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Influence of the semisynthetic bile acid (MKC) on the ileal permeation of gliclazide in healthy and diabetic rats

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Abstract:
The aim of this study is to investigate how the semisynthetic bile acid; 3α,7α-dihydroxy-12-keto-5β-cholanate, also known as 12-monoketocholic acid (MKC) influences the ileal permeation of the antidiabetic drug gliclazide in healthy and diabetic rats. Male Wistar rats were divided into 10 groups (n = 32), of which 5 comprised healthy rats (1 to 5) and 5 diabetic rats (6 to 10). Group 1 was used to measure the permeation of gliclazide (200 μg/ml) alone (control) while in groups 2 to 5 gliclazide permeation was measured in the presence of MKC (50 μg/ml), glibenclamide (100 μg/ml), rifampicin (100 μg/ml) and verapamil (30 μg/ml), respectively, using Ussing chambers. Groups 6 to 10 were treated in the same way, after the induction of type 1 diabetes with alloxan (*iv* 30 mg/kg). In tissues from healthy rats, there was a 9-fold reduction in the mucosal to serosal permeation of gliclazide in the presence of MKC ($p < 0.001$) while glibenclamide and rifampicin reduced the permeation of gliclazide in both directions; mucosal to serosal and serosal to mucosal and verapamil had no effect. In contrast, in diabetic rats, there was no net transport of gliclazide alone or after the addition of MKC, glibenclamide, rifampicin or verapamil. The lack of any net flux of gliclazide in diabetic rats suggests the lack of action of drug transporters involved or the suppression of their expression. Furthermore, MKC-induced inhibition of mucosal to serosal unidirectional flux of gliclazide, in healthy rats, can be the result of the selective inhibition of Mrp3.

Key words:
bile acid, gliclazide, glibenclamide, rifampicin, verapamil, permeation, transporters, diabetes

Abbreviations: Mdr – multiple drug resistance, MKC – monoketocholic acid (semisynthetic bile acid, 3α,7α-dihydroxy-12-keto-5β-cholanate), Mrp – multidrug resistance associated protein

Introduction

Diabetic patients differ considerably in their response to antidiabetic drugs due to such factors as ethnicity, drug interactions and disease state [6, 48]. Hence, a thorough understanding of the pharmacokinetics and pharmacodynamics of antidiabetic drugs is essential to optimize individualized drug therapy. Drug optimization can also lead to fewer diabetic complications and a better quality of life. Antidiabetic drugs are generally administered orally and vary considerably in their bioavailability and metabolism which play a significant role in their efficacy and safety [6, 19, 49, 60].

Gliclazide is a second generation sulfonylurea used to treat non-insulin dependent diabetes mellitus (Type II diabetes) [21, 47]. Its primary mode of action is to in-

duce insulin secretion by pancreatic β-cells [8, 55], and as a result, it is relatively ineffective in the treatment of insulin-dependent or type 1 diabetes (T1D) [33, 56]. Gliclazide has highly variable systemic bioavailability which may result from variability in the first-pass metabolism and/or absorption [14, 35, 50, 53]. Changes in gliclazide pharmacokinetics in rats could arise due to variability in bile excretion and thus bile salt production. Bile salts have been shown to increase intestinal as well as nasal absorption of insulin [22, 24, 34], cefpirom [42] and many other drugs [36]. The semisynthetic bile salt sodium 3α,7α-dihydroxy--12-oxo-5β-cholanate (MKC) has been shown to improve the absorption and blood brain barrier penetration of morphine and quinidine [37] as well as the hypoglycemic effect of insulin [29, 30, 35] (Fig. 1).

