1 **Gut microbial transformation of the dietary mutagen MeIQx may**

2 **reduce exposure levels without altering intestinal transport**

3 Jianbo Zhang,[1, 7] Michael T. Empl,[2, 8] Mirjam Schneider,[1] Bernd Schröder,[3] Julita Stadnicka-

4 Michalak,[4, 5] Gerhard Breves,[3] Pablo Steinberg,[2, 6] Shana J. Sturla[1]

5 [1] Department of Health Sciences and Technology, ETH Zürich, Zürich, Switzerland

6 [2] Institute for Food Toxicology, University of Veterinary Medicine Hannover, Hannover,

7   Germany

8 [3] Department of Physiology, University of Veterinary Medicine Hannover, Hannover,

9   Germany

10 [4] Eawag, Dübendorf, Switzerland

11 [5] School of Architecture, Civil and Environmental Engineering, EPF Lausanne, Switzerland

12 [6] Current address: Max Rubner-Institut, Federal Research Institute of Nutrition and Food,

13   Karlsruhe, Germany

14 [7] Current address: Department of Biological Engineering, Massachusetts Institute of

15   Technology, Cambridge, United States

16 [8] Current address: Fraunhofer Institute for Toxicology and Experimental Medicine ITEM,

17   Nikolai-Fuchs-Straße 1, 30625, Hannover, Germany.

18 Address correspondence to:

19 Shana J. Sturla, Ph.D

20 E-mail: sturlas@ethz.ch

21 Phone: +41 44 632 91 75

22 Fax: +41 44 632 11 23

23 Pablo Steinberg, Ph.D

24 E-mail: pablo.steinberg@mri.bund.de

25 Phone: +49 721 6625-201

26 Fax: +49 721 6625-111

28 **Abstract**

29   The mutagen and probable human carcinogen 2-amino-3,8-dimethylimidazo[4,5-

30 *f*]quinoxaline (MeIQx) is metabolized in the colon to 9-hydroxyl-2,7-dimethyl-7,9,10,11-

31 tetrahydropyrimido[2',1':2,3]imidazo[4,5-*f*]quinoxaline (MeIQx-M1) by conjugation with

32 microbially generated acrolein. However, whether this microbiota-controlled process alters

33 systemic exposure and hepatotoxicity of MeIQx remains unclear. The physiological relevance

34 of this microbial transformation on the systemic exposure of MeIQx was investigated using an

35 *in vitro-in vivo* extrapolation approach. To address whether microbial transformation influences

36 intestinal transport of MeIQx, the intestinal uptake of MeIQx and its metabolite MeIQx-M1

37 was quantified using Ussing chambers mounted with different intestinal segments from male

38 Fischer 344 rats. Up to 0.4 % of both MeIQx and MeIQx-M1 were transported from the

39 mucosal side to the serosal side of intestinal tissue within 90 min, suggesting that the intestinal

40 uptake of both compounds is similar. With the uptake rates of both compounds, physiologically

41 based pharmacokinetic (PBPK) modeling of the fate of MeIQx in the human body including

42 microbial transformation of MeIQx was performed. Results indicate for the first time that high

43 levels of microbe-derived acrolein would be required to significantly reduce systemic exposure

44 of MeIQx in humans. Finally, neither MeIQx nor MeIQx-M1 were cytotoxic towards human

45 liver HepaRG cells at dietary or higher concentrations of MeIQx. In summary, these findings

46 suggest that gut microbial transformation of heterocyclic amines has the potential to influence

47 systemic human exposure to some extent, but may require significant gut microbial production

48 of acrolein and that further investigations are needed to understand physiological levels of

49 acrolein and competing biotransformation pathways.

Introduction

52

53   The association of well-cooked red meat with increased colorectal cancer risk is in part

54   attributed to exposure to carcinogenic chemicals such as heterocyclic aromatic amines (HCAs;

55   Ushiyama et al. 1991; Sinha et al. 2001; Bouvard et al. 2015) like 2-amino-1-methyl-6-

56   phenylimidazo[4,5-*b*]pyridine (PhIP) (Felton et al., 1986), 2-amino-3,8-dimethylimidazo[4,5-

57   *f*]quinoxaline (MeIQx) (Jägerstad et al., 1984), and 2-amino-3-methylimidazo[4,5-*f*]quinoline

58   (IQ) (Kasai et al., 1980). Upon metabolic activation, HCAs form DNA adducts (Nauwelaers et

