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INHIBITION OF CHOLINE UPTAKE IN SYNCYTIAL MICROVILLUS MEMBRANE VESICLES OF HUMAN TERM PLACENTA

SPECIFICITY AND NATURE OF INTERACTION

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Abstract—The potency and nature of the inhibitory effect of various cationic drugs on the transport of choline across the placental syncytial microvillus membrane was investigated. Tetraethylammonium, a model substrate for organic cation transport, was a poor inhibitor. Enlarging the degree of alkylation of the quaternary ammonium increased the inhibitory effect, in proportion with increasing lipophilicity. Log concentration vs % control uptake curves showed marked differences in inhibitory potency for the different cationic drugs. Hemicholinium-3 inhibited mediated choline uptake in the micromolar range, whereas atropine and mepiperphenidol were less potent. The H_2-receptor antagonists cimetidine, ranitidine, and famotidine inhibited choline uptake in the millimolar ranges. Dixon analysis revealed a competitive nature of inhibition for hemicholinium-3 and atropine (K_i = 40 μM and 1.2 mM, respectively). Cimetidine interacted noncompetitively (K_i = 3.4 mM). Since relatively high concentrations were needed to reach half maximal inhibition, impairment of fetal choline supply due to maternal drug use during pregnancy is not to be expected.

Key words: choline transport; inhibition; cationic drugs; human placenta; syncytial microvillus membrane vesicles

Choline, a cationic quaternary ammonium compound, is an essential substrate for adequate growth and development of the fetus. Choline serves as a substrate for the synthesis of phospholipids and acetylcholine. Since the human placenta and fetus do not synthesize choline, fetal supply is highly dependent on the proper transfer of this nutrient from maternal to fetal circulation [1]. The initial step in placental transfer involves uptake across the syncytial microvillus membrane of the trophoblast. In the perfused human placental cotyledon, *trans*-placental transfer was slow in contrast to the fast and high placental uptake [2]. Since there was a small excess *trans*-placental choline transfer in comparison with the extracellular marker mannitol, preferentially towards the fetal circulation, the human placenta possesses a unidirectional pathway for choline in the maternal to fetal direction. Placental choline uptake at the maternal side was inhibited by choline itself and at the fetal side by HC-3,‡ a well-known competitive inhibitor of choline transport, suggesting the existence of specific transport systems at both sides of the trophoblast. Evidence for mediated trophoblastic choline uptake was also found in human placental fragments, which accumulated choline against a concentration gradient, inhibitable by HC-3 with a K_i of 0.45 mM. However, because uptake in fragments is the net result of transport across both the syncytial microvillus and basal membranes, it is impossible to differentiate between inhibition of the choline transporter at both sides of the trophoblast. Isolated vesicles of these membranes are a more appropriate tool for investigating the location and nature of such interactions. Recently, we described the mechanisms of choline uptake into isolated SMMV of human term placenta [3]. Uptake was not sodium-dependent or coupled to proton transport. An inside negative membrane potential enhanced choline uptake, showing that a negatively charged inner membrane surface acts as a driving force for trophoblastic choline uptake. Mediated transport was confirmed by the *trans*-stimulatory effect of unlabeled choline and *cis*-inhibitory effects of HC-3, the organic cation transport inhibitor mepiperphenidol, and H_2-receptor antagonists. The model substrates for organic cation transport, TEA and NMN, did not inhibit choline transport. Furthermore, we found that uptake under *trans*-stimulation conditions was saturable with a K_m of 550 μM. Our results were confirmed in a study by Grassl, who used the same approach [4]. In addition he showed, by studying the inhibitory potency of a large number of organic cations, that at least two sites of interaction with the placental choline transporter can be postulated: a negative site that binds with the positively charged nitrogen and a site of hydrogen bonding that interacts with the primary alcohol. Furthermore, the degree of nitrogen group alkylation appeared to be of importance.

