12]. The resistance of RCC to anticancer-induced apoptosis has primarily been related to the expression of P-glycoprotein and to effective drug detoxification mechanisms. Treatment of RCC cell lines with topotecan (Hycamtin), a novel topoisomerase I inhibitor with little substrate affinity for P-glycoprotein, led to the induction of apoptosis and a significant reduction in cell numbers [30].

Materials and methods

Animals

Investigations were performed on female Wistar rats (Han:Wist) from our Institute's own out-bred stock. The animals were 2 months old and the average body weight was 172 ± 8 g (mean ± SEM). Rats were kept in plastic cages under identical conditions (12/12 h light/dark cycle), in environmentally controlled rooms (22 ± 2°C, 50% ± 10% humidity), including free access to standard diet Altromin 1316R (Altromin, Lage, Germany) and tap water. The experiments were permitted in accordance with the German law for the humane treatment of research animals.

Patients

Between July 1997 and June 2001, ex vivo accumulation experiments were performed on human kidney slices. Intact renal cortical tissue (control), i.e. macroscopically inconspicuous material from tumour bearing kidney as well as RCC tissue, was obtained from 41 patients undergoing radical nephrectomy of the tumour in the Department of Urology of the University of Jena. This study was performed in accordance with internationally accepted ethical standards for human experimentation and was approved by the Ethical Review Committee of the Medical Faculty of the University of Jena. RCC cases were classified as described by Fleck et al. [8]. The patients comprised 24 men and 17 women with an average age of 63.3 ± 12.1 years. Tissue samples (about 1 g) were stored in normal saline on ice (4°C) immediately after kidney removal. After transportation (30–60 min), the slices were prepared as described below.

Accumulation experiments

Acute experiments (2-h incubation time)

Renal slices with pool sizes of about 100 mg (~1 mm thick) were prepared free-hand and incubated in 50 ml-flasks with the anticancer drug of interest under bidirectional shaking (about 100 rpm) in 3 ml Cross-Taggart buffer (pH 7.4, 30°C, oxygen gassing at 2.5 l/h/sample and incubation time 120 min). To identify acute hormonal effects, the medium was supplemented with either dexamethasone, T_3, or EGF as described below. Following incubation, the uptake of the substance was expressed by the ratio between the drug concentration in the tissue and in the medium (slice to medium concentration ratio = $Q_{S/M}$) according to [34].

Sub-chronic experiments (24-h incubation time)

Experiments were performed with 24-h-incubation of renal slices in 50 ml-flasks containing 10 ml William's medium E (WE medium, Bio Whittaker) which was supplemented with glutamine (292 mg/l), insulin (1 μM), gentamicin (50 mg/l), and different concentrations of dexamethasone, T_3 (Sigma, St. Louis, USA) or EGF [Des-Leu26/Cys(Acm)20/31)EGF (20–31)] (BACHEM Bubendorf,

Switzerland). Dexamethasone and T_3 were used at a concentration of 10^{-8} μM and 10^{-9} μM, respectively, which were found to be optimal in previous experiments [6, 9, 10]. EGF was added to the incubation medium at a concentration of 1 μg/ml. This concentration was indirectly calculated from in vivo data reported in the literature to influence various organ functions effectively [42]. The 24-h experiments were performed under carbogen gassing (95% O_2 + 5% CO_2; 2.5 l/h/sample) adjusted to pH 7.4 and 25°C. Thereafter, the slices were placed in Cross-Taggart buffer (pH 7.4) containing the drug of interest. The uptake of this drug was measured as described above.

Determination methods

P-aminohipurate was determined after Bratton and Marshall [3]. To estimate cisplatin concentrations (Jenapharm, Jena, Germany) the platinum content was measured by flameless atomic absorption spectrometry (AAS; model EA 4, Carl Zeiss, Jena, Germany). MTX (Serva, Heidelberg, Germany) was estimated by HPLC described first by Nürnberg et al. [28]. For topotecan (Hycamtin; SmithKline Beecham Pharma, München, Germany) both lactone and ring-opened form (hydroxy acid) concentrations were determined with a reversed-phase HPLC technique (HPLC LC10 system, Shimadzu, Japan) according to Loos et al. [22]. For the measurement of raltitrexed (Tomudex; Zenica, Plankstadt, Germany) a RP-HPLC method was adapted by Hilger et al. [13].