59   al., 2011), are mutagenic (Kim and Guengerich, 2005; Rendic and Guengerich, 2012; Sugimura,

60   1988), induce malignant cell transformation (Pfau et al., 1999) as well as mutations in

61   oncogenes of rodent and human fibroblasts (Sugimura et al., 2004), and form tumors in multiple

62   organs in rodent models (Ito et al., 1991; Kato et al., 1988; Ohgaki et al., 1987; Sugimura et al.,

63   2004). We recently showed that complex human-derived gut microbial communities can reduce

64   the mutagenicity of HCAs by conversion to their acrolein conjugates (i.e. conversion of MeIQx

65   to 9-hydroxyl-2,7-dimethyl-7,9,10,11-tetrahydropyrimido[2',1':2,3]imidazo[4,5-*f*]quinoxaline

66   or MeIQx-M1) (**Figure 1**) (Zhang et al., 2017). Similar microbial metabolites were observed

67   for other HCAs, including PhIP (Fekry et al., 2016; Vanhaecke et al., 2006), IQ (Zhang et al.,

68   2017) and 2-amino-9H-pyrido[2,3-*b*]indole (AαC) (Beer et al., 2017). However, our

69   understanding of this process raises several questions concerning its physiological relevance of

70   this process and whether it can impact on the disposition of HCAs in humans.

71   The mechanism of microbial conversion of MeIQx to MeIQx-M1 is well established.

72   Bacteria that harbor the *pduCDE*-gene encoding for the glycerol to 3-hydroxypropionaldehyde

73   (3-HPA)-catalyzing enzyme glycerol/diol dehydratase (De Angelis et al., 2014; Engels et al.,

74   2016; Fekry et al., 2016), such as *Enterococcus spp.*, *Lactobacillus reuteri* (Vanhaecke et al.,

75   2008b), *Lactobacillus rossiae*, and *Eubacterium hallii* (Fekry et al., 2016; Zhang et al., 2017),

76   are responsible for this metabolic transformation. 3-HPA chemically decomposes to release

77   acrolein, which then binds to HCAs to form HCA-M1 metabolites (Beer et al., 2017; Engels et

78   al., 2016; Zhang et al., 2017). The transformation blocks the metabolic activation site of HCAs,

79   thereby avoiding their capacity to bind DNA and thereby significantly reducing their

80   mutagenicity (Vanhaecke et al., 2008a; Zhang et al., 2017). Bacteria-mediated glycerol

81   conjugation has therefore been proposed as a detoxification pathway for HCAs in the human

82   lower digestive tract, however, the physiological relevance of this process depends on whether

83   HCA and/or HCA-M1 are absorbed.

84   It has been shown that gut microbial transformation, due to a potential alteration of

85   absorption, can change the systemic levels of drugs and toxicants and thereby their efficacy and

86   toxicity, respectively (Behr et al., 2017; Haiser et al., 2013; Spanogiannopoulos et al., 2016).

87   In the case of HCAs, Humblot et al. (2007) have shown that bacterial hydrolysis of IQ-

88   glucuronide increased urine levels of IQ in rats mono-colonized with β-glucuronidase-positive

89   *Escherichia coli*. MeIQx has been quantified in plasma of healthy individuals and the clearance

90   rates were highly variable among individuals (Malfatti et al., 2016), highlighting the importance

91   of understanding the currently unknown influence of microbial conversion on intestinal

92   transport and toxicokinetics of MeIQx and MeIQx-M1.

93   The aim of this study was to investigate how bacterial transformation of MeIQx may lead

94   to a physiologically relevant reduction of the systemic exposure to MeIQx. We determined the

95   capacity of rat intestinal tissue to transport MeIQx and MeIQx-M1. This information led us to

96 develop a physiologically based pharmacokinetic (PBPK) model to describe the impact of

97 MeIQx-M1 formation on the disposition of MeIQx. Finally, we evaluated the toxicological

98 relevance of intestinal uptake by characterizing the cytotoxic potential of MeIQx and MeIQx-

99 M1 towards a human liver cell line *in vitro*. The relevance of the findings is discussed in the

100 context of whether microbial transformation may modulate systemic HCA exposure in humans.

