Toxicology Research

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/toxicology

Participation of $b^{0,+}$ and $B^{0,+}$ systems in the transport of mercury bound to cysteine in intestinal cells

Vázquez M., Vélez D., Devesa V.*

Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Avenida Agustín Escardino 7, 46980 - Paterna (Valencia), Spain.

* To whom correspondence should be addressed (telephone (+34) 963 900 022; fax (+34) 963 636 301; e-mail: vdevesa@iata.csic.es)

ABSTRACT

The main source of exposure to mercury (Hg) as divalent inorganic Hg [Hg (II)] and methylmercury (CH_3Hg) is the diet, in which complexes with the amino acid cysteine (Hg-Cys) may be found.

The present study explores the participation of the $b^{0,+}$ and $B^{0,+}$ amino acid transporters in the intestinal transport of mercuric conjugates with cysteine [Hg(II)-Cys and CH_3Hg-Cys], using Caco-2 cells as a model of the intestinal epithelium. For this purpose, the effects of $b^{0,+}$ and $B^{0,+}$ gene silencing upon Hg(II)-Cys and CH_3Hg-Cys uptake were evaluated. In addition, after treatment with Hg-Cys complexes, we evaluated the differential expression of $b^{0,+}$ and $B^{0,+}$, and of transporters belonging to the LAT and y^+LAT systems, which have also been described as possible participants in the Hg-Cys transport.

The results show that $b^{0,+}$ and $B^{0,+}$ silencing reduces intracellular accumulation of Hg(II)-Cys (39 ± 8%) and CH_3Hg-Cys (16 ± 3%), respectively. These results, together with the existing literature, suggest that these transporters are involved in the transport of Hg-Cys in intestinal cells. On the other hand, the differential expression studies reflect an increase in LAT and y^+LAT transporters after treatment with Hg-Cys, which could indicate their participation in the cellular elimination of such complexes, as has been previously suggested.

KEYWORDS: Mercury, $b^{0,+}$ system, $B^{0,+}$ system, LAT system, y^+LAT system, intestinal transport.

INTRODUCTION

Mercury (Hg) is a prevalent environmental contaminant that poses significant health concerns to humans and the environment. The main source of Hg exposure in humans is the diet, where it is fundamentally found as divalent inorganic Hg [Hg (II)] and methylmercury (CH_3Hg). The principal source of CH_3Hg is seafood, specifically large predators such as swordfish, tuna and sharks, while Hg(II) is prevalent in food matrixes such as plants and cereals.[1] CH_3Hg is regarded as a potent neurotoxic agent, and is also classified by the International Agency for Research on Cancer as possibly carcinogenic for humans (Group 2B).[2] Hg(II) in turn mainly affects the renal system, though it reportedly can also cause dermatological, gastrointestinal and immunological disorders.[3]

The intestinal absorption ratios of these two mercurial forms are very different. CH_3Hg is practically fully absorbed after intake in an aqueous solution or in food.[4] In contrast, Hg(II) absorption is considerable more limited (< 20%).[5] Using X-ray absorption spectroscopy (XAS), Harris et al.[6] found that CH_3Hg in seafood forms complexes with an aliphatic thiol – possibly cysteine. Information on Hg(II) is lacking in this regard, however taking into account its affinity for thiol groups,[7] probably it may form the same type of complex in food. Following intake, the gastrointestinal digestion process may modify the chemical form of an element or compound. Although studies are available on other trace elements,[8,9] it is not clear how the gastrointestinal digestion conditions affect the chemical forms

of Hg found in food. It has been suggested that the acidic high chloride conditions in the human stomach may convert CH_3Hg-Cys species to CH_3HgCl.[6] Therefore, in the intestinal lumen Hg solubilized during the digestion of food may be in the form of complexes with thiol groups or as salts.

The molecular structure of Hg has important toxicological implications, since the absorption mechanisms and ratios differ in each case. A recent study has shown that CH_3HgCl can be absorbed by a transcellular passive diffusion mechanism.[10] It has also been suggested that CH_3Hg bound to the thiol groups may be transported by amino acid transporters through a mechanism of molecular mimicry.[11,12] Because of its less hydrophobic nature, Hg(II) does not undergo important passive transcellular transport.[13] *In vitro* studies also suggest that Hg(II) bound to thiol groups may be transported by amino acid transporters.[11] However, despite the existing evidence, no study has firmly identified such amino acid transporters as being involved in the intestinal transport of Hg.