Since trophoblastic uptake is the rate-limiting step in fetal choline supply, interaction of maternally adminis-

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‡ Abbreviations: HC-3, hemicholinium-3; Hepes, 2-aminohydroxyethylpiperazine-N-2-ethanesulfonic acid; Mes, 2(N-morpholino)ethanesulfonic acid; NMN, n-methylnicotinamide; pH_i, pH inside the vesicle; pH_o, pH outside the vesicle; SMMV, syncytial microvillus membrane vesicles; TMA, tetramethylammonium-bromide; TEA, tetraethylammonium-bromide; TPrA, tetrapropylammonium-bromide; TBA, tetrabutylammonium-hydrogensulfate; TPeA, tetrapentylammonium-bromide; THA, tetrahexylammonium-bromide; val, valinomycin.

tered drugs with the choline transporter at the microvillus membrane could have clinical implications for fetal growth and development. This study was designed to investigate further the specificity of the choline transporter at the syncytial microvillus membrane of the human placental trophoblast, by characterizing the inhibitory potency and nature of the interaction of various cationic drugs.

MATERIALS AND METHODS

Chemicals

[^{3}H]-Choline was obtained from Amersham (Buckinghamshire, U.K.). Cimetidine was kindly donated by Smith, Kline & French (Welwyn Garden City, Herts, U.K.), and mepiperphenidol and famotidine by Merck, Sharp & Dohme (Rahway, NJ, U.S.A.). All other chemicals were purchased from either Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany), or Boehringer Mannheim (Mannheim, Germany), and were of analytical grade. GF/F filters were obtained from Whatman Int. Ltd. (Maidstone, U.K.).

Preparation of SMMV

SMMV were prepared from fresh human term placentae according to an established method [5], which was further improved upon in our laboratory [3, 6]. Briefly, tissue was minced in a Waring blender and stirred for 30 min to loosen the microvilli. After $MgCl_2$ aggregation and differential centrifugation, SMMV were harvested and suspended in the appropriate intravesicular buffer for uptake studies, to a final protein concentration of 10–15 mg/mL. Vesicles were frozen in liquid nitrogen and stored at –80° for four weeks at the maximum. This freezing and storage procedure did not influence choline uptake. The alkaline phosphatase enrichment of SMMV compared to starting mince, measured according to Mircheff and Wright [7], was 24-fold ($M_0 = 70 \pm 15$ and SMMV = 1690 ± 310 μmol/hr/mg, $N = 14$). Protein was assayed with a Coomassie blue kit (Biorad, Munich, Germany).

Uptake studies

Uptake of [^{3}H]-choline into SMMV was measured in quadruplicate at 37° using a rapid filtration technique [8]. The samples were filtered through Whatman GF/F filters (average pore size 0.7 μm), and the radioactivity remaining on the filters counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for nonspecific filter binding. The exact conditions of the transport experiments are given in the legends. Uptake is expressed as pmol or nmol/mg protein or % of control uptake (mean ± SD), N representing the number of experiments with different placentae.

Data analysis

From concentration vs % uptake curves of three concentrations of choline below K_m (50, 125, and 250 μM), the concentration required to reach half maximal inhibition (IC_{50}) of several cationic drugs was estimated by least-squares nonlinear regression analysis, using the computer program GraphPad Inplot 4.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). The weighted residual sums of squares of one- and two-site models were compared using the F-test. Transformation of the data according to Dixon revealed the nature of inhibition. The inhibitory constant (K_i) for a competitive inhibitor was estimated according to the equation of Cheng-Prusoff: $K_i = IC_{50} / (1 + S/K_m)$, where S = choline concentration [9]. For K_m and V_{max} of choline, previously determined values were used, viz. 550 μM and 10 nmol/mg/10 sec, respectively [3]. In case of a noncompetitive inhibitor, IC_{50} is independent of S, and consequently K_i equals IC_{50}. Paired Student's t-test was used to determine statistical significance ($P < 0.05$).