101 **Materials and Methods**

102 **Chemicals**

103   MeIQx was purchased from Chemie Brunschwig (Basel, Switzerland). Acrolein, 2-amino-

104 1-methylbenzimidazole (AMBI), and the constituents of the culture media were purchased from

105 Sigma-Aldrich (Buchs, Switzerland). HPLC-grade acetonitrile and methanol were purchased

106 from Merck (Darmstadt, Germany) and Sigma-Aldrich, respectively. Formic acid was

107 purchased from Fisher Scientific (Geel, Belgium), and water was purified with a Milli-Q

108 Integral Water Purification System (Millipore, Billerica, MA, USA). 9-Hydroxyl-2,7-dimethyl-

109 7,9,10,11-tetrahydropyrimido[2',1':2,3]imidazo[4,5-*f*]quinoxaline   (MeIQx-M1)   and   2-

110 hydroxyl-10-methyl-2,3,4,10-tetrahydrobenzo[4,5]imidazo[1,2-*a*]pyrimidine   (AMBI-M1)

111 were prepared by reaction of MeIQx or AMBI with acrolein using a procedure described

112 elsewhere (Zhang et al., 2017).

113 **Animals**

114   7-9 weeks old male Fischer 344 rats with a body weight of 239-273 g (**Figure S1**) were

115 obtained from Charles River (Sulzfeld, Germany). They were housed for 7 days in the animal

116 facility of the Department of Physiology at the University of Veterinary Medicine Hannover

117 (Hannover, Germany) prior to sacrifice. During this period, the animals had free access to water

118 and standard rodent maintenance feed and were kept under a 12/12 h light/dark lighting regime.

119 The study was performed under approval no. 33.9-42502-05-12A219 of the Animal Welfare

120 Service of the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES;

121 Oldenburg, Germany).

Preparation of the intestinal segments and Ussing chamber experiments

123 Intestinal segments were prepared according to the method described by Tóth (2007).

124 Briefly, all rats were sacrificed using CO_2. The whole gut was removed and then kept in ice-

125 cold Krebs-Henseleit solution continuously gassed with carbogen (95 % O_2, 5 % CO_2) for

126 approximately 5 min. Then, they were prepared for the Ussing chamber experiments. The initial

127 part of the colon was thereby defined as the beginning of the large intestine connected to the

128 caecum (identified by a characteristic v-forming pattern along the mesentery artery). The

129 jejunum was defined as the segment distal to the ligament of Treitz and was removed

130 completely until the beginning of the ileum. The specified location of different segments used

131 for the experiments was as follows: The duodenal sample was taken 1 cm below the pylorus,

132 the proximal jejunal sample 15 cm distal of the duodenum, the distal jejunal sample 10 cm

133 proximal of the ileum, the ileal sample directly proximal to the caecum and the caecal sample

134 from the *corpus caeci*. The tissue excised up to 3 cm distal to the junction of the caecum and

135 the colon was defined as proximal colon, tissue immediately distal to this segment as distal

136 colon, and the remaining segment as rectum. Once the gut was removed and segmented, the

137 *tunica serosa* and *tunica muscularis* of the tissue segments were removed by stripping on ice,

138 prior to them being mounted into the Ussing chambers. The area of a single chamber aperture

139 was 0.5 cm^2 for all tissues as described in detail elsewhere (Breves et al., 2000). Two silicon

140 rings were used to ensure a good connection between the two halves of the chambers and to

141 prevent damage to the epithelium.

142   The Ussing chamber experiments were performed using procedures described by Breves

143 et al. (2000) and Nicken et al. (2015). Briefly, after tissues were mounted into the Ussing

144 chambers, the whole system was equilibrated with carbogen gas for 30 min. Thereafter, 10 mM

145 stock solutions of MeIQx or MeIQx-M1 were added to the mucosal compartments (final

146 concentration: 10 µM). One and 90 min after the addition of the compounds (longer incubation

147 times were not tested because of tissue deterioration), samples for LC-MS analysis (400 µl)

148 were removed from each compartment and immediately snap-frozen in liquid nitrogen. In order

149 to determine unspecific binding of MeIQx and MeIQx-M1 to the surface of the Ussing

150 chambers, a silicone membrane was additionally mounted. The components of the buffer

151 solutions added to the Ussing chambers are listed in **Table S1**.