Statistics

The results are given as arithmetic means ± SEM of four to six independent slice preparations. For each patient, three to four slice preparations per medium concentration were used in order to minimise methodological variances. Statistically significant differences between the experimental groups were analysed using the Mann-Whitney U-test or the Student's *t*-test. The significance level was set as $P < 0.05$.

Results

In *acute* PAH accumulation experiments on intact, healthy human kidneys, a mean $Q_{S/M}$ of 3.12 ± 0.24 ($n = 31$) was measured (Fig. 1). In RCC, PAH accumulation was completely abolished $Q_{S/M} \sim 1$. Interestingly, the accumulation capacity in the "intact" renal tissue seems to be inversely correlated to tumour grading (pT1–3) and tumour diameter. For further background information see Fleck et al. [9].

The addition of dexamethasone into the incubation medium increased PAH accumulation in the renal tissue of rats and in intact human kidney tissue (Fig. 2, left) as a sign of ex vivo stimulation. This stimulatory effect was concentration and time dependent. The optimal dexamethasone concentration was between 10^{-8}–10^{-9} mM in the incubation WE medium. Acute incubation (2 h) with dexamethasone or T_3 had no effect on PAH accumulation, either in intact kidney tissue or in RCC, whereas after 12 h and, more pronounced, after 24 h a significant increase in PAH accumulation capacity could be demonstrated. This disappeared after 48-h incubation (not shown). In intact human renal tissue, the PAH accumulation capacity dropped from $Q_{S/M} = 5.3 \pm 0.5$ in acute, 2-h accumulation experiments to 2.8 ± 0.4 after a 24-h incubation time. It can be increased by dexamethasone by up to 156%. On the other hand, T_3 decreased PAH uptake in intact human kidney tissue.

In RCC, the PAH uptake could be stimulated significantly after 24 h incubation in dexamethasone by about 90%, while T_3 was without effect on PAH accumulation (Fig. 2, right). It should be mentioned that RCC's can be differentiated into "dexamethasone-responders" and "non-responders" [10]. For the presentation of data given in Fig. 2 only responders were chosen.

The accumulation of MTX was significantly lower than for PAH, especially in intact rat and human kidney tissue, and the concentration dependency was of minor importance (Fig. 3). Evidently, this indicates a passive

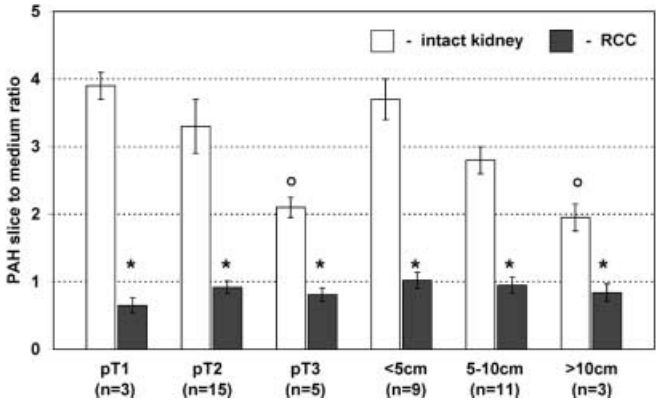

Fig. 1. Correlation between RCC grading (pT_1–pT_3) and tumour diameter (< 5– > 10 cm) on PAH accumulation in RCC and in intact human renal tissue of the tumour bearing kidney. Arithmetic means ± SEM; n = 3–4 measurements per patient; number of patients in *parentheses*; an *asterisk* indicates significant differences between intact renal tissue and RCC; ^{o} indicates a significant difference between two subsequent experimental groups ($P \leq 0.05$)