The oral route is the most popular means for drug administration, since dosing is convenient, non-invasive and many drugs are well absorbed by the gastrointestinal tract [58]. However, the gastrointestinal mucosa represents a physical and a biochemical barrier to the systemic availability of orally ingested, pharmacologically active molecules [31]. The function of the biochemical barrier depends largely upon the metabolism of drugs by intracellular enzymes and specific membrane transport systems. The efficacy of many drugs depends mostly on their ability to cross cellular barriers to reach their target. However, the extent to which a drug accumulates within a tissue is frequently limited not so much by its ability to enter cells but by its tendency to depart. This may arise from active efflux mechanisms present in the plasma membrane. These efflux mechanisms drug transporters such as multiple drug resistance (Mdr)1, multidrug resistance associated protein (Mrp)2 and Mrp3, play a critical role in limiting the absorption and accumulation of potentially toxic substances and can effectively confer resistance to a diverse range of compounds [10]. The influence of efflux drug transporters on drug permeation, in humans and animals, can be studied by using Ussing chambers [32]. Transcellular absorption from lumen to blood requires drug uptake across the apical membrane, followed by transport across the cytosol, then exit across the basolateral membrane into blood [25]. Drugs that cross the apical membrane may be substrates for apical efflux transporters, which extrude compounds back into the lumen [16, 17]. These efflux transporters are principally ABC transport proteins, such as Mdr1, Mrp2, and Mrp3. Mdr1 and Mrp2 are located in the apical membrane of the intestinal epithelial cells and act as the first line of defense by limiting the absorption of potentially toxic compounds. Other efflux transporters such as Mrp3, are situated in the basolateral membrane and prevent their substrates from reaching the lumen [10]. Mdr1, Mrp2 and Mrp3 act as regulatory transporters and are present in the intestinal wall, liver, the blood-brain barrier, and kidneys [26, 51]. A number of drugs have been identified that can affect the efflux transporters including verapamil, glibenclamide, and rifampicin. When administered, these compounds can modify the permeation of other drugs through the intestine [24, 55], which can result in a significant change in the oral bioavailability of these drugs [15]. Glibenclamide has been shown to be an inhibitor of Mrp2 and Mrp3 while rifampicin is an inhibitor of Mrp2 [10, 15, 18, 20, 21]. Verapamil has been established as an inhibitor of Mdr1 and used in Ussing chambers to investigate xenobiotic interactions with specific protein transporters [44, 54]. Mrp2 and Mrp3 have been shown to recognize bile salts as their substrates [59, 62] whereas monovalent bile acids, such as cholic acid, are Mrp3 inhibitors [63].

The aim of our study was to investigate the mechanism by which MKC influences the ileal permeation of gliclazide in tissues from healthy and diabetic rats, at the level of the efflux drug transporters.

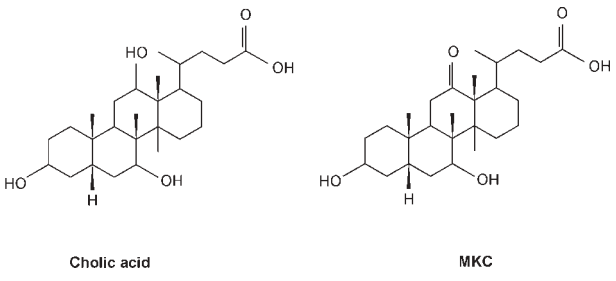

Fig. 1. The chemical structures of cholic acid and monoketocholic acid (MKC). MKC has a ketone group on carbon atom 12 replacing the hydroxyl group in the cholic acid

Materials and Methods

Materials

Gliclazide (99.92%), glibenclamide (99%), (±) verapamil (99%), rifampicin (97%), and alloxan (98%)

were purchased from Sigma Chemical Co., St Louis, MO, USA. MKC was synthesized and purified in the Department of Pharmacy, University of Novi Sad, Serbia, by the method of Miljkovic et al. [39]. All chemicals and solvents were of HPLC grade.

Drug preparations

A gliclazide stock suspension (20 g/l) was prepared by adding gliclazide powder to 10% Ultra water-soluble gel. The MKC sodium salt stock solution (5 g/l) was prepared by adding MKC powder to Ringer buffer (in mmol/l: 147.2 mM Na^+, 5.1 mM K^+, 1.25 mM Ca^+, 1.2 mM Mg^{2+}, 115.2 mM Cl^-, 15 mM HCO_3^-, 0.4 mM $H_2PO_4^-$, 1.8 mM HPO_4^{2-}, 4.9 mM L-glutamate$^-$, and 4.9 mM pyruvate$^-$). The glibenclamide stock solution; 100 g/l, was prepared by adding glibenclamide powder to 100% EtOH while rifampicin 100 g/l, and verapamil 30 g/l stock solutions were prepared by adding the drug powder to MeOH. All drug preparations were mixed thoroughly at room temperature, for 6 h, placed in a refrigerator, and used within 48 h of preparation.

Animal protocol

The study was approved by the Otago University Animal Ethics Committee, New Zealand.