152 **Quantification of MeIQx and MeIQx-M1 in the mucosal and serosal compartments**

153 **of the Ussing chambers**

154   The concentration of MeIQx and MeIQx-M1 was measured by nanoLC-mass spectrometry

155 using a nanoACQUITY UPLC™ system (Waters Corporation, Milford, MA, USA) equipped

156 with a capillary column and a triple quadruple (QqQ) mass spectrometer. The capillary column

157 (75 µm × 10 cm D × L, 15 µm orifice) was hand-packed using a commercially available fused-

158 silica emitter (New Objective, Woburn, MA; USA) with 5 µm Luna C18-bonded separation

159 media (Phenomenex, Torrance, CA, USA). The mobile phase A consisted of 0.1 % formic acid

160 in ultrapure water, while the mobile phase B consisted of 0.1 % formic acid in acetonitrile. The

161   separation was achieved using the following gradients: 0-5 min, flow rate (FL) 1 µl min⁻¹,

162   isocratic 13 % B; 0-5.5 min, FL convex 1-0.3 µl min⁻¹, isocratic 13 % B; 5.5-6 min, FL

163   0.3 µl min⁻¹, linear 13-25 % B; 6-8 min, FL 0.3 µl min⁻¹, isocratic 25 % B; 8-8.5 min, FL 0.3 µl

164   min⁻¹, linear 25-98 % B; 8.5-9 min, linear 0.3-1 µl min⁻¹, isocratic 98 % B; 9-11 min, FL

165   1 µl min⁻¹, isocratic 98 % B. The column was then returned to the initial conditions and re-

166   equilibrated for 5 min. The eluent was ionized by nano-electro spray ionization (nano-ESI) in

167   the positive mode and the compounds were monitored by a QqQ mass spectrometer with the

168   following transition reactions: MeIQx, 214 → 199, 214 → 172; MeIQx-M1, 270 → 252,

169   270 → 214; AMBI, 148 → 133; AMBI-M1, 204 → 186, 204 → 148 (**Figure S2**).

170   2-Amino-3-methyl benzimidazole (AMBI) and 10-methyl-2,3,4,10-

171   tetrahydrobenzo[4,5]imidazo[1,2-*a*]pyrimidin-2-ol (AMBI-M1) (**Figure 1**) were added to

172   samples as internal reference standards for MeIQx and MeIQx-M1, respectively. The mixture

173   was concentrated under vacuum, the residue was dissolved in acetonitrile diluted appropriately

174   with water and filtered using a 0.2 μm polyvinylidene difluoride membrane. The linear range

175   of the calibration curve was 1-100 nM (**Figure S3**). The limit of quantification for this method

176   was determined to be 1 nM for both MeIQx and MeIQx-M1 based on a signal to noise ratio of

177   10.

178   **Calculation of the apparent permeability coefficient (P$_{app}$) for MeIQx and MeIQx-M1**

179   The apparent permeability coefficient for intestinal transport was estimated according to the

180   following equation (Yee, 1997):

181
$$P_{app} = \frac{\Delta C_s \times V_s}{\Delta t \times A \times C_{m0} \times V_m}$$

182 Where V_s and V_m = volume of medium (in L) perfusing the serosal and mucosal reservoir,

183 respectively,

184 ΔC_s = concentration change (nmol L^{-1}) in the serosal reservoir at the time interval between t_2

185 and t_1,

186 Δt = time interval (in seconds) between t_2 and t_1,

187 A = area of the tissue/membrane = 0.5 cm^2,

188 P_{app} = unidirectional flux (in nmol cm^{-1} h^{-1}) from mucosa to serosa,

189 C_{m0} = initial concentration (in nmol L^{-1}) in the mucosal reservoir.

190 **Physiologically based pharmacokinetic kinetic (PBPK) modeling**

191   To evaluate the influence of the bacteria-derived acrolein-mediated metabolic

192 transformation of MeIQx on its disposition in humans, we developed a PBPK model with

193 perfusion rate-limited kinetics (**Figure S4**). The model developed in this study was adapted

194 from PBPK models reported by Al-Malahmeh et al. (2017) for rats and humans. The model

195 consists of six compartments, lung, liver, gut tissue, adipose tissue (fat), richly perfused tissue,

196 and slowly perfused tissue, connected by the systemic circulation. Physiological parameters

197 (i.e. body weight, tissue volumes, cardiac output, and tissue blood flows) of individuals were

198 derived from literature (**Table S2**; Brown et al. 1997). Partition coefficients describing the

199 distribution of MeIQx in different compartments were estimated based on the method described

200 by DeJong et al. (1997; **Table S3**). The uptake of MeIQx from the gut could be described as a

201 first order kinetic process, assuming direct uptake by the liver with an absorption rate constant