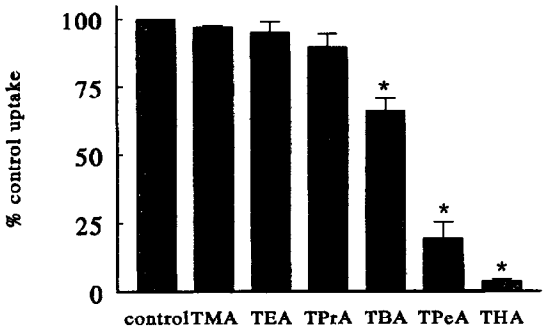

Fig. 1. Effect of 1 mM *cis* concentrations of a series of homologous quaternary ammonium compounds on uptake of 250 μM [^{3}H]-choline at 10 sec. Vesicles, suspended in 100 mM mannitol, 100 mM KCl, and 10 mM Hepes-Tris, pH = 7.4, were pre-equilibrated with 5 mM unlabeled choline and 20 μM valinomycin at 37°C. Five μL vesicle suspension was added to 195 μL extravesicular medium. Extravesicular media consisted of 100 mM mannitol, 100 mM KCl, 20 μM valinomycin, 10 mM Hepes-Tris, pH = 7.4, inhibitor at a specified concentration and unlabeled choline to achieve an extravesicular concentration of 250 μM in the final solution. Values are expressed as % of representative control (without inhibitor) uptakes (mean ± SD, $N = 3$). Control uptake was 5115 ± 625 pmol/mg protein. *$P <$ 0.05.

RESULTS

Inhibitory potency of tetraalkylammonium compounds

In our previous study it was found that 5 mM TEA did not inhibit choline (250 μM) uptake into SMMV [3]. We now investigated whether variation in the degree of alkylation of TEA influences inhibitory potency. Enlarging the alkyl chains at the quaternary ammonium resulted in a higher percentage of inhibition (Fig. 1). The

Table 1. Apparent inhibitory constants of cationic drugs on choline uptake

Compound	IC_{50} (mM)	K_i (mM)
Choline	0.55 ± 0.11	–
HC-3	0.069 ± 0.004	0.039 ± 0.009*
Atropine	1.45 ± 0.30	1.24 ± 0.10*
Mepiperphenidol	0.85 ± 0.55	–
Cimetidine	3.39 ± 0.47	3.39 ± 0.47†
Ranitidine	4.04 ± 0.62	–
Famotidine	3.80 ($N = 1$)	–
TEA	>50 ($N = 1$)	–

IC_{50} values determined from inhibition curves at a choline concentration of 250 μM and inhibitory constants K_i (* competitive; † noncompetitive) determined from inhibition curves at 50, 125, and 250 μM choline. Values are presented as means ± SD, $N = 3$, except for famotidine and TEA.