Male Wistar rats (age 2–3 months, weight 350 ± 50 g) were maintained in an experimental animal facility and were given standard diet and water *ad libitum*. Temperature and light were controlled to mimic the natural habitat. Healthy (control) rats were administered saline intravenously (*iv*) (1 mg/kg) while diabetes was induced by injecting alloxan (30 mg/kg) *iv* into the tail vein [9, 28]. Rats were considered diabetic if blood glucose concentration was > 20 mmol/l, serum insulin < 0.04 μg/l, 2 to 3 days after alloxan injection [1, 2, 27] and showed the following signs of diabetes: polydipsia (abnormal thirst), polyuria (increased urination), weight loss (due to lean mass loss), and asthenia (weakness due to inability to utilize glucose) [9].

In vitro transport studies

Rats were sacrificed 7 days after alloxan/saline administration, by CO_2 asphyxiation then a midline longitudinal incision was made and the distal ileum (10 cm) was immediately removed, flushed free of luminal contents with Ringer's buffer then placed in a beaker containing ice-cold Ringer's buffer. The Ringer's buffer was then warmed to 37°C in a water bath and bubbled with carbogen ($O_2 : CO_2$ as 95:5) for 20 min to obtain a pH of 7.4 ± 0.05 prior to use [7]. The isolated ileum was mounted on a glass rod and the adherent tissues were carefully removed using a pair of blunt forceps. The excised section was opened along the mesenteric border, stripped of the underlying muscular layer, glued to plastic rings then cut, and mounted into Ussing chambers as flat sheets (exposed area 0.7 cm^2). The mounted pieces were placed between the two halves of the chambers and bathed by 10 ml of Ringer's buffer on each side, while maintained at 37°C in water-jacketed reservoirs circulating in the chambers. A 20 min gliclazide-equilibration period was allowed before the addition of MKC, glibenclamide, rifampicin or verapamil. 20 μl samples were taken at –20, –19, 0, 10, 20, 30, 40, 60, 90, 120 and 180 min. Samples taken were immediately replaced by the same volume of Ringer's buffer. Cumulative corrections were made for previously removed samples.

The steady-state flux (Jss) was based on the appearance of drug in the receiver (recipient) chamber under sink conditions:

$$Jss = [(dCr \cdot Vr) \div dt \times A]$$

Where dCr/t is the change in drug concentration in the receiver chamber at steady-state over time, Vr is the volume of receiver buffer (10 ml), A is the cross-sectional area of the exposed tissue (0.7 cm^2). The flux ratio was calculated as the ratio of Jss of the mucosal to serosal over the serosal to mucosal, at equal drug concentrations.

Measurements of electrophysiological parameters of the mounted segments were made [4]. Transmural potential difference (PD) was short circuited by a short circuit current (Isc) delivered by a dual voltage-clamp system. Isc was corrected for fluid resistance. PD was expressed in mV and Isc in μA/cm^2. At the end of each experiment, tissues viability was tested by adding 30 mM glucose solution into the mucosal chamber and reporting the change in resistance (Ω·cm^2) and Isc (μA/cm^2). All analyzed data came from viable tissues with resistance and current > 30 throughout the period of the experiment, and a highly significant ($p < 0.01$) increase in Isc observed after the addition of glucose.

At the end of each experiment, the amount of gliclazide retained in the tissues in the ring of Ussing chambers, was measured. The amount of gliclazide

retained in tissues, when only gliclazide was added, was considered as a control. Two different controls were obtained, one for healthy tissues and one for diabetic tissues. The ratio of the amount of gliclazide retained after the addition of gliclazide followed by MKC, glibenclamide, rifampicin or verapamil, to the amount in the control was calculated. This ratio called Gliclazide Retention Ratio (GRR) was used as an index of the drug transporters ability to remove gliclazide from tissues through efflux, and was calculated for each direction mucosal to serosal and serosal to mucosal in the healthy and diabetic tissues. The higher GRR, the more gliclazide retained in the tissues, the less gliclazide removed by efflux drug transporters and thus the less functional these efflux transporters are.

HPLC analysis

Gliclazide concentration in Ringer's buffer was measured using an HPLC method based on Park et al. [45] and Rouini et al. [49]. Samples were mixed with acetonitrile at a 2:1 ratio, and after vortexing for 10 s then centrifuging, the supernatant (20 μl) was injected into the HPLC system. At the end of each experiment, the tissues in the ring was removed, weighed (20 mg), placed in a 1.7 ml PCR tube (Axygen-PCR tubes, CA 94587, USA), then diluted with HPLC water (1:10), vortexed for 20 s, ultra-sonicated for 5 s (using Portable Scientz-111 Ultrasonic Cell Crusher, China), added to acetonitrile (1:2), vortexed for 20 s, centrifuged (15,000 rpm) for 15 min, then supernatant was analyzed for drug concentration.