202 k_a (**Table S4**). The catalytic parameters of glucuronidation, sulfation, and oxidation in the liver

203 were derived from *in vitro* data obtained in the course of the incubation of substrates with

204   microsomal/cytosol proteins, i.e. uridine 5'-diphospho-glucuronosyltransferase (UGT),

205   sulfotransferase, and cytochrome P450 enzymes (**Table S2**; Ozawa et al. 1995; Malfatti and

206   Felton 2004; Zhou et al. 2004). The rate of formation of MeIQx-M1 is a second order reaction

207   of MeIQx with acrolein and the second order rate constant was derived from the direct reaction

208   of acrolein with MeIQx as previously reported (Zhang et al., 2017). The mass balance equations

209   were expressed as differential equations and solved using Berkeley Madonna (Macey and

210   Oster, UC Berkeley, CA, USA) with Rosenbrock's algorithm for stiff systems (version 8.3.18)

211   or Euler's method (version 9.1.9). To test the impact of acrolein on the plasma concentration

212   of MeIQx, we applied the PBPK model to fit the above-mentioned parameters (**Tables S2-S4**)

213   to the measured plasma concentration in the presence of acrolein. Thereafter, these fitted

214   parameters were used to simulate the impact of acrolein on the MeIQx plasma concentration

215   by comparing the plasma level of MeIQx in the presence of acrolein with those in the absence

216   of this compound. More details on modeling equations, codes, sensitivity analysis, and

217   performance evaluation are available in the Supplementary Information.

218   **Cytotoxicity assay**

219   HepaRG^{TM} cells (Biopredic International, Rennes, France) were exposed to increasing

220   concentrations of MeIQx and MeIQx-M1 and their impact on cell viability characterized as

221   previously described (Zhang et al., 2017). Briefly, HepaRG cells were cultured in William's E

222   medium (Thermo Scientific, Reinach, Switzerland) supplemented with 10 % (v/v) fetal calf

223   serum, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin, 5 µg ml^{-1} insulin and 5×10^{-5} M

224   hydrocortisone hemisuccinate. The cells were seeded in a 96-well-plate and grown for two

225   weeks in media. For the following two weeks the cells were differentiated in medium containing

226 2 % DMSO and then treated with MeIQx or MeIQx-M1 (0, 10, 50, 100, 150, 200, 350, 500,

227 and 1250 µM in 0.5 % DMSO from a 250-mM stock). The CellTiter-Glo® luminescent cell

228 viability assay (Promega, Dübendorf, Switzerland) was then performed according to the

229 manufacturer's protocol. As a positive control, 2 % (v/v) Triton X-100 was used and the

230 experiment was performed three times in total. The 10 %-response benchmark dose (BMD_{10})

231 was calculated with PROAST (version 38.9) in R by following the manual provided by the

232 European Food Safety Authority (EFSA; EFSA Scientific Committee 2009).

233 **Statistics**

234 All statistical analyses were performed using GraphPad Prism (version 7.03; GraphPad,

235 La Jolla, CA, USA). Multiple *t*-tests ($\alpha = 0.05$) were performed to compare the transport of

236 MeIQx and MeIQx-M1 in different intestinal segments using the Holm-Sidak method. 2-way

237 ANOVA multiple comparisons were performed to compare the cytotoxicity of MeIQx and

238 MeIQx-M1. Outliers of apparent permeability (P_{app}) and fraction were identified using the

239 ROUT method implemented in GraphPad Prism.

240 **Results**

241 **Low transport of both MeIQx and its microbial metabolite through intestinal**

242 **segments**

243 To test the capacity of the rat intestine to transport MeIQx and its microbial metabolite

244 MeIQx-M1, we added each to the mucosal compartment of an Ussing chamber and determined

245 the amount of the chemical transported to the serosal compartment. The compounds were

246 transported by all tissue segments. For MeIQx, levels in the serosal chamber ranged from 14.2

247   to 37.4 nM after 90 min (**Figure 2A** and **B**) and proximal regions of the small and large intestine

248   seemed to absorb more MeIQx when compared to their distal counterparts. For example, in the

249   small intestine, the highest mean concentrations of MeIQx were observed in the proximal

250   duodenum, followed by the proximal jejunum, ileum, and distal jejunum (**Figure 2A** and **B**).

