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Effect of ketoprofen and its enantiomers on the renal disposition of methotrexate in the isolated perfused rat kidney

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to inhibit the renal tubular secretion of methotrexate. However, the relative contribution of the active *S*- and inactive *R*-enantiomers is unknown. This study examined the effect of racemic ketoprofen and its enantiomers on the renal disposition of methotrexate in the isolated perfused rat kidney (IPK). Nineteen kidneys were divided between a control and three treatment groups. Controls were perfused with methotrexate alone (25 μg mL^{-1}, n = 5) over three 30-min periods. Treatment groups were perfused with methotrexate (25 μg mL^{-1}) for the first period, followed by a second period of methotrexate (25 μg mL^{-1}) plus *R*- (n = 5), *S*- (n = 5) or *RS*-ketoprofen (n = 4) at 25 μg mL^{-1}, and a third period of methotrexate (25 μg mL^{-1}) plus *R*-, *S*- or *RS*-ketoprofen (50 μg mL^{-1}). Perfusate and urine were collected over 10-min intervals. Methotrexate was measured by HPLC and its binding in perfusate by ultrafiltration. The clearance ratio (CR) for methotrexate was obtained by dividing the renal clearance by the product of its fraction unbound and the glomerular filtration rate. During control experiments, there was no significant change in the CR over 90 min. *R*-, *S*- and *RS*-ketoprofen at 50 μg mL^{-1} reduced the CR of methotrexate significantly, but there was no difference between the three groups. While the enantiomers of ketoprofen reduced the renal excretion of methotrexate, the interaction was not enantioselective.

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Introduction

As well as its role in chemotherapy, the chemotherapeutic agent methotrexate is increasingly being used for the treatment of rheumatoid arthritis (Kremer 1996). In both instances it is often administered in combination with a non-steroidal anti-inflammatory drug (NSAID). Unfortunately, in man NSAIDs can increase the risk of adverse effects from methotrexate (Thyss et al 1986; Stewart & Evans 1990). Due to the potential severity of side effects resulting from the altered and unpredictable disposition of methotrexate, there have been considerable investigations attempting to characterize the mechanism(s) of its interaction with NSAIDs (Statkevich et al 1993; Masuda et al 1997).

After administration, methotrexate exists predominantly as an organic anion at physiological pH and is eliminated from the body primarily by excretion of the unchanged drug into urine (Shen & Azarnoff 1978). Its renal clearance involves mainly tubular secretion, and it has been suggested that NSAIDs may compete with methotrexate for a secretory transport system (He et al 1991; Statkevich et al 1993; Masuda et al 1997).

Ketoprofen is one of the NSAIDs that has been reported to interact with methotrexate, potentially leading to severe and even life-threatening situations (Thyss et al 1986; Statkevich et al 1993). In most therapeutic preparations ketoprofen exists as a racemic mixture, despite the *S*-enantiomer alone being responsible for the inhibition of prostaglandin synthesis (Hayball et al 1992; Suesa et al 1993). Stereospecific protein binding (Dubois et al 1993; Sakai et al 1996; Zhivkova & Russeva 1998) and liver

metabolism (Carabaza et al 1996) have been reported for ketoprofen, but the ability of the enantiomers to alter the renal secretion of other compounds has not been examined. If the *R*-enantiomer of ketoprofen was an inhibitor of the renal tubular secretion of methotrexate, this would represent an example of the administration of an inactive enantiomer, as part of a racemic mixture, contributing to a clinically significant drug–drug interaction.

Previous studies have shown the isolated perfused rat kidney (IPK) to be a useful experimental model for examining renal interaction between drugs (Statkevich et al 1993). Therefore, this study used the IPK to examine the effect of the racemic mixture and, more specifically, compare the effect of the enantiomers of ketoprofen on the disposition of methotrexate.

Materials and Methods

Chemicals

Methotrexate (>98%, HPLC grade, Lot No. 57H0576) was obtained from Sigma Chemical Co. (St Louis, MO) and Methotrexate Injection BP from FH Faulding & Co. Ltd (Mulgrave, Victoria, Australia). Racemic ketoprofen was obtained from Sigma, and *R*(−)- and *S*(+)-ketoprofen from Laboratorios Menarini S.A. (Barcelona, Spain). [^{14}C-Carboxyl]inulin was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK) and Pentex Bovine Serum Albumin fraction V from Miles Inc. (Pittsburgh, PA). All other chemicals, used for the kidney viability assays, the preparation of the perfusion media, and for the analysis of methotrexate, were of analytical grade and from commercial suppliers, as described previously (Mancinelli et al 1995).