The present study explores the possible participation of the amino acid transporters $b^{0,+}$ and $B^{0,+}$ in the transport of Hg(II)-Cys and CH_3Hg-Cys across the intestinal epithelium. For this purpose, the effects of $b^{0,+}$ and $B^{0,+}$ gene silencing upon Hg(II)-Cys and CH_3Hg-Cys uptake were evaluated in a Caco-2 cell model of the intestinal epithelium. In addition, after treatment of the cells with Hg-Cys complexes, we evaluated the differential expression of $b^{0,+}$ and $B^{0,+}$, and of the amino acid transporters belonging to the LAT (*LAT1* and

LAT2) and y^+LAT (*y^+LAT1* and *y^+LAT2*) systems, which have also been described as possible participants in the transport of Hg-Cys.

RESULTS

Caco-2 cells between days 2-15 post-seeding were used for different assays. It was confirmed that the transporters of interest ($b^{0,+}$, $B^{0,+}$, LAT1, LAT2, y^+LAT1 and y^+LAT2) were expressed during the study period. These transporters were selected on the basis of other studies in which they were suggested to participate in the transport of Hg(II)-Cys and CH_3Hg-Cys.[10, 13-21]

On the other hand, viability assays showed that gene silencing and Hg exposure did not affect the cell viability, with values over 80%.

Inhibition of *$b^{0,+}$* and *$B^{0,+}$* by transient gene silencing and its effect upon the cellular uptake of Hg(II)-Cys and CH_3Hg-Cys. For each transporter we performed individual transfections with two siRNA and with a mixture of both. The differences in silencing between the two siRNA were only detectable for *$B^{0,+}$* (Figure 1). On the other hand, siRNA mixture did not result in greater transporter gene silencing (data not shown). Both transporters were significantly silenced (Figure 1), though the decrease in expression of *$b^{0,+}$* (11.0- to 13.9-fold) was less pronounced than that of *$B^{0,+}$* (14.3- to 72.2-fold).

The effect of silencing of the *$b^{0,+}$* and *$B^{0,+}$* system upon the accumulation of Hg-Cys was evaluated using the cells treated with the

siRNA associated with greater transporter silencing. Table 3 shows the percentage of cellular accumulation of Hg corresponding to the transfected and the control cell cultures. Silencing of $b^{0,+}$ produced a significant decrease in the amount of Hg present in the cells treated with Hg(II)-Cys versus the control cells (39 ± 8%). However, no changes in CH_3Hg-Cys accumulation were recorded. In turn, the silencing of $B^{0,+}$ resulted in a significant decrease in the accumulation of CH_3Hg-Cys (16 ± 3%), but not in the accumulation of Hg(II)-Cys.

Relative expression of the transporters in cells exposed to Hg(II)-Cys and CH_3Hg-Cys. After exposing the Caco-2 cells to the Hg-Cys complexes during 1, 2, 8 and 24 hours, we evaluated the changes in the expression of the amino acid transporters located on the apical side, which had been used in the gene silencing experiments ($B^{0,+}$ and $b^{0,+}$), and of the amino acid transporters located on the basal side, LAT (*LAT1* and *LAT2*) and y^+LAT (y^+*LAT1* and y^+*LAT2*) systems. Exposure to both forms of mercury causes significant changes in the expression of the genes studied only after 24 hours (Figure 2). The results show that both Hg-Cys complexes produced over-expression of these transporters compared with the cells not treated with Hg-Cys, with the exception of $B^{0,+}$, which tended to decrease with both treatments.

DISCUSSION

The cellular transport of Hg(II) and CH_3Hg has been studied in different *in vitro* and *in vivo* models. However, the transporters in charge of the intestinal transport of mercurial forms, and which conditions facilitate the entry of these toxic species to the systemic circulation, have not been defined to date. In our study, the identification of the intestinal transporters responsible for the absorption of Hg(II) and CH_3Hg was centered on those transporters located on the apical side of human enterocytes, and which may intervene in the transport of the toxic species from the lumen to the interior of the intestinal epithelium. Taking into account the existing literature, we selected the $b^{0,+}$ and $B^{0,+}$ amino acid transporter systems.