251   A similar trend was observed in the large intestine, suggesting that the proximal region of the

252   intestine might primarily contribute to the uptake of MeIQx. MeIQx-M1 was also slowly

253   transported from the mucosal to the serosal compartment, and low levels were detected in the

254   serosal chamber after 90 min of incubation, with the mean concentration values ranging from

255   7.9 to 10.8 nM (**Figure 2**). No detectable amounts of MeIQx-M1 were measured in the serosal

256   compartment after only 1 min of incubation. The concentrations of MeIQx-M1 in the mucosal

257   compartments tended to decrease over time, although there were no significant differences in

258   the levels of MeIQx-M1 in the mucosal compartment between 1 and 90 min after incubation

259   (**Figure 2C and D**). The tissues are viable throughout the 90-min experiment based on no

260   significant changes on electrophysiological parameters such as the short-circuit current (I_{sc}) and

261   tissue conductance (G_t; **Table S5**).

262

263   To address whether the microbial metabolism of MeIQx impacts its uptake, we

264   quantitatively compared the intestinal uptake of MeIQx to MeIQx-M1 by calculating the

265   fraction of the compounds transported and the apparent permeability (P_{app}) for each in the

266   different intestinal segments. The transport rate of MeIQx (0.1-0.4 %) in all intestinal segments

267   was slightly higher than for MeIQx-M1, which ranged from 0.06 to 0.09 % (**Table 1**).

268   Correspondingly, the P_{app} of MeIQx (0.6-1.3 $\times$ 10^{-6} cm s^{-1}) is greater than that of MeIQx-M1

269   (0.3-0.4 $\times$ 10^{-6} cm s^{-1}; **Table 1**).

Impact of MeIQx-M1 formation on the disposition of MeIQx via PBPK modeling

271   Having established that MeIQx and MeIQx-M1 are transported at similar rates, we aimed

272   to estimate whether the microbial conjugation process has a significant influence on MeIQx

273   disposition in humans. Therefore, we established a PBPK model wherein parameters were fit

274   to *in vivo* plasma concentration data for MeIQx previously reported in 6 human subjects

275   (Malfatti et al., 2016). We evaluated the performance of the model by comparing the fitted

276   values of MeIQx plasma concentrations and the area under the curve (AUC) values with

277   measured values from human subjects. The model satisfactorily simulated the kinetic profile of

278   MeIQx in the different individuals (**Figure S5** and **Figure S6**). Sensitivity analyses indicate

279   that the AUC of MeIQx was highly sensitive to absorption rate, acrolein levels, glucuronidation,

280   volume of GI tract, and body weight (**Figure S7**). In the parameter fitting, we derived an

281   estimated acrolein level for each individual that, based on the experimental uptake rates, could

282   account for the measured MeIQx clearance rates (**Table S6**). Thereafter, batch runs were

283   performed to compare the plasma levels of MeIQx in the presence and absence of acrolein.

284   While the maximal plasma concentration (C_{max}) and AUC of MeIQx predicted using the model

285   was not dramatically affected by the acrolein-mediated MeIQx transformation (up to 4-fold

286   change, **Table 2, Table S6**), the plasma concentrations of MeIQx 4 or 8 h after its oral

287   administration were dramatically decreased by up to 48-fold for all individuals.

288   Correspondingly, up to 4005 μmoles acrolein were predicted to be required in these individuals

289   for the microbial transformation to fully account for the loss pathway of the chemical.

Cytotoxicity of MeIQx and MeIQx-M1 in HepaRG cells

In a previous study, we found that MeIQx was more cytotoxic than MeIQx-M1 toward human colon epithelial cells, however, these cells are not metabolically competent and do not represent a physiologically relevant tissue site pertinent to when the compounds are taken up. Therefore, to test whether the microbial transformation has any impact on the cytotoxicity of MeIQx on metabolically competent liver cells, we exposed HepaRG cells to increasing concentrations of both compounds and evaluated cell viability. After 24 h exposure, neither induced cytotoxic effects at concentrations below or equal to 500 μM (**Figure S8**). At the highest concentration tested, i.e. 1250 μM, MeIQx impaired cellular viability to a significantly greater extent than MeIQx-M1. Using these data, we calculated the benchmark dose (BMD), the no observed adverse effect level (NOAEL), and the lowest observed adverse effect level (LOAEL; **Table 3**), which were similar or slightly higher for MeIQx-M1 when compared to the values for MeIQx. The cytotoxicity data for the parent compound is consistent with data reported for MeIQx in HepaRG cells (Dumont et al., 2010), human liver cancer cells (Hep G2; Pezdirc et al. 2013), and non-malignantly transformed human colon epithelial cells (HCECs; Zhang et al. 2017)).