Rat IPK model

The study was approved by the Institute of Medical and Veterinary Science Animal Ethics Committee (Adelaide, SA, Australia). Male Sprague-Dawley rats (350–400 g; Gilles Plains Animal Resource Centre, Adelaide, SA, Australia) were housed at 21 °C on a 12-h light/dark cycle with food and water freely available. The IPK preparation was based on a method described by Mancinelli et al (1995). The rats were anaesthetised with an intraperitoneal injection of 60 mg kg^{-1} sodium pentobarbitone (Nembutal, Boehringer Ingelheim, North Ryde, Australia) before surgery. The isolated kidneys were perfused in a recirculating manner in a thermostatically controlled cabinet maintained at 37 °C. An erythrocyte-free modified Krebs–Henseleit bicarbonate buffer solution (containing bovine serum albumin, glycine, L-cysteine, L-glutamic acid and glucose (Mancinelli et al 1995)) in a reservoir was pumped through the kidney via an in-line filter (8 μm), a membrane oxygenator, glass bubble trap, flow meter, and finally a glass arterial cannula inserted into the renal artery, and returned to the reservoir. Throughout the perfusion, carbogen was supplied to the membrane oxygenator to maintain a constant concentration of oxygen in perfusate at a value greater than 0.6 mM and the pH was maintained at 7.4. The viability of the kidney was monitored by measuring glomerular filtration rate (GFR), and the reabsorption of water, sodium and glucose (Mancinelli et al 1995). The organs were considered viable if the respective values were greater than 0.4 mL min^{-1}, 75%, 95%, and 99%, respectively.

Experimental design

To examine the influence of ketoprofen and its enantiomers on the renal disposition of methotrexate, kidneys were divided into four groups. The control group consisted of five kidneys perfused with methotrexate alone at an initial concentration of 25 μg mL^{-1} for three periods of 30 min. The three treatment groups consisted of kidneys perfused with methotrexate (25 μg mL^{-1}) for an initial period of 30 min, after which time racemic (n = 4), *R*- (n = 5) or *S*-ketoprofen (n = 5) was added to the perfusate at a concentration of 10 μg mL^{-1}. After a further 30 min, the concentration of racemic, *R*- or *S*-ketoprofen was increased to 50 μg mL^{-1}.

Urine was collected over successive 10-min intervals (10–20, 20–30, 40–50, 50–60, 70–80 and 80–90 min) from the start of the perfusion with methotrexate; samples of perfusate were collected from the reservoir at the midpoint times of these intervals.

Analytical method

The concentrations of methotrexate in perfusate and urine were measured by reversed-phase HPLC with detection by UV absorption. Perfusate (500 μL) was mixed with 500 μL acetonitrile, centrifuged, and 525 μL of the supernatant extracted with 640 μL isoamyl alcohol/ethyl acetate (12.5/87.5). The upper organic layer was aspirated and 50 μL of the lower phase containing methotrexate was injected directly onto a reversed-phase analytical column (C_8 10 μm, 250 mm × 3.8 mm, Alltech Assoc., Baulkham Hills, Australia). A mobile phase consisting of 13% acetonitrile in 0.05 M phosphate buffer (pH 7.4) was pumped (Model LC-10AT, Shimadzu, Japan) through the column at 1 mL min^{-1} and the eluent monitored for UV absorption at 320 nm (SPD-10A, Shimadzu). Urine samples were injected directly onto the column. Six calibration standards over the range of 5–80 μg mL^{-1} and three quality controls of 7, 30 and 60 μg mL^{-1} were prepared in perfusate and urine, and were placed randomly within the analytical run. Intra-day values for six quality controls at the respective concentrations were 6.8 ± 0.2, 28.1 ± 0.7 and 54.2 ± 2.2 μg mL^{-1}; corresponding values from an assessment of inter-day accuracy (n = 6) were 6.4 ± 0.4, 28.5 ± 1.3 and 54.5 ± 1.5 μg mL^{-1}. The limit of quantification was 6.0 μg mL^{-1}.

The concentration of [^{14}C-carboxyl]inulin was determined in a liquid scintillation analyser (Packard Model 2200C, Packard Bioscience, Australia) after mixing 100 μL perfusate or 50 μL urine with 3 mL aqueous scintillant (Amersham, Arlington Heights, IL). Concentrations of glucose in perfusate and urine were determined by the glucose oxidase method using a diagnostic glucose kit

(Sigma Diagnostics, St Louis, MO); concentrations of sodium were determined using atomic absorption spectrophotometry (Mancinelli et al 1995).