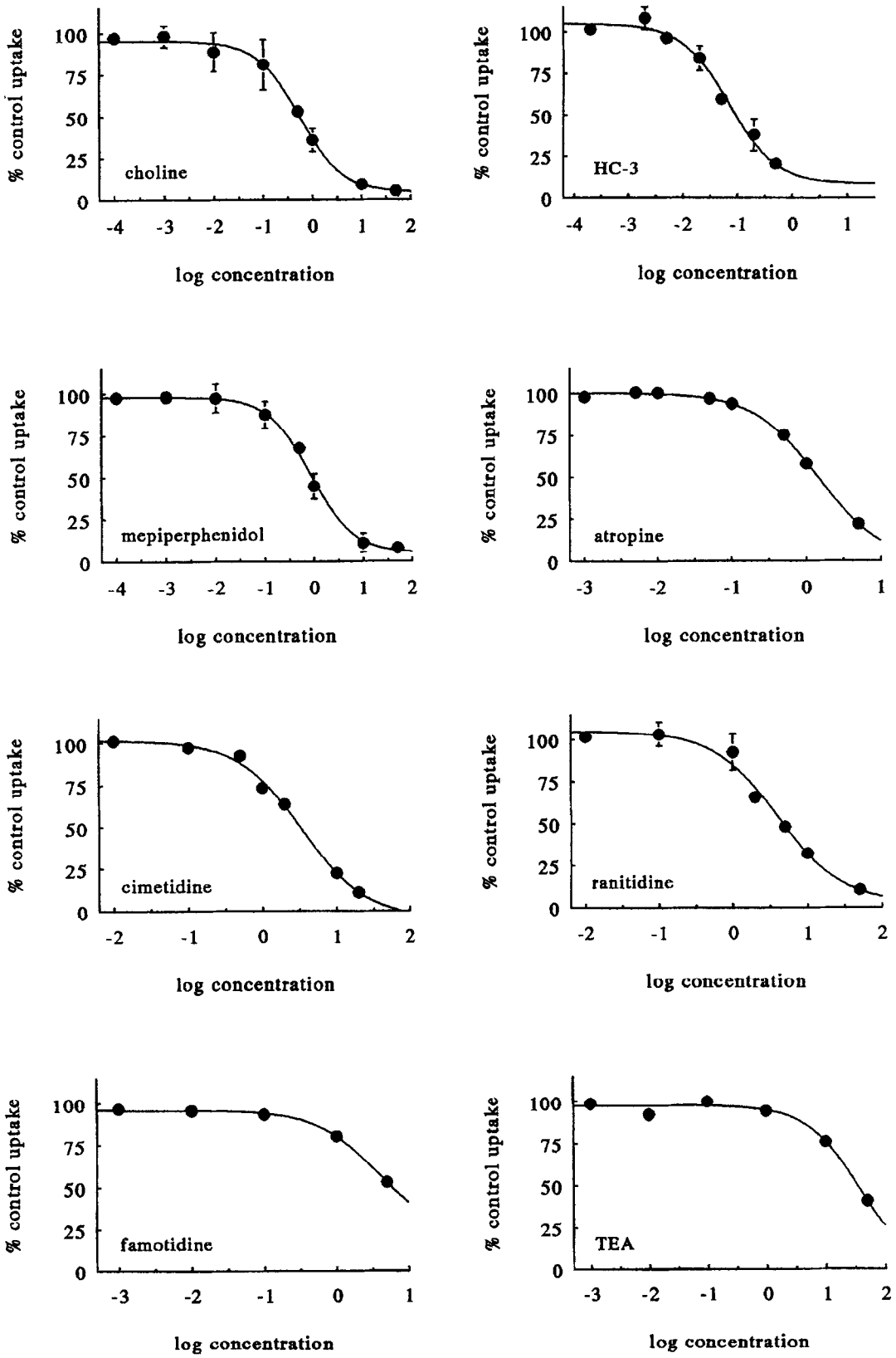

Fig. 2. Inhibition of 250 μM [^{3}H]-choline uptake into SMMV at 10 sec under trans-stimulation conditions by various organic cations. Experimental conditions were the same as described in the legend of Fig. 1. Values are expressed as % of representative control uptakes vs log concentration inhibitor (mM). Each point represents the mean ± SD of three experiments with three placentae, except for famotidine and TEA (N = 1).

increase in inhibitory potency of the compounds corresponded well with the increase in lipophilicity as given by their calculated log *P* values [10]. Because of the surface tension lowering properties of tetraalkylammonium compounds, we verified whether the vesicles stayed intact in the presence of the inhibitors. Only in the presence of THA was the equilibrium uptake of 250 μM choline at 60 min significantly reduced as compared with control uptake. The other compounds did not interfere with the membrane integrity.

Inhibition of choline uptake by several organic cations

Plots of log concentration inhibitor vs % of control uptake of 250 μM choline are shown in Fig. 2. The results of the nonlinear regression analysis of the typically sigmoid shaped curves are summarized in Table 1. Marked differences in inhibitory potency can be seen between the organic cations tested. HC-3 inhibited choline uptake for 50% at a relatively low concentration, whereas the organic cation transport inhibitor mepiperphenidol and the anticholinergic drug atropine were less potent inhibitors. The H_2-receptor antagonists cimetidine, ranitidine, and famotidine showed IC_{50} values only in the mM ranges. For TEA only a rough estimate of the IC_{50} value could be made, because of the very high concentrations (>10 mM) necessary to achieve half maximal inhibition. In all cases a two-site model did not fit the data better than a one-site model ($P > 0.2$).

Nature of interaction with the choline transporter

Although IC_{50} values provide a good measure of inhibitory potency, they cannot explain the nature of the interaction. We used the method of Dixon analysis to evaluate the type of choline transport inhibition by HC-3, atropine, and cimetidine. The concentration-dependent inhibition of these compounds was measured at three choline concentrations: 50, 125, and 250 μM. Increasing the substrate concentration decreased the inhibitory effectiveness of HC-3 and atropine, resulting in a lower IC_{50} value at a lower choline concentration. Transformation of the data according to Dixon showed that the lines intersected above the *X* axis and to the left of the *Y* axis, indicating a competitive mode of interaction of HC-3 and atropine with the choline transporter (Fig. 3). K_i values for competitive inhibition, calculated from the Cheng-Prusoff equation for the three choline concentrations, were 40 μM for HC-3 and 1.2 mM for atropine (Table 1). In contrast, the IC_{50} value for cimetidine was independent of the choline concentration. Dixon analysis resulted in an intersection of the lines on the *X* axis, consistent with a noncompetitive type of interaction (Fig. 3). Consequently, the K_i for cimetidine equalled the IC_{50} value of 3.4 mM (Table 1).