Discussion

The food carcinogen MeIQx can be metabolized to MeIQx-M1 by the microbial community in the human intestine. However, the influence of this microbial transformation process on systemic exposure remains unclear. The results of this study suggest that small percentages of MeIQx and MeIQx-M1 are transported from the mucosal to the serosal side of intestinal tissues. In addition, combining these transport values with previously reported plasma

312   levels of MeIQx in humans, PBPK modeling showed that high levels of microbe-derived

313   acrolein are required to reduce the systemic exposure of MeIQx in humans. Finally, it was

314   confirmed that conversion of MeIQx to MeIQx-M1 does not likely induce liver toxicity after

315   being taken up

316   **Conversion of MeIQx to MeIQx-M1 does not alter its transport rate**

317   To evaluate the relevance of the MeIQx concentration used in the mucosal compartment,

318   we considered the daily intake data available for MeIQx. The daily intake of MeIQx was

319   estimated to range from 72 ng per day in Sweden (Augustsson et al., 1997) to 3.9 µg per day in

320   Japan (Wakabayashi et al., 1997). The volume of the colon typically ranges from 160 to 203 ml

321   in healthy subjects (Pritchard et al., 2014). Therefore, concentrations of MeIQx in the colon are

322   estimated to range from 1.7 to 114 nM. Similarly, the concentration of dietary PhIP found in

323   the colon is approximately 1.5-1.9 µM when considering a single dose of 70-84 µg

324   administered to human subjects (Malfatti et al., 2006) as well as the above-mentioned colon

325   volume. Thus, the actual concentration of HCAs in the human gut after dietary intake of cooked

326   meat is anticipated to be 1-3 orders of magnitude lower than the 10 µM used in the present

327   study. Nevertheless, using 10 µM as the initial concentration allows for direct comparison with

328   previous studies using 10 µM PhIP and PhIP-M1 to investigate the intestinal uptake of these

329   compounds (Dietrich et al., 2001; Nicken et al., 2016, 2015, 2013, 2010).

330   The amount of MeIQx and MeIQx-M1 taken up after 90 min in the Ussing chamber

331   model ranged from 0.1 to 0.4 % and from 0.06 to 0.09 %, respectively. These values are

332   similar to what has been previously observed in the same model for the HCA PhIP and its

333   analogous microbial metabolite PhIP-M1, i.e. 0.1-0.2 % and 0.03-0.3 %, respectively

334   (Dietrich *et al.*, 2001; Nicken *et al.*, 2015, 2016;). While the model does not allow a direct

335   comparison with in vivo data due to the small area of mucosal surface, limited period of

336   time, and species-related differences, if the data is extrapolated on the basis of estimates

337   for time of human intestinal transit (35 h) (Metcalf et al., 1987), and assuming similar

338   transport rates for rat and human, the measured transport percentages would translate

339   to up to 9 % and 5 % of MeIQx or PhIP, respectively, being taken up. In previous studies

340   involving analysis of rat urine following oral MeIQx, Turesky et al. (1993) and Sjödin et al.

341   (1989) reported slightly higher values of up to 20% and 41 %, respectively. In these

342   examples, the remaining chemical was excreted in the feces of the animals (Turesky et al.,

343   1993, Sjödin et al., 1989). Similarly, Vanhaecke et al. found up to 21 % of PhIP (unchanged

344   plus acid-labile conjugates) excreted in urine, while up to 42 % of PhIP was excreted in

345   the feces from humans exposed to PhIP in food (Vanhaecke et al., 2008a). The potential

346   underestimation of transport by the Ussing chamber model is consistent with a reported

347   potential for underestimation by up to 5-fold observed in some cases that compared to

348   single perfusion in vivo (Lennernäs, 2007). Despite the potential for underestimation of

349   transport rate, the model is particularly effective for comparing between anticipated

350   effects of chemicals vs. metabolites.Apparent permeability coefficient of MeIQx and

351   MeIQx-M1 relative to other chemicals

352   The apparent permeability coefficients (P_{app}) for a wide range of compounds have been

353   determined using the Ussing chamber to predict uptake or transport in the human intestine

354   (Lennernäs, 2007). The P_{app} for HCAs and their microbial metabolites range from 0.3 to