Binding in perfusate

The unbound fraction (fu) of methotrexate in perfusate was determined in samples containing 25 μg mL^{-1} methotrexate alone and in the presence of 50 μg mL^{-1} racemic ketoprofen. After equilibration to 37 °C, quadruplicate 1-mL samples of perfusate were subjected to ultrafiltration using the MPS-1 Micropartition system with YMT-10 membranes (Amicon Corp., Danvers, MA). The fu of methotrexate in perfusate was calculated by dividing the mean concentration of methotrexate in the ultrafiltrate by the mean concentration of methotrexate in the unfiltered perfusate, both measured by HPLC.

Pharmacokinetics and statistical analysis

The renal clearance (CL_R) of methotrexate in each 10-min urine collection interval was determined as the rate of excretion into urine divided by the concentration of methotrexate in perfusate at the mid-point of the urine collection interval. A clearance ratio (CR) for methotrexate was calculated by dividing CL_R by the product of its fraction unbound (fu) and the GFR. For all experiments, the percentage tubular reabsorption (%TR) of water, glucose and sodium was calculated as described by Mancinelli et al (1995).

Statistical comparison of values for the CR between the four different treatments was performed by two-way repeated measures analysis of variance. Period was set as the within-subject factor with three levels, while treatment was set as the between-subject factor with four levels. Differences between the treatments within the time intervals were considered significant if the *P*-value was less than or equal to 0.05. Where a significant difference was found, comparisons were performed using multiple *t*-tests.

Results

Across all experiments, initial concentrations of methotrexate ranged from 23.5 to 38.6 μg mL^{-1}. However, there were no significant trends in the concentrations with time ($P > 0.05$). Mean values from each experiment for the pH of perfusate and urine ranged from 7.43 to 7.59 and from 6.82 to 7.85, respectively. Figure 1 shows the mean values of GFR over time for individual kidneys. Mean values for GFR were 0.45 mL min^{-1} in the control group and from 0.49 to 0.60 mL min^{-1} in the three treatment groups; there were no significant differences between the groups ($P = 0.80$) nor were there significant changes in the values over time ($P = 0.31$).

Figure 2 shows that the mean values for the CR in each period during perfusion with methotrexate alone (control) ranged from 1.72 to 1.90, and there was no significant difference between the three periods ($P > 0.05$). Similarly, there were no differences between the four groups in the values for CR during period 1 ($P > 0.05$). However, during period 3 there were significant differences in the values of CR for methotrexate between control kidneys and kidneys co-perfused with all three forms of ketoprofen at a concentration of 50 μg mL^{-1} (Figure 2). During this period, there were no significant differences in the values of CR for methotrexate between the three groups co-perfused with *R*-, *S*- or *RS*-ketoprofen; furthermore, the 95% confidence intervals for the three ratios included 1.0. The reductions in each value from the control group perfused during the same period were 37%, 40% and 36%, respectively. During co-perfusion with *R*-, *S*- or *RS*-ketoprofen at a concentration of 10 μg mL^{-1}, the reduction in values ranged from 15% to 22%, but were not significant ($P > 0.05$).

At a concentration of approximately 25 μg mL^{-1}, the fraction of methotrexate unbound in perfusate in the absence and presence of 50 μg mL^{-1} racemic ketoprofen was 14.7% and 14.4%, respectively.

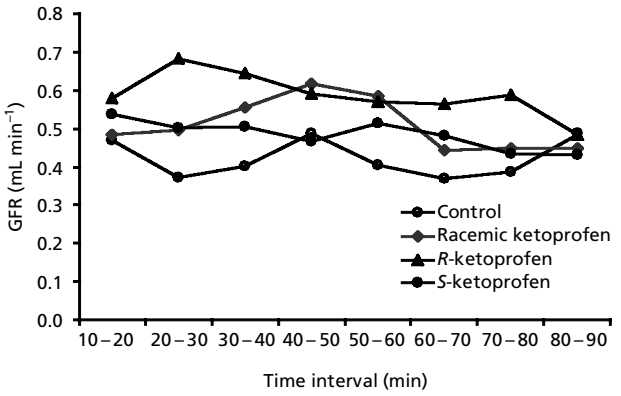

Figure 1 Mean values of GFR over time for kidneys perfused with methotrexate (control), and those perfused with methotrexate plus racemic ketoprofen, *R*-ketoprofen or *S*-ketoprofen. Bars for s.d. were omitted to improve clarity.