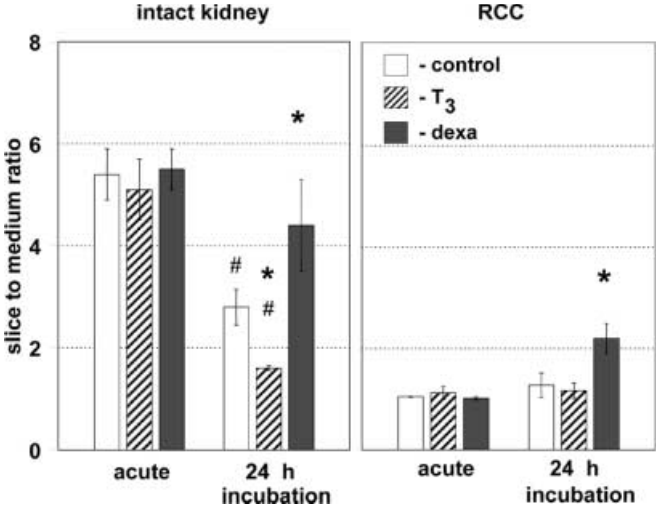

Fig. 2. Influence of dexamethasone or T_3 (acute effects and influence of 24-h incubation) on PAH accumulation (initial PAH concentration: 5×10^{-8} M) in intact human kidney tissue and in RCC. Arithmetic means ± SEM; n = 6 patients, 3–4 measurements per patient; an *asterisk* indicates a significant hormone effect ($P \leq 0.05$); # indicates a significant difference between the 2- and 24-h-incubation ($P \leq 0.05$)

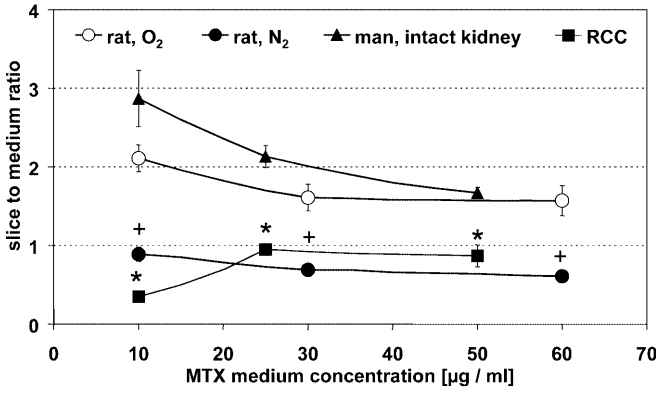

Fig. 3. Concentration dependence and energy dependence (O_2 or N_2-gassing) of MTX accumulation ($Q_{S/M}$) in intact rat and human kidney tissue and in RCC during 2-h incubation experiments. Arithmetic means ± SEM; $n = 6$; an *asterisk* indicates a significant difference between intact human kidney tissue and RCC ($P \leq 0.05$); a *plus* indicates a significant difference between oxygen and nitrogen atmosphere ($P \leq 0.05$)

influx of the lipophilic MTX into the kidney tissue. The difference between intact human kidney tissue and RCC shown for PAH could be confirmed for MTX as well. RCC was unable to enrich MTX. Therefore, the passive uptake shown for tissue human kidney is not as effective compared to the efflux of MTX in RCC cells if one assumes nearly similar passive enrichment in intact kidney and in RCC.

Finally, in rat kidney it could be shown that MTX uptake is not only passive, but is also an energy dependent oxygen consuming process. The uptake of MTX in rat kidney reached a steady state after about 2 h (Fig. 4). The efflux of MTX was not energy consuming. This means that there is evidently no active transport