The column was a Luna 5 μm C18 (2) 100 × 2.00 mm from Phenomenex with a guard column (4 × 2.0 mm) also from Phenomenex. The detector was a Shimadzu, UV-V15 detector set at 229 nm. The mobile phase was acetonitrile 49% and water 51%, pH 2.7 at a flow rate of 0.4 ml/min. The retention time for gliclazide was 2.9 min. A gliclazide standard curve was constructed using standard solutions of 0.5, 1, 2.5, 5, 10, 20, 40, 100, and 200 μg/ml. The within-day coefficient of variation ranged from 1.2% at 200 μg/ml to 2.9% at 0.5 μg/ml. The limit of detection was 0.4 μg/ml and the limit of quantitation was 0.8 μg/ml. The recovery rate of gliclazide from Ringers' buffer was 89 ± 4.1%.

Statistical analysis

Results were analyzed using the analysis of variance (ANOVA) by Minitab (Minitab, Version 14; Minitab Inc., USA). Data were reported as the mean ± standard deviation. Differences were considered significant if $p < 0.05$ and highly significant if $p < 0.01$.

Results

Permeation studies

The gliclazide unidirectional flux in healthy tissues was higher in the mucosal to serosal (Jss $^{M\ to\ S}$) than serosal to mucosal (Jss $^{S\ to\ M}$) direction, suggesting the involvement of drug efflux transporters. In contrast, in diabetic tissues, gliclazide unidirectional flux; Jss $^{M\ to\ S}$ and Jss $^{S\ to\ M}$ were the same, suggesting the lack of action of drug efflux transporters due to either their dysfunction or suppression of their expression.

Tab. 1. Comparison between the mucosal to serosal (M to S) and the serosal to mucosal (S to M) flux of gliclazide, at steady state (Jss), in tissues from healthy and diabetic rats. Tissues resistance through the course of the experiments, and the current change after glucose challenge, are shown. Data are the mean ± SD

	Jss $^{(M\ to\ S)}$ (μg/min/cm^2) n = 16 chambers	Jss $^{(S\ to\ M)}$ (μg/min/cm^2) n = 16 chambers	Resistance (· cm^2)		Δ Short Circuit current (Isc) after glucose challenge (μA/cm^2)	
			M to S n = 16	S to M n = 16	M to S n = 16	S to M n = 16
Healthy (control)	1.32 ± 0.24	0.74 ± 0.12	41.03 ± 8.4	46.17 ± 10.6	20.36 ± 7.91$^{\$\$}$	23.14 ± 9.7$^{\$\$}$
Diabetic	0.41 ± 0.08**	0.43 ± 0.07*	44.25 ± 9.97	45.39 ± 8.4	24.08 ± 6.23$^{\$\$}$	22.47 ± 8.14$^{\$\$}$

* $p < 0.05$ gliclazide flux at steady state compared with control, ** $p < 0.01$ gliclazide flux at steady state compared with control; $^{\$\$}$ $p < 0.01$ Isc after glucose challenge compared with zero difference