It has been shown that Hg(II)-Cys acts as a substrate of the $b^{0,+}$ transporter system in transfected kidney cells.[18,21] It has also been suggested that the $B^{0,+}$ system intervenes in the transport of Hg(II)-Cys in cells of the retinal epithelium .[22] In turn, in oocytes of *Xenopus laevis*, CH_3Hg bound to cysteine or homocysteine has been found to be a substrate of the $B^{0,+}$ system.[19] On the other hand, substrate competition studies in Caco-2 cells[10,13] also indicate possible participation of these systems in the transport of mercurial forms into the cell. The mentioned authors report that the transport of Hg(II) and CH_3Hg in the intestinal epithelial monolayer decreases in the presence on the apical side of neutral and cationic amino acids, which constitute substrates of the $b^{0,+}$ and $B^{0,+}$ transport systems.[23]

The gene silencing results obtained in our study show that a decrease in the mRNA levels of the $b^{0,+}$ system produces a significant reduction in Hg(II)-Cys uptake by the Caco-2 cell monolayer (Table 3). Hg(II) bound to cysteine has a structure very similar to that of cysteine,[18] an amino acid for which the $b^{0,+}$ system shows strong affinity.[24] It therefore has been postulated that Hg(II)-Cys is transported via a molecular mimicry mechanism. Regarding the $B^{0,+}$ system, although other authors have suggested its intervention in the transport of Hg(II)-Cys,[22] our own findings do not support its intervention in intestinal cells.

In relation to CH_3Hg, a study using Caco-2 cells and a parallel artificial membrane permeability assay (PAMPA) system has shown that in form of CH_3HgCl part of the transport of this mercurial species takes place through passive diffusion.[10] However, carrier mediated transport has also been observed, part of which is inhibited in the presence of amino acids on the apical side.[10] The results of the gene silencing assays of the $B^{0,+}$ and $b^{0,+}$ amino acid transporter systems carried out in our study show that a decrease in $B^{0,+}$ expression leads to a significant decrease in the accumulation of CH_3Hg bound to L-Cys (Table 3). The gene silencing results allow us to point to this system as a mediator of CH_3Hg-Cys transport into the intestinal epithelium. The participation of this system again could be explained in terms of molecular mimicry, since CH_3Hg–Cys is structurally similar to the amino acid methionine,[11] which is transported by the $B^{0,+}$ system.[25] It must be pointed out that Caco-2 cell exposure to

CH_3Hg-Cys implies a decrease in the mRNA levels of the $B^{0,+}$ system. Taking into account that this system intervene in the entry of this chemical form of Hg, the above could be interpreted as a defense mechanism designed to avoid an excessive increase in the toxic mercurial form within the cell.

In addition to the results corresponding to identification of the transporters involved in the absorption of mercurial forms, it is also interesting to mention the changes in expression of other transporter systems in Caco-2 cells exposed to Hg-Cys. In general terms, we can speak of an increase in the LAT (*LAT1*, *LAT2*) and y^+LAT (y^+LAT1, y^+LAT2) amino acid transporter systems, independently of the treatment applied. The isoforms of the LAT system are preferentially located in the basolateral membrane of the intestinal epithelial cell,[26-29] though some authors have indirectly localized LAT1 in the apical membrane.[30,31] Both isoforms transport neutral amino acids via an Na^+-independent mechanism.[32] The y^+LAT system in turn is responsible for Na^+-independent transport of basic amino acids and Na^+-dependent transport of neutral amino acids, though with low affinity.[25] In the same way as the LAT system, it is preferentially located in the basolateral membrane of the epithelial cells.[27,33,34]

These transporters have been postulated as possible participants in the transport of mercurial species from the interior of the cell to the basolateral side in studies involving Caco-2 cultures, in view of the decrease in apical-basolateral transport observed in the presence of amino acids.[10,13] The possible implication of these systems in the

transport of mercurial forms has also been suggested in earlier studies involving other cell lines or models.[14-18,35,36] In fact, in oocytes of *Xenopus laevis*, the LAT system has been shown to be implicated in the transport of CH_3Hg-Cys.[17] A more detailed analysis, similar to that made in the present study, is needed in order to confirm the participation of these systems in the transport of mercurial forms towards the basal side of the enterocytes. If such participation is confirmed, then the increased expression of the LAT and y^+LAT systems found in our study could again represent a cell defense mechanism characterized by an increase in the transporters involved in eliminating the toxic mercurial species from the cell.