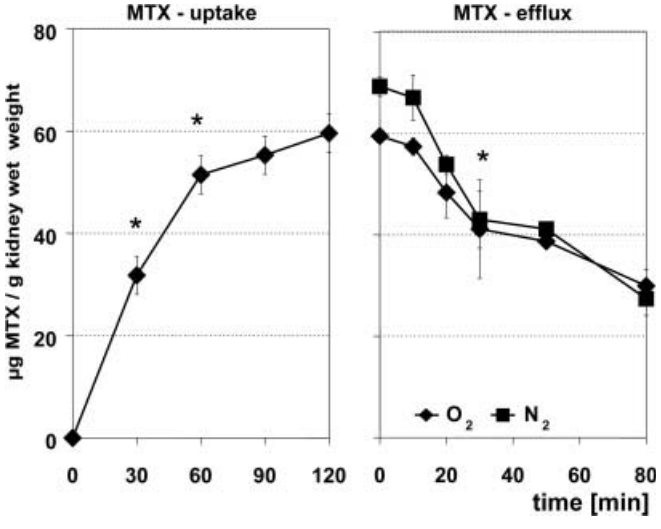

Fig. 4. Time dependence of MTX uptake and MTX efflux under aerobic (O_2) and anaerobic (N_2) incubation conditions in rat kidney tissue. Initial MTX medium concentration: 10 µg/ml. Arithmetic means ± SEM; $n = 4$ per time; an *asterisk* indicates significant differences between two subsequent times ($P \leq 0.05$)

out of the renal cells of rats. For economic reasons, comparable experiments were not performed on intact human kidney tissue or RCC.

The maximal acute accumulation (2-h-incubation) of anticancer drugs is concentration dependent and distinctly lower compared to PAH in intact rat and human renal tissue and in RCC (Fig. 5). In general, anticancer drug accumulation is significantly lower in RCC in relation to intact renal tissue. However, the uptake of topotecan is more effective in intact human kidney tissue compared to the rat. On the other hand, the maximal accumulation of raltitrexed is quite comparable to that of PAH in the rat, but not in humans.

In principle, the maximal accummulation capacity for anticancer drugs can be stimulated in intact rat and human kidney tissue (Fig. 6). However, in contrast to PAH, T_3 is effective whereas dexamethasone is not in the case of MTX. For RCC, however, the accumulation of MTX is slightly enhanced only after dexamethasone use. In contrast to PAH and MTX, there is no stimulatory effect of either dexamethasone or T_3 on the accumulation of CP, raltitrexed, and topotecan into intact kidney tissue, either in rats or humans, or in RCC.

Based on the hypothesis that the cellular EGF effect is mediated by T_3 and dexamethasone [31], combination studies were done on rat kidney tissue. EGF alone seems have no effect on renal transport capacity (Fig. 7). After the additional incubation of rat renal cortical slices in dexamethasone plus EGF, the stimulatory effect of dexamethasone on PAH uptake completely disappeared. Because of the lack of EGF effect in rats, experiments on human tissue were not performed.

Discussion

A stimulation of tubular PAH accumulation can be induced ex vivo in intact human kidney tissue and in RCC. Ex vivo experiments are qualified to characterise the renal accumulation capacity of anticancer drugs in human kidney as shown in detail for PAH [9]. Discrepancies between the effects of dexamethasone and T_3 and/or EGF indicate the different modes of action of these substances at the cellular level. Evidently the transport of anticancer drugs out of the kidney cells is more effective than their uptake, also after stimulation of the latter. Therefore, the stimulation of renal tubular secretion capacity is not a suitable therapeutic strategy to overcome MDR in RCC and to increase the anticancer drug concentration within RCC cells.

PAH was investigated as a substrate for the apical MDR protein MRP2 [20], for mdr 1 or mdr 3 [26], and a lipoprotein receptor-related protein [21]. These ATP-dependent export pumps for anionic conjugates and amphiphilic anions were cloned and localised to the apical membrane of the proximal tubules in human and rat kidney. MRP2 has a similar substrate specificity as MRP1, and both may contribute to the MDR of RCC [32].