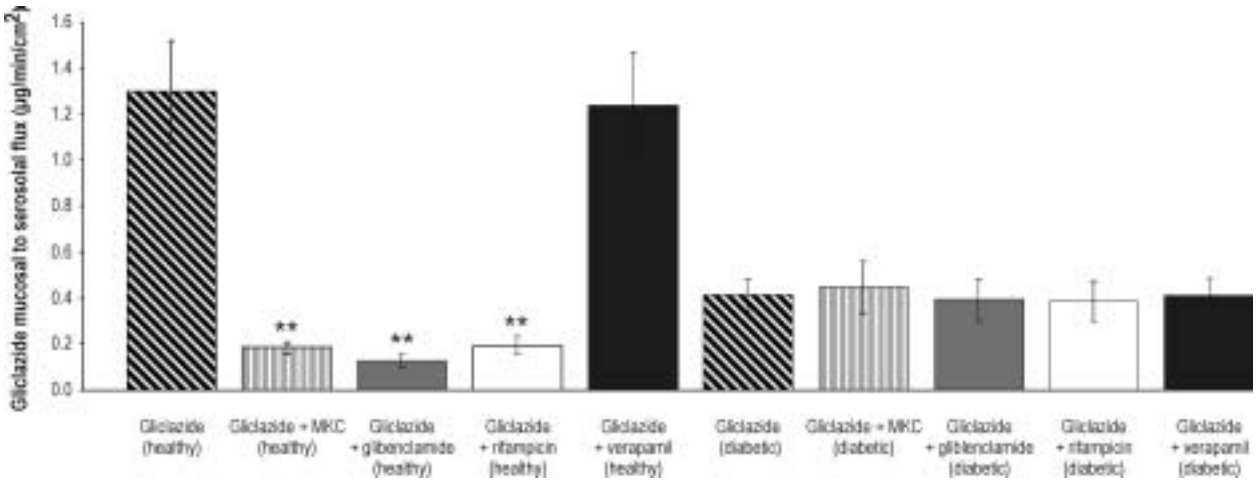

Fig. 2. Mucosal to serosal gliclazide flux, alone, with monoketocholic acid (MKC) and with multidrug resistance associated protein (Mrp)2, Mrp3, and multiple drug resistance (Mdr1) inhibitors, glibenclamide, rifampicin and verapamil in healthy rats. Glibenclamide, rifampicin, and MKC significantly inhibited mucosal to serosal gliclazide flux. Data are the mean ± SD and n = 16. ** $p < 0.01$ gliclazide flux at steady state compared with control

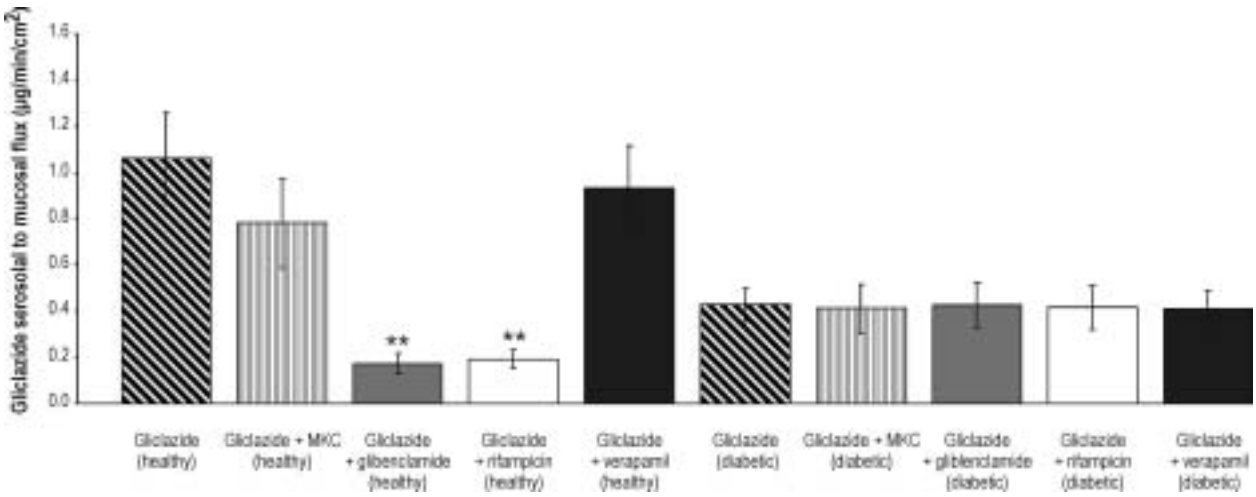

Fig. 3. Serosal to mucosal gliclazide flux, alone, with monoketocholic acid (MKC) and with multidrug resistance associated protein (Mrp)2, Mrp3, and multiple drug resistance (Mdr1) inhibitors, glibenclamide, rifampicin and verapamil in healthy and diabetic rats. Glibenclamide and rifampicin significantly inhibited serosal to mucosal gliclazide flux. Data are the mean ± SD and n = 16. ** $p < 0.01$ gliclazide flux at steady state compared with control

Overall, gliclazide unidirectional flux in diabetic tissues was lower compared with healthy tissue, possibly due to impaired functional drug transporters. The resistance of tissues, throughout the course of the experiment, and the change in current after glucose challenge, in diabetic and healthy tissues, were the same, confirming tissues viability (Tab. 1).