The involvement of amino acid transporters in the transport of mercury can have different physiological implications. The presence of mercury in the intestinal lumen may affect amino acid transport by competing for the same carrier. Contrarily, the presence of amino acids may reduce the absorption of mercury. Consequently, in food matrices rich in proteins, such as seafood products, absorption of mercury may be reduced. This fact has already been shown in *in vitro* studies using Caco-2 cells.[37] If so, new variables should be considered in the assessment of the risk associated with the ingestion of mercury, which are not normally taken into account, such as diet composition. On the other hand, if the components of a food product affect the transport of mercury, this could be used as a basis for the design of dietary strategies aimed at reducing the bioavailability of the trace element.

CONCLUSIONS

This study contributes relevant information on the transporters involved in Hg(II) and CH_3Hg absorption by the intestinal epithelium. The Hg present in the bowel lumen bound to low molecular weight thiol groups such as cysteine may enter the intestinal epithelium via the $b^{0,+}$ and $B^{0,+}$ amino acid transporter systems. Once within the cell, it may be transported towards the basal side by the LAT and y^+LAT amino acid transporter systems - though this possibility requires confirmation by more detailed studies. Mercury in the form of salts (chlorides, nitrates) has other cell transport mechanisms. The transcellular transport of CH_3Hg salts through the intestinal epithelium may take place by simple passive diffusion.[10] The transport of Hg(II) requires more detailed investigation, though some authors suggest the possible participation of a divalent cation transporter.[13]

EXPERIMENTAL

Mercurial species. The Hg(II) and CH_3Hg standards were prepared from the dilution of $Hg(NO_3)_2$ (1000 mg/L, Merck, Spain) and CH_3HgCl (1000 mg/L, AlfaAesar, Spain), respectively.

The Hg-Cys complexes were formed following the protocol described by Zalups and Ahmad,[37] with minor modifications. Five µM L-Cys (Merck) were kept in contact at room temperature during 10 minutes with 2.5 µM Hg(II) or CH_3Hg (equivalent to 0.5 mg/L)

prepared in Minimal Essential Medium with Earle's salts (MEM) (HyClone) supplemented with 1 mM sodium pyruvate and 10 mM HEPES.

Human cell culture. Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were maintained in 75 cm^2 flask to which 10 mL of Dulbecco's Modified Eagle Medium (DMEM) with glucose (4.5 g/L) and glutamine (0.87 g/L) were added. The DMEM was supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.0025 mg/L amphotericin B (DMEMc). The cells were incubated at 37ºC in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. The medium was changed every 2-3 days. When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 g/L) and EDTA (0.2 g/L), and reseeded at a density of 1-1.5 $\times$ 10^4 cells/cm^2. The assays were performed with cultures between passages 25 and 40. All the reagents used for cell maintenance were obtained from HyClone Laboratories (Spain).

Gene expression of transporters in Caco-2 cells exposed to Hg(II)-Cys and CH_3Hg-Cys. Caco-2 cells were grown in 6-well plates at a density of 6.4 $\times$ 10^4 cells/cm^2 in DMEMc. After 12-15 days of

differentiation, the cells were exposed to Hg(II)-Cys or CH_3Hg-Cys in supplemented MEM during 1, 2, 8 and 24 hours. After exposure, the cells were washed twice with phosphate buffered saline (PBS, Hyclone) and recovered for gene expression. Cells not exposed to Hg-Cys were used as control.

Levels of mRNA were quantified by Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR). RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel, Germany). The extracted RNA was quantified spectroscopically in a Nanodrop ND-1000 (NanoDrop Technologies, USA), adjusting the samples with RNase-free water in order to work with the same concentrations. First-strand complementary DNA (cDNA) was obtained from 200 ng of total RNA using a Reverse Transcriptase Core Kit (Eurogentec Headquarters, Belgium).

qPCR was performed using the LightCycler® 480 Real-Time PCR Instrument (Roche Diagnostics, USA). Reactions were carried out in a 10 µL final volume containing 5 µL LightCycler® 480 SYBR Green I Master Mix (2X) (Roche), 2.5 µL cDNA (20 ng/µL), 1 µL of each forward and reverse primer (10 µM) (Biolegio, The Netherlands) (Table 1) and nuclease-free water. No-template controls were run to verify the absence of genomic DNA. 18S ribosomal RNA was employed as reference gene (F: GTAACCCGTTGAACCCCATT; R: CCATCCAATCGGTAGTAGCG). PCR efficiency curves for each gene were calculated using triplicates of a two-fold dilutions curve.