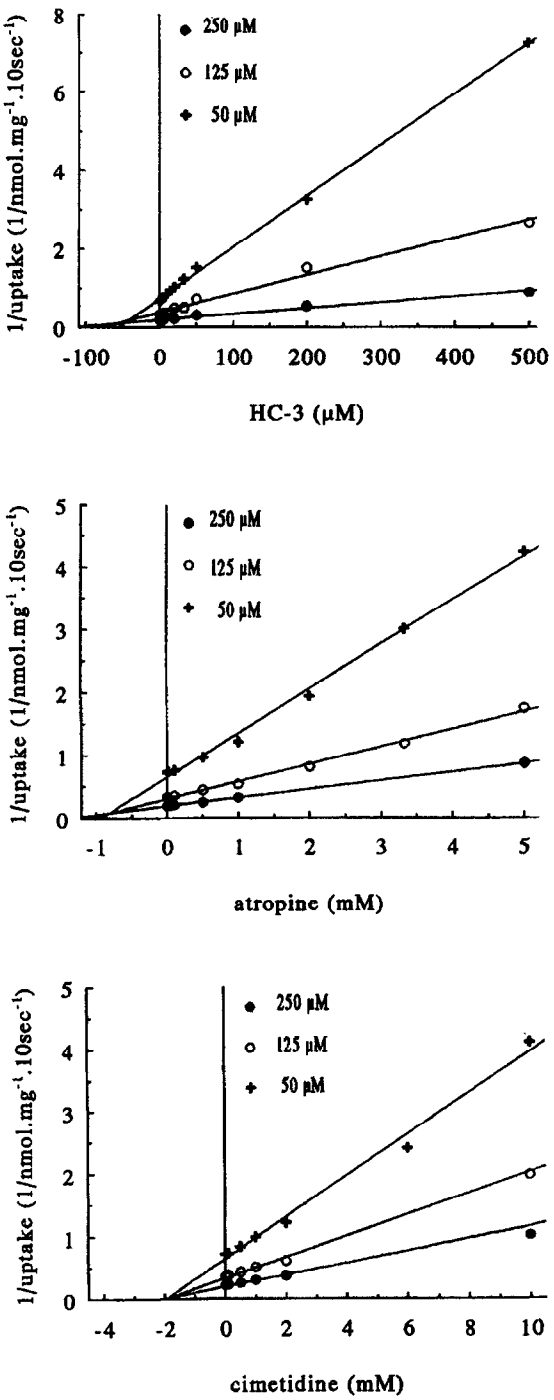

Fig. 3. Dixon plots of the interaction of HC-3, atropine, and cimetidine with choline. Concentrations of extravesicular [^{3}H]-choline were 50, 125, and 250 μM. Experimental conditions were the same as described in the legend of Fig. 1, except that in the case of 50 μM choline, 5 μL vesicle suspension was added to 495 μL extravesicular medium.

DISCUSSION

The present study demonstrates that several cationic drugs inhibited human placental choline transport across the syncytial microvillus membrane with different inhibitory potencies. HC-3 and atropine appeared to be competitive inhibitors, whereas cimetidine interacted noncompetitively. TEA, a model substrate for organic cation transport in various tissues, only inhibited choline transport at very high concentrations. Enlarging the degree of alkylation of this quaternary ammonium compound increased the inhibitory effect in proportion with increasing lipophilicity.