The influence of drug transporter inhibitors on gliclazide flux

In healthy tissues, glibenclamide (100 µg/ml) and rifampicin (100 µg/ml) inhibited the absorption of gliclazide. This was a consequence of the inhibition of both the mucosal to serosal and the serosal to mucosal

flux of gliclazide. Verapamil however, had no effect on either the absorption or the unidirectional fluxes of gliclazide. In contrast, in the diabetic tissues, none of the inhibitors had an effect on the unidirectional fluxes of gliclazide, although it was notable that in the presence of rifampicin and glibenclamide both the mucosal to serosal and the serosal to mucosal fluxes of gliclazide were greater than the comparable fluxes in the tissues from healthy animals. Collectively, these data suggest that in healthy animals gliclazide is transported across the intestinal epithelium by both Mrp2 and Mrp3, but not Mdr1 transporters. Furthermore, these transporters do not appear to be functional in the diabetic animals (** $p < 0.01$ gliclazide flux at steady state compared with control – Fig. 2; ** $p < 0.01$ gliclazide flux at steady state compared with control – Fig. 3).

The influence of MKC on gliclazide flux

The addition of MKC (50 μg/ml), in healthy tissues, inhibited the unidirectional flux of gliclazide, in only the mucosal to serosal direction suggesting a selective inhibitory effect of MKC on Mrp3 (which is an efflux drug transporter on the serosal side) but not on Mdr1 or Mrp2 (which are efflux drug transporters on the mucosal side). Furthermore, MKC was shown to exert a net secretory effect on gliclazide flux; which is the consequence of inhibiting the absorptive flux of gliclazide, by inhibiting the efflux of gliclazide to the serosal side, by Mrp3 (** $p < 0.01$ gliclazide flux at steady state compared with control – Fig. 2; ** $p < 0.01$ gliclazide flux at steady state compared with control – Fig. 3).

Gliclazide retention in tissues

Our data show an increase in GRR associated with the inhibition of the unidirectional flux of gliclazide, in healthy tissues, after the addition of glibenclamide and rifampicin (both directions) and MKC (mucosal to serosal). GRR obtained after the addition of MKC to the mucosal side of healthy tissues was found to be 5 times higher than the GRR of the control. However,

Tab. 2. The quantities of gliclazide trapped in the tissue (μg of gliclazide per mg of ring tissue) in healthy rats

Drugs	Mucosal to serosal flux (gliclazide retention in μg/g of ring tissue)	Serosal to mucosal flux (gliclazide retention in μg/g of ring tissue)	Gliclazide retention ratio (GRR) compared with the corresponding control retention	
	Mean ± SD	Mean ± SD	Mucosal to serosal	Serosal to mucosal
Gliclazide (control)	0.21 ± 0.03	0.20 ± 0.02		
Gliclazide + MKC	1.09 ± 0.25**	0.20 ± 0.03	5.2	1.0
Gliclazide + glibenclamide	0.98 ± 0.03**	0.96 ± 0.03**	4.7	4.8
Gliclazide + rifampicin	0.93 ± 0.04**	0.84 ± 0.31**	4.4	4.2
Gliclazide + verapamil	0.19 ± 0.06	0.21 ± 0.03	0.95	1.1

** $p < 0.01$ gliclazide quantity compared with control

Tab. 3. The quantities of gliclazide trapped in the tissue (μg of gliclazide per mg of ring tissue) in diabetic rats

Drugs	Mucosal to serosal flux (gliclazide retention in μg/g of ring tissue)	Serosal to mucosal flux (gliclazide retention in μg/g of ring tissue)	Gliclazide retention ratio (RR) compared with the corresponding control retention	
	Mean ± SD	Mean ± SD	Mucosal to serosal	Serosal to mucosal
Gliclazide (control)	0.95 ± 0.1	0.90 ± 0.21		
Gliclazide + MKC	0.88 ± 0.19	0.93 ± 0.05	0.93	1.0
Gliclazide + glibenclamide	0.94 ± 0.06	0.90 ± 0.08	0.98	1.0
Gliclazide + rifampicin	0.93 ± 0.07	0.93 ± 0.05	0.98	1.0
Gliclazide + verapamil	0.92 ± 0.09	0.92 ± 0.10	0.97	1.0

GRR obtained after MKC addition to the serosal side of healthy tissues was found to be the same as the control one suggesting the lack of accumulation of gliclazide when added to the serosal side (Tab. 2). In contrast with healthy tissues, diabetic tissues showed high GRR due to larger amount of gliclazide trapped in the tissues with the inhibitors of the drug transporters having no effect on gliclazide unidirectional flux and GRR, compared to control. This supports further our hypothesis that in T1D tissues, drug transporters either lack functionability or their expression is significantly suppressed (Tab. 3).