The PCR conditions were 95ºC for 5 min, followed by 40 cycles:

10 s denaturation at 95ºC, 10 s annealing at 55ºC, and 20 s elongation at 72ºC. The melting curve of each sample was analyzed after every PCR run to confirm PCR product specificity. Data were analyzed using the Relative Expression Software Tool (REST 2009, QIAGEN) in standard mode.

Small interfering RNA (siRNA) transfections in Caco-2 cells: effect on $b^{0,+}$ and $B^{0,+}$ gene expression and Hg-Cys accumulation. Cells were trypsinized, counted and then diluted in DMEMc without antibiotics or antifungals. On average, 5×10^4 cells/cm^2 were seeded in a 24-well plate and incubated overnight under normal growth conditions. siRNA (Table 2) was obtained from Qiagen (Hs_ SLC7A9_3, Hs_ SLC7A9_7 and Hs_ SLC6A14_3) and Life Technologies (s22220), and diluted to a concentration of 60 nM in DMEM without supplementation. Lipofectamine transfection reagent (Life Technologies, 3 µL in 50 µL DMEM without supplementation) was added to the diluted siRNA in 1:1 proportion, incubating the mixture for 5 min to allow the formation of transfection complexes. After this time, the medium of the cultures was eliminated and 100 µL of the mixture siRNA/lipofectamine were added dropwise to the cells and incubated under normal growth conditions. Gene silencing was monitored at 24 hours after transfection at mRNA level by RT-qPCR, using the conditions described above (section 2.3). Gene silencing was also monitored visually by observing the effect upon a cell death control (All Start HS Cell Death Control siRNA, Qiagen) at different

timepoints (2, 4, 6, 24, and 48 hours). Control samples with non-transfected cells, a mock transfection control with only transfection reagent, and a negative control siRNA without transfection reagent were also used.

Cellular Hg-Cys uptake studies were carried out at 24 hours post-transfection. Transfected and control cells were exposed during 1 hour to Hg(II)-Cys and CH_3Hg-Cys, formed as described above (section 2.1). After exposure, the medium was withdrawn, and the culture was washed with PBS and detached by EDTA-trypsin treatment. Cells recovered after centrifugation were used for the determination of Hg.

Cell viability. After transfection and at the end of exposure assays, the number of viable cells was quantified using the trypan blue exclusion technique (Trypan Blue Solution, 0.4%, Sigma).

Determination of Hg. Cells were placed in a Teflon perfluoroalkoxy vessel and treated with 1 mL of HNO_3 (14 M, Merck) and 1 mL of H_2O_2 (30% v/v, Panreac, Spain). The Teflon vessel was irradiated at 800 W (15 min at 180ºC) in a microwave accelerated reaction system (MARS) from CEM (Vertex, Spain). At the end of the digestion program, the digest was placed in a tube and allowed to rest overnight to eliminate the nitrous vapor. It was then made up to volume with 0.6 M HCl.

For Hg quantification, a continuous flow cold vapor generation-

atomic fluorescence spectrometer (CV-AFS) (PSA 10.025 Millennium Merlin, PS Analytical, UK) was used. The analytical conditions were the following: reducing agent 2% (w/v) $SnCl_2$ (Scharlau, Scharlab, Spain) in 1.8 M HCl, 4.5 mL/min flow rate; carrier solution 0.6 M HCl, 9 mL/min flow rate; carrier gas argon, 0.3 L/min flow rate; dryer gas air, 2.5 L/min flow rate; specific Hg lamp; fixed 254 nm filter. The analytical performance of the equipment used for Hg quantification was evaluated by the analysis of water certified reference standard (RTC, QCI-049-1) supplied by LGC Standards (Spain) (Hg certified value = 40.8 ± 1.19 µg/L).

Statistical analysis. All tests were performed at least in sextuplicate in independent cultures. The results were subjected to statistical analysis by one-factor analysis of variance (ANOVA) with the Tukey HSD *post hoc* multiple comparison test (SigmaPlot version 12.0). Statistical significance was accepted for $p<0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work received support from the Spanish Ministry of Economy and Competitiveness (AGL2012-33461), for which the authors are

deeply indebted. Marta Vázquez received a Personnel Training Grant from the Spanish Ministry of Science and Education to carry out this study.