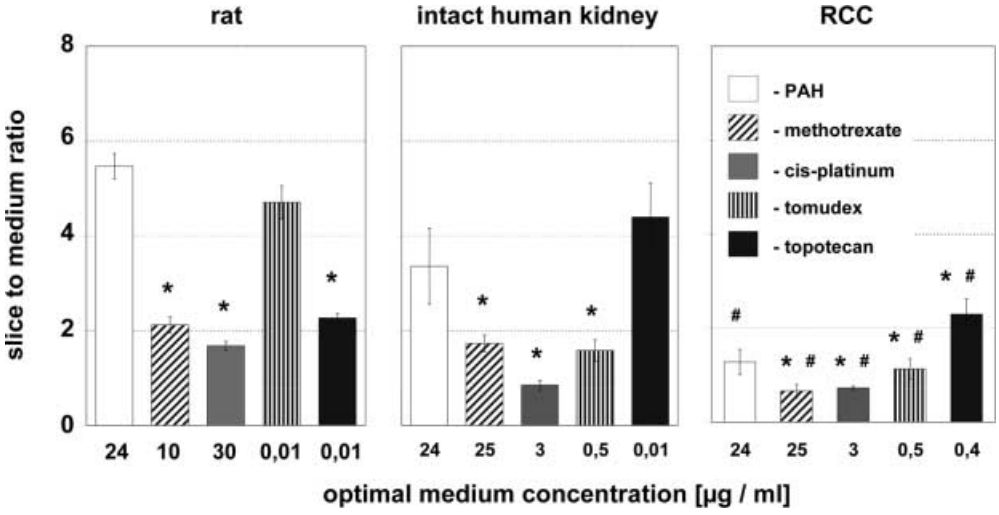

Fig. 5. Comparison between intact rat and human kidney tissue and RCC in relation to the maximal accumulation of PAH, MTX, CP, raltitrexed, and topotecan during acute, 2-h incubation. The optimal drug concentration in the medium is given at the bottom of the columns. Arithmetic means ± SEM; $n = 6$ rats or 3–6 patients with 3–4 measurements per patient; an *asterisk* indicates significantly different from PAH ($P \leq 0.05$); # indicates a significant difference between intact human kidney tissue and RCC ($P \leq 0.05$)

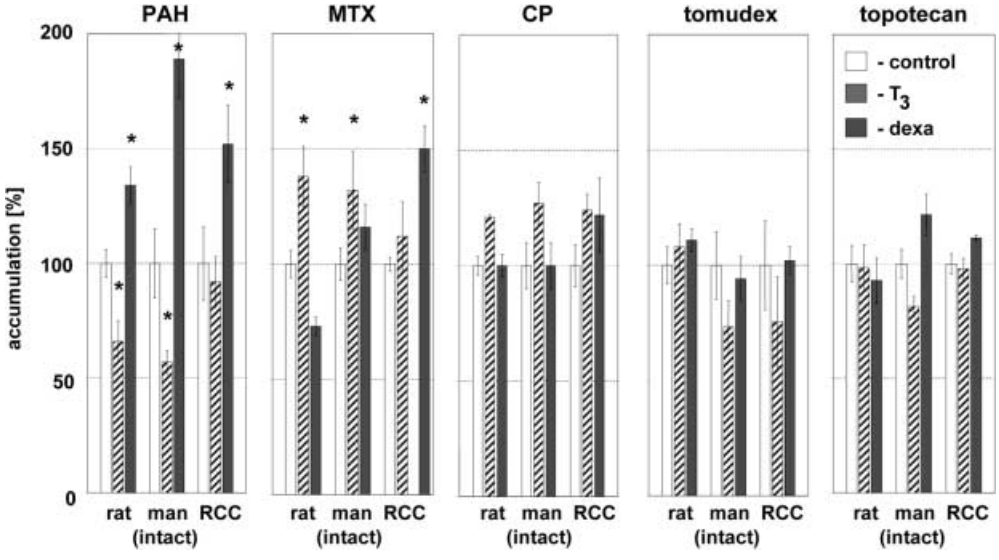

Fig. 6. Effects of dexamethasone or T_3 on the accumulation of PAH, MTX, CP, raltitrexed, and topotecan in intact renal tissue of rats and humans and in RCC. Maximal changes after 24-h incubation are given in % of corresponding controls (=100%). Arithmetic means ± SEM; $n = 6$ patients, 3–4 measurements per patient; an *asterisk* indicates a significant hormonal effect ($P \leq 0.05$)