Wright *et al.* postulated a set of structural elements important for interaction with the choline transporter: (a) a terminal hydroxyl group; (b) the positive charge of the nitrogen; and (c) the presence of at least two free methyl

groups at the positive nitrogen [11]. The placental choline transporter appeared to have a similar substrate specificity [4]. Small quaternary ammonium compounds such as TMA, acetylcholine, and N-methylnicotinamide showed affinity for the rat intestinal choline carrier [12], whereas TMA showed no affinity for the human placental and rabbit renal choline transporter [4, 11]. The inability of large molecular quaternary ammonium compounds to interact with the choline transporter was assumed to be due to steric hindrance, viz. the masking of the positive nitrogen [11, 12]. The failure of TEA, for instance, to interact with the choline transporter was further confirmed by the finding that TEA was not able to *trans* stimulate choline uptake into renal and intestinal brush-border membrane vesicles [11–13]. In the present study TMA, TEA, and TPrA did not inhibit or poorly inhibited placental choline transport. In spite of a lack of determinants important for interaction with the choline transporter, further enlargement of the alkyl chains resulted in a significant inhibition of placental choline transport by TBA, TPeA, and THA. For TPeA this was also observed in rabbit renal brush-border membranes [11], and in the study by Grassl [4] a higher degree of inhibition of choline uptake was found in the presence of trimethylphenylammonium, as compared to no significant inhibition with TMA. The reduced choline uptake in the presence of the more lipophilic quaternary ammonium compounds is likely to be of noncompetitive nature [14]. The higher degree of alkylation probably facilitates a nonspecific interaction (e.g. solubilization into the membrane or interaction with an allosteric binding site at the transport protein, thereby interfering with the ability of the carrier to transport choline).

Cimetidine has affinity for the organic cation-proton antiporter [15], but to our knowledge no data concerning a possible interaction with the choline transporter are currently available. However, in renal brush-border membranes an affinity of choline for the organic cation-proton antiporter has been demonstrated, since choline inhibited TEA transport [11, 16]. Our data provide evidence that H_2-receptor antagonists inhibit choline uptake noncompetitively. The measured IC_{50} values were in the mM range, and the inhibitory potency of cimetidine was not dependent on choline concentration, resulting in an intersection of lines on the abscissa after Dixon transformation (Fig. 3). It therefore seems unlikely that clinically significant interactions of these drugs with choline uptake *in vivo* will occur. Cimetidine is the highest-dosed H_2-receptor antagonist, but maternal effective plasma concentrations (90% inhibition of gastric acid production) are only approximately 15 μM [17]. For the lower-dosed drugs, famotidine and ranitidine, IC_{50} values in the same range as for cimetidine were observed. Significant interactions *in vivo* of these nowadays more frequently prescribed H_2-receptor antagonists are therefore not to be expected.

The different IC_{50} values for HC-3 at different choline concentrations indicate that the inhibitory potency of HC-3 was dependent on choline concentration. The same pattern of inhibition was found for atropine, which is indicative of competitive interaction. HC-3 is an effective inhibitor, as its affinity for the choline transporter was more than 10-fold higher than for choline itself (K_i for HC-3 = 40 μM and K_m for choline = 550 μM), whereas the affinity for atropine was more than 2-fold lower (K_i = 1.2 mM). The Y coordinates of the intersections in the Dixon plot (HC-3 = 0.12, atropine = 0.07) corresponded well with the $1/V_{max}$ value of 0.10, providing further confirmation that both inhibitors indeed interacted competitively [18]. Although Grassl did not report a K_i value for HC-3, a value of 92 μM can be calculated from his data [4]. In human placental fragments a K_i of 450 μM for HC-3 was reported [1], which is almost 10-fold higher than the value we estimated in isolated human placental SMMV. This discrepancy is most likely due to the different experimental techniques used. An inhibition constant determined in fragments is merely a hybrid parameter from interaction with choline transporters at the microvillus and basal side of the trophoblast, whereas the inhibitory effect in purified microvillus membranes can only be ascribed to an interaction with the choline transporter at this membrane. Therefore, estimating inhibitory parameters of drugs on transport proteins is more appropriate in purified membranes. However, the net effect on the transfer of choline in maternal to fetal direction should be determined in more physiologically-based models, such as trophoblast cell culture or cotyledon perfusion models.

We conclude that several cationic drugs are able to interact with human placental choline transport across the syncytial microvillus membrane in a competitive and noncompetitive way. Competitive inhibition was seen in the μM and low mM ranges, whereas noncompetitive inhibition was seen in the high mM ranges, indicating a nonspecific interaction with the biomembrane. Since relatively high concentrations were needed to reach half maximal inhibition, impairment of fetal choline supply *in vivo* due to maternal drug use during pregnancy is not to be expected.

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