REFERENCES

1. EFSA European Food Safety Authority, *EFSA J.*, 2012, **10**, 2985.
2. IARC (International Agency for Cancer Research), Monographs on the evaluation of carcinogenic risks to humans. Overall Evaluations of Carcinogenicity to Humans, 58, 1993.
3. NTP (National Toxicology Program), NTP TR 408, NIH publication no. 91–3139, U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC, 1993.
4. T. Rahola, T. Hattula, A. Korolainen and J. K. Miettinen, *Ann. Clin. Res.*, 1973, **5**, 214.
5. T. W. Clarkson and L. Magos, *Crit. Rev. Toxicol*., 2006, **36**, 609.
6. H. H. Harris, I. J. Pickering and G. N. George, *Science*, 2003, **301**, 1203.
7. R. K. Zalups, *Pharmacol. Rev*., 2000, **52**, 113.
8. P. Alava, G. Du Laing, M. Odhiambo, A. Verliefde, F. Tack and T. R. Van de Wiele, *J. Environ. Sci. Health A. Tox. Hazard. Subst. Environ. Eng*., 2013, **48**, 656.
9. M. Calatayud, E. Bralatei, J. Feldmann, V. Devesa and D. Vélez,

J. Agric. Food Chem., 2013, **61**, 12164.

10. M. Vázquez, D. Vélez and V. Devesa, *Chem. Res. Toxicol*., 2014, **27**, 254.
11. N. Ballatori, *Environ. Health Perspect*., 2002, **110**, 689.
12. C. C. Bridges and R. K. Zalups, *Toxicol. Appl. Pharmacol*., 2005, **204**, 274.
13. M. Vázquez, V. Devesa and D. Vélez, *Toxicol. In vitro*, 2014, DOI: 10.1016/j.tiv.2014.09.013.
14. M. Aschner, N. B. Eberle, S. Goderie and H. K. Kimelberg, *Brain Res*., 1990, **521**, 221.
15. L. E. Kerper, N. Ballatori, T. W. Clarkson, *Am. J. Physiol*., 1992, **262**, R761.
16. E. M. Mokrzan, L. E. Kerper, N. Ballatori and T. W. Clarkson, *J. Pharmacol. Exp. Ther*., 1995, **272**, 1277.
17. T. A. Simmons-Willis, A. S. Koh, T. W. Clarkson and N. Ballatori, *Biochem. J.*, 2002, **367**, 239.
18. C. C. Bridges, C. Bauch, F. Verrey and R. K. Zalups, *J. Am. Soc. Nephrol.*, 2004, **15**, 663.
19. C. C. Bridges and R. K. Zalups, *Chem. Res. Toxicol*., 2006, **19**, 1117.
20. L. Heggland, P. Kaur and T. Syversen, *Toxicol. In vitro*, 2009, **23**, 1020.

21. C. C. Bridges and R. K. Zalups, *Am. J. Pathol*., 2004, **165**, 1385.

22. C. C. Bridges, J. R. Battle and R. K. Zalups, *Toxicol. Appl. Pharmacol.*, 2007, **221**, 251.

23. M. S. Kilberg, B. R. Stevens and D. A. Novak, *Annu. Rev. Nutr*., 1993, **13**, 137.

24. W. C. Wang, W. T. Gu, X.F. Tang, M. M. Geng, M. Fan, T.J. Li, W. Y. Chu, C. Y. Shi, R. L. Huang, H . F. Zhang and Y. L. Yin. Comp. Biochem. Physiol. B, 2009, **154**, 157.

25. R. Martín-Venegas, M. J. Rodriguez-Lagunas, Y. Mercier, P. A. Geraert and R. Ferrer, *Am. J. Physiol. Cell. Physiol.,* 2009, **296**, C632.

26. G. Rossier, C. Meier, C. Bauch, V. Summa, B. Sordat, F. Verrey and L. C. Kuhn, *J. Biol. Chem*., 1999, **274**, 34948.

27. C. Bauch, N. Forster, D. Loffing-Cueni, V. Summa and F. Verrey, *J. Biol. Chem*., 2003, **278**, 1316.

28. F. Verrey, *Pflugers Arch*., 2003, **445**, 529.

29. T. V. Nguyen, D. E. Smith and D. Fleisher, *Pharm. Res*., 2007, **24**, 353.

30. B. R. Stevens, H. J. Ross and E. M. Wright, *J. Membr. Biol*., 1982, **66**, 213.

31. V. Berger, Y. Larondelle, A. Trouet, Y. J. Schneider, *J. Nutr*., 2000, **130**, 2780.

32. H. Segawa, Y. Fukasawa, K. Miyamoto, E. Takeda, H. Endou and

Y. Kanai, *