Discussion

In this study, we investigated the change in ABC-mediated transport of gliclazide, alone or with MKC, in healthy and diabetic rats, with the proposition that the key transporters involved are Mdr1, Mrp2, and Mrp3. The effect of the drug transporter inhibitors, glibenclamide, rifampicin, and verapamil, on the ileal permeation, of gliclazide was observed in healthy and diabetic rats using Ussing chambers.

In our study, we have shown the lack of action of drug transporters in diabetic tissues compared with healthy tissues even though tissue resistance, being an index of flux, remained constant throughout the course of the experiment. There was no change in gliclazide flux after the addition of different inhibitors to diabetic tissues while there was their significant effect on healthy tissues through the suppression of gliclazide permeation by glibenclamide and rifampicin (in both directions; mucosal to serosal and serosal to mucosal) and MKC (in one direction, mucosal to serosal). This lack of transport of gliclazide, after the induction of diabetes with alloxan, suggests either a suppressed or impaired drug transporters. Furthermore, in the presence of rifampicin and glibenclamide both the mucosal to serosal and the serosal to mucosal fluxes of gliclazide were greater than the comparable fluxes in the tissues from healthy animals. This can be explained by the increase in paracellular permeability of the tight junction observed in T1D [3, 43].

We have also confirmed that, in healthy tissues, the unidirectional transport of gliclazide from the mucosal to the serosal direction of the rat ileum was significantly ($p < 0.001$) higher than from serosal to mucosal direction. This implicates a more active role of efflux transporters rather than simple diffusion in gliclazide transport. Taking into account the significant expression of Mrp2, Mrp3 and Mdr1 in the rat ileum [10, 26, 51] in healthy rats, the suppression of gliclazide flux in both directions; mucosal to serosal and serosal to mucosal by glibenclamide and rifampicin (Mrp2 and Mrp3 inhibitors), suggests gliclazide as a substrate for Mrp2 and Mrp3 while, the lack of effect by verapamil suggests the lesser involvement of Mdr1 in gliclazide transport or the simultaneous inhibition of uptake transporters, e.g. organic anion transporting polypeptides whose inhibitor verapamil is [13]. This lack of inhibitory effect of gliclazide flux, by verapamil, in healthy rats, was further supported by the fact that the addition of verapamil made no difference in GRR value compared with control. On the other hand, the addition of glibenclamide and rifampicin resulted in larger GRR values due to the suppression of gliclazide flux and the subsequent accumulation in tissues thus raising GRR values. Accordingly, the measured reduction in gliclazide permeation in both directions of the chamber, when combined with glibenclamide or rifampicin in healthy rats, can be explained by the inhibition of Mrp2 (resulting in less gliclazide being removed from enterocytes to the mucosal side) and Mrp3 (resulting in less gliclazide being removed from enterocytes to the serosal side). Collectively, the higher amount of gliclazide retained in healthy tissues, after the addition of MKC (in the mucosal to serosal direction), and the addition of glibenclamide and rifampicin (in the mucosal to serosal and serosal to mucosal directions) supported the findings that gliclazide removal from tissues was severely impaired as a result of Mrp2 and Mrp3 inhibition, while the amount of gliclazide retained in tissue was unchanged after the addition of verapamil which supports further the assumption that Mdr1 has no significant involvement in gliclazide removal from the healthy ileal enterocytes.

Mrp1 and Mrp4 efflux transporters are also present in the ileal enterocytes [5, 10, 15, 52, 59] (Fig. 4). Mrp1 drug transporters are located on the mucosal side of the enterocytes 5. They share common inhibitors with Mrp2, and Mrp3, such as ampicillin [11]. In a previous study, MKC increased the bioavailability of ampicillin [38]. Accordingly, gliclazide transport can also be influenced by Mrp1, in addition to Mrp2 and Mrp3 and thus the possibility of Mrp1 involvement in gliclazide permeation needs to be investigated