355 1.2×10^{-6} cm s^{-1}, which is extremely low when compared to nutrients and some oral drugs such

356 as D-glucose ($P_{app} = 100 \times 10^{-6}$ cm s^{-1}; human jejunal tissue; Sjöberg *et al.*, 2013), naproxen

357 ($P_{app} = 45 \times 10^{-6}$ cm s^{-1}; female Sprague-Dawley rat jejunum; Lennernäs et al. 1997)) and

358 ketoprofen ($P_{app} = 48 \times 10^{-6}$ cm s^{-1}, male Wistar rat jejunal tissue; Sjögren et al. 2016)). In

359 contrast, the P_{app} of some oral drugs such as fexofenadine and candesartan, which are only

360 absorbed to a very limited extent, range from 0.8 to 1.2×10^{-6} cm s^{-1} (Sjögren et al., 2016),

361 which is comparable to the P_{app} of MeIQx and MeIQx-M1 presented herein. While the intestinal

362 transport is expected to occur in a manner that leads to a minor fraction impacting liver and

363 other organs/tissues, due to the mutagenic basis of the mode of action of MeIQx following

364 hepatic bioactivation, even low exposures may be physiologically relevant.

365 **Bacteria-derived acrolein alters the MeIQx disposition in humans**

366   Combining the transport values with previously reported plasma levels of MeIQx in

367 humans, we established a PBPK model to evaluate the influence of bacteria-derived acrolein

368 on the disposition of MeIQx in humans. Based on the prediction, the acrolein-mediated

369 metabolic transformation of MeIQx in the intestine would decrease the AUC of MeIQx by 2 to

370 4-fold in six human individuals (**Table 2**). These results suggest that bacteria-derived acrolein

371 may have a moderate influence on systemic exposure of MeIQx in human individuals. This

372 prediction is consistent with an earlier observation by Vanhaecke et al. (2008b), that the

373 efficiency of human fecal microbiota in converting PhIP to PhIP-M1 ranged from 2 to 98 %. In

374 addition, a great individual variation was also observed regarding the disposition and excretion

375 of PhIP and PhIP-M1 in human subjects: While some individuals excreted a comparable

376 amount of PhIP and PhIP-M1 in the urine, others excreted much more PhIP than PhIP-M1.

377   Drawbacks of the present PBPK model might include the fact that the model calibration was

378   only based on average anatomical parameters, albeit considering individual variations (Brown

379   et al., 1997), as there is no information available on the physiological and anatomical

380   parameters of the actual human subjects tested (Malfatti et al., 2016). Moreover, the established

381   PBPK model did not include the detailed fate of acrolein in the gastrointestinal tract and

382   considered the effective acrolein, but not free acrolein, available in that compartment. It has

383   been shown that HCAs can be converted to M1 metabolites even when there is no detectable

384   free acrolein in the solution (Engels et al., 2016), indicating that acrolein can exist in a

385   conjugated form and retain reactivity. Hence, the calculated 4005 μmoles acrolein should be

386   considered as acrolein equivalent, and thus a lower amount of free acrolein may be required to

387   significantly influence MeIQx disposition. Moreover, the aforementioned potential for

388   underestimation of transport rates in the Ussing chamber model translates to the potential for

389   the present PBPK model to overestimate the required physiological acrolein equivalents

390   required. Although there are many pieces of evidence indicating that acrolein is formed in the

391   human gastrointestinal tract (Zhang et al., 2018), actual information on the levels and fate of

392   this compound are not available.

393   **Conclusions**

394   In summary, we have demonstrated that MeIQx and its metabolite MeIQx-M1 have a

395   similar intestinal transport rate and are not cytotoxic towards human liver hepaRG cells. PBPK

396   modeling predicted that high levels of microbe-derived but not free acrolein may alter the

397   systemic levels of MeIQx in humans by up to 4-fold. However, further studies are needed to

398   validate the presented PBPK modeling on the basis of physiological acrolein levels, MeIQx

399 transformation, and potentially higher absolute transport rates. The results infer that

400 transformation by gut microbiota should be considered as a factor governing the disposition of

401 dietary mutagenic HCAs in the human body. The PBPK model described here could be applied

402 to other toxicants that are subjected to microbial transformation in order to be able to perform

403 a more comprehensive and robust risk assessment of such compounds.

410 **Compliance with ethical standards**

411   **Conflict of interest**: The authors declare no competing financial interests.

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