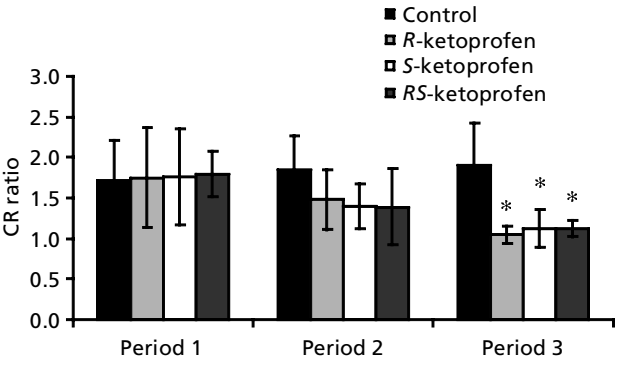

Figure 2 Mean (± s.d.) values of CR for methotrexate during perfusion of the rat IPK with methotrexate alone (control, n = 5), or in the presence of *R*-ketoprofen (n = 5), *S*-ketoprofen (n = 5) and *RS*-ketoprofen (n = 4), at a concentration of 10 μg mL^{-1} (period 2) and 50 μg mL^{-1} (period 3). *Significantly different from the corresponding control in period 3.

Discussion

Previous studies have shown the rat IPK to be an appropriate model for studying renal interactions between drugs (Statkevich et al 1993). Functional parameters demonstrating the viability of the perfused kidneys in this study were within ranges established previously in our laboratory as being acceptable (Mancinelli et al 1995).

The initial concentrations of methotrexate in perfusate differed slightly between the experiments (ranging from 23.5 to 38.6 $\mu g\,mL^{-1}$), and while it was shown in earlier studies that the renal handling of methotrexate was dependent on the concentration perfusing the isolated rat kidney (Statkevich et al 1993), those studies found that there was non-saturated net secretion of methotrexate over a range of unbound concentrations (approximately 2–7 $\mu g\,mL^{-1}$), which included those observed in this study (3.3–5.4 $\mu g\,mL^{-1}$). This study also demonstrated net tubular secretion of methotrexate in the rat IPK in the absence of ketoprofen; values for CR of between 1.72 and 1.90 were comparable with a mean value of 1.52 observed by Statkevich et al (1993). The effect of racemic ketoprofen on the fu of methotrexate in perfusate was negligible, and it is likely that the impact of each enantiomer on the binding of methotrexate in perfusate was also negligible. Although the low concentrations of racemic ketoprofen, *R*- and *S*-ketoprofen (10 $\mu g\,mL^{-1}$) decreased the CR of methotrexate by 22.3%, 15.1% and 20.5%, respectively, none of the decreases were statistically significant. However, the respective compounds at a concentration of 50 $\mu g\,mL^{-1}$ caused significant reductions (between 36%–40%) in the CR observed for the control group in period 3. There was no evidence for any differences in the extent of reduction caused by the enantiomers of ketoprofen. The values of CR for the treatment groups in period 3 were not significantly different from 1.0, which indicated that the renal clearance of unbound methotrexate approximated GFR and that the net tubular secretion of methotrexate in period 3 of the control group was inhibited to the same degree by ketoprofen and its enantiomers. Such observations were in agreement with those from a previous study with two other NSAIDs, indometacin and the chiral NSAID, flurbiprofen, both of which decreased the value of CR for methotrexate in the rat IPK (Statkevich et al 1993). However, that study did not examine for differences in effect between the enantiomers of flurbiprofen.

Kremer (1996) suggested that the interaction between methotrexate and NSAIDs might be due to a NSAID-induced inhibition of prostaglandin synthesis, resulting in a decrease in GFR and reduced renal clearance of methotrexate. In this study, the GFR of the perfused rat kidney was found to vary within and between experiments. Importantly, there was no evidence that ketoprofen lowered the GFR of the IPK (Figure 1).

Nierenberg (1983) examined the impact of selected NSAIDs on the uptake of methotrexate into slices of kidney from the rabbit, with the conclusion that the accumulation of methotrexate in the slices occurred via an active process that was inhibited competitively by a range of NSAIDs. Methotrexate is transported by the organic anion transporter, OAT1 (Sekine et al 1997; Uwai et al 1998), which is located in the basolateral membrane of renal tubules (Hosoyamada et al 1999). *Xenopus laevis* oocytes expressing OAT1 were used to demonstrate competitive inhibition of the transport of methotrexate by probenecid, indometacin and salicylic acid (Uwai et al 2000). Using a similar system, indometacin was a substrate for uptake as well as an inhibitor of the transport of p-aminohippuric acid by OAT1 (Apiwattanakul et al 1999). A number of other NSAIDS, including ketoprofen, were found to inhibit the transport of p-aminohippuric acid, with complete inhibition by 1 mM ketoprofen (Apiwattanakul et al 1999). Unfortunately, ketoprofen was not examined over a range of concentrations, but other NSAIDs inhibited transport competitively. While ketoprofen itself was not efficiently transported by human OAT1 expressed in Chinese hamster ovary cells, it was a potent inhibitor of the OAT1-catalysed transport of adefovir (Mulato et al 2000). Therefore, it seems most likely that the observed decrease in the renal clearance of methotrexate in this study was due to competitive inhibition by the enantiomers of ketoprofen of the transport mechanisms responsible for the tubular uptake and secretion of methotrexate, most probably OAT1.