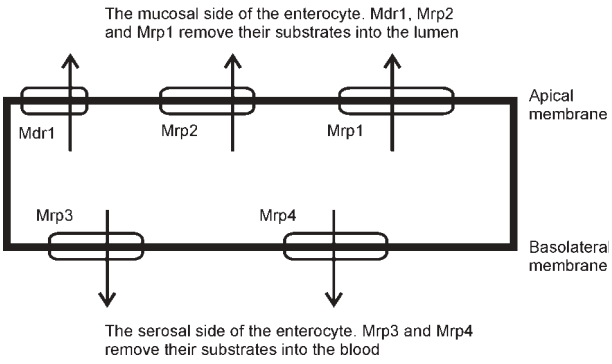

Fig. 4. A simple cartoon showing the distribution of multidrug resistance associated protein (Mrp)1, Mrp2, Mrp3, Mrp4, and multiple drug resistance (Mdr1) in enterocytes

further. On the other hand, Mrp4 drug transporters are located on the serosal side of the enterocytes [10]. These efflux transporters share similar inhibitor selectivity to Mrp3 [12, 46] including the monovalent bile salt [61, 63] and so their involvement in MKC transport is possible and needs to be revealed.

After the addition of MKC to ileal tissues from healthy rats, the inhibitory effect on gliclazide permeation was noticed in only one direction; mucosal to serosal. This implicates MKC as an Mrp3 inhibitor. The higher amount of gliclazide accumulated in the tissue after MKC addition supports that further. Moreover, Mrp3 inhibitors include monovalent bile salts [23, 57, 63] and MKC is a semisynthetic cholic acid derivative with monovalent charge, which makes MKC eligible as an Mrp3 inhibitor. Relating our results with our previous findings, we postulate that this unexpected reduction of gliclazide permeation by MKC can be explained by the *in vivo* metabolic activation of MKC, by its transformation into different bile acids derivatives, which enhanced gliclazide absorption through the ileal mucosa of healthy rats.

There is a certain level of ambiguity in literature regarding inhibitor selectivity for these drug transporters. Accordingly, we have hypothesized the selectivity of Mrp2, Mrp3, and Mdr1 inhibitors based on some recent publications and then we drew a comprehensive conclusion based on our results. Verapamil has been known as an Mdr1 inhibitor while in a recent study, glibenclamide has shown to be an Mrp2 and Mrp3 inhibitor. Even though a recently published literature has shown rifampicin as an Mrp2 inhibitor, its involvement with Mrp3 has not been determined. Since glibenclamide and rifampicin have been shown to be inhibitors of the same drug transporters, NTCP (Sodium Taurocholate Cotransporting Polypeptide) and BAEP (Bile Acid Exporting Pump) [40, 41], the similar results of our experiments on gliclazide flux in healthy and diabetic rats, after glibenclamide and rifampicin administration, has lead us to conclude that rifampicin and glibenclamide share the same drug transporters, Mrp2 and Mrp3.

In contrast to our findings, in a recent study [50], Mrp3 has been shown to have a low affinity for monovalent bile acids. With the present high level of transport of bile acids in humans, Mrp3 has been proposed to have a limited role in the transport of bile acids into blood. Furthermore, ileum has been shown to be the main site of bile acid reabsorption not *via* Mrp3 but rather an unknown basolateral transporter.

In conclusion, alloxan-induced diabetic rats have shown the lack of active control over the ileal unidirectional flux of gliclazide. This can be explained by the suppression of drug transporters involved or their impaired function as a result of the diabetes induced by a toxic compound. Furthermore, the unchanged gliclazide flux in diabetic rats, after the addition of the different agents, was higher than the suppressed gliclazide flux in the comparable healthy tissues after the addition of glibenclamide and rifampicin (both directions) or MKC (mucosal to serosal). This can be the result of the increase in paracellular permeability of the tight junction as a result of the alloxan-induced diabetes. In healthy rats, gliclazide uptake by enterocytes is significantly controlled by the efflux drug transporters Mrp2 and Mrp3 but not Mdr1, while MKC reduced gliclazide permeation through the mucosal side of ileal enterocytes by the competitive inhibition of Mrp3. The reduction of MKC of the ileal flux of gliclazide can be explained by the metabolic activation of MKC into other bile acids derivatives, which reduced gliclazide ileal permeation. Further research is needed to clarify MKC metabolism and the effect of diabetes on MKC permeation through the gut wall and the possible influence of other protein transporters such as Mrp1 and Mrp4 on both, gliclazide and MKC ileal flux.

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