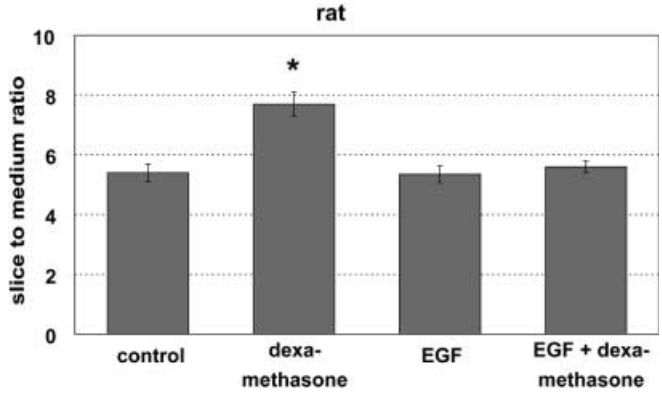

Fig. 7. Accumulation of PAH in intact rat renal cortical slices after 24-h incubation in WE medium containing EGF (1 µg/ml) or dexamethasone (10^{-8} µM) alone and EGF in combination with dexamethasone. Arithmetic means ± SEM; $n = 6$. an *asterisk* indicates a significant hormonal effect ($P \leq 0.05$)

Organic anion transporters in the kidney proximal tubule play an essential role in eliminating a wide range of organic anions including anticancer drugs and their metabolites [43]. MDR is explained first of all by an increase in the transport of cytostatics out of RCC [19]. Therefore, a stimulation of the uptake of cytostatic drugs could be helpful in the treatment of RCC and its metastases. In further experiments, results obtained on RCC have to be demonstrated with tissue of RCC metastases as described for the combination between interferon and dexamethasone [1]. The final goal of the experiments presented here was to stimulate the uptake of cytostatics into RCC to overcome MDR. Tobe et al. reported that the comparison of MDR 1 levels between different histological types revealed that the mean MDR 1 level of the more undifferentiated clear-cell tumours was significantly lower than the mean MDR 1 level of adjacent normal kidney [36]. Higher MDR 1 expression in the invasive tumours compared with non-invasive

tumours suggests that MDR 1 expression and invasiveness may be linked [14].

The question remains open as to why the co-administration of dexamethasone plus EGF does not stimulate the in vitro PAH uptake in rats whereas in vivo a significant increase in PAH excretion was found after EGF application [7].

If it would be possible to enhance the uptake of anticancer drugs in vivo, this could improve the efficacy of chemotherapy. Doubtless this is only one side of the coin: the enhanced uptake of anticancer drugs could be followed by an increase in their export out of the tumour cell (for transport of MTX see Fig. 4). Therefore, the results of this study may reflect the sum of two different influences: hormones could both increase drug uptake into RCC and could accelerate their efflux. These two aspects can not be differentiated by our experimental approach: MDR can be created by the membrane-bound MDR-associated protein, the detoxifying glutathione metabolism, the antiapoptotic protein BCL2, and changes in levels or activity of the topoisomerase enzymes [41].

A second difficult problem involves the chemical differences between the weak organic acid PAH and most anticancer drugs (alkaloids, nucleotides). It has to be taken into consideration that, in the case of anticancer drugs, hormones other than dexamethasone, T_3, or EGF could enhance enrichment in RCC because they could increase the uptake as well as reduce the efflux of the drugs out of RCC [5] in a way different from the hormone influence measured on the PAH transport. Own preliminary (unpublished) PCR data show that evidently PAH carrier synthesis seems to be enhanced by dexamethasone and T_3. Dexamethasone is a substrate to the mdr1 gene product [33]. Therefore, it cannot be excluded that ex vivo dexamethasone interferes with the transport proteins evacuating drugs from the tubular cell [39].

Anticancer chemotherapy is focussed firstly on RCC metastases, which are more susceptible to anticancer drugs [25]. In addition, the administration of high-dose dexamethasone could disturb the organism's metabolism. Therefore, the effect of anticancer drugs could be enhanced, but side effects of high-dose hormone therapy may occur. In summary, the addition of high doses of dexamethasone or T_3 are not able to modulate the MDR in RCC to a practically relevant extent. Nevertheless, combination therapy seems to be a method of choice in the future: the combination of various drugs as reported by Naito et al. and Punt et al. significantly increased the life span of mice inoculated with drug-resistant tumour cells without any significant side effects [27, 29].

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