Comparisons of renal clearances between enantiomers have been conducted most extensively with weak bases. Differences in values have been reported in man for the unbound enantiomers of pindolol (Hsyu & Giacomini 1985; Somogyi et al 1992), disopyramide (Lima et al 1985) and amphetamine (Hutchaleelaha et al 1994); all underwent secretion in addition to filtration and, presumably, the observations reflected differences in secretory transport between isomers. However, there were no differences in the renal clearance of the unbound enantiomers of methadone (Foster et al 2000) and bisoprolol (Horikiri et al 1997), nor in inhibition by the enantiomers of pindolol, disopyramide and bupivacaine on the transport of tetraethylammonium across vesicles prepared from the apical membrane (referred to as brush-border membrane vesicles by the authors) (Gross & Somogyi 1994). Furthermore, inhibition by pindolol of the uptake of N-methylnicotinamide by these vesicles was not stereoselective (Ott et al 1991). From observations with the four stereoisomers of norephedrine and ephedrine (Gross & Somogyi 1994), it was proposed that differences in inhibition may be dependent on the distance between the cationic function and the asymmetric carbon of the inhibitor. Very few studies have compared the renal clearances of the enantiomers of secreted weak acids. Differences were observed in Cynomolgus monkeys between the isomers of a uricosuric agent, S-8666, which contains a carboxylic acid moiety (Nakano & Kawahara 1992), but there were no differences for sulbenicillin (Itoh et al 1998) or carbenicillin (Itoh et al 1993) in man. The authors are not aware of any studies comparing the renal clearance of the unbound enantiomers of NSAIDs.

Data for the enantiospecific binding of ketoprofen to proteins in plasma are contradictory (Hayball et al 1991; Dubois et al 1993; Sakai et al 1996; Brink 1998; Zhivkova & Russeva 1998). From the majority of studies, it would

seem appropriate to assume non-enantioselective binding (Hayball et al 1991; Brink 1998) and, therefore, it is unlikely that there were differences in the renal clearances of unbound ketoprofen in the rabbit (Abas & Meffin 1987). It was apparent that a high degree of hydrophobicity and the presence of a carboxyl function enhanced the competitiveness of NSAIDs for the transport of p-aminohippuric acid (Apiwattanakul et al 1999) and adefovir (Mulato et al 2000) by OAT-1. The carboxyl group of ketoprofen is adjacent to the chiral center. Therefore, given the competitive inhibition of the transport of methotrexate by ketoprofen (Masuda et al 1997) and of p-aminohippuric acid by a range of NSAIDs (Apiwattanakul et al 1999), it is somewhat surprising that in this study no difference between the enantiomers of ketoprofen in the potency with which they inhibited the renal excretion of methotrexate by the IPK was observed.

Methotrexate is also transported in a bi-directional manner by the kidney-specific organic anion transporters, OAT-K1 and OAT-K2, which are more likely located in the apical membrane of renal tubular cells (Masuda et al 1997, 1999). NSAIDs were found to reduce the accumulation of methotrexate in LLC-PK_1 cells expressing rat OAT-K1, with indometacin and ketoprofen demonstrating competitive inhibition of the accumulation (Masuda et al 1997). However, given the current lack of certainty in regard to the cellular location of these transporters, and their role in the renal excretion of methotrexate, it was difficult to ascertain the significance of these observations to the findings with the IPK.

Conclusions

This study examined the influence of ketoprofen, and compared the effect of its enantiomers, on the disposition of methotrexate in the isolated perfused rat kidney. At the concentrations of methotrexate used, there was net secretion of methotrexate in the absence of ketoprofen, and this was inhibited by co-perfusion with racemic ketoprofen. However, while the individual enantiomers inhibited secretion, there was no difference in their degree of inhibition.

Overall, our findings, combined with those from other studies, were in keeping with the conclusion that there is a renal interaction between ketoprofen and methotrexate. This is the first study examining whether the interaction is enantioselective; the lack of selectivity suggested the potential for a therapeutically inactive enantiomer to reduce the clearance of a co-administered drug. If interactions caused by a chiral drug (given as a racemate) are clinically important, consideration should be given to the potential benefits of administering the active enantiomer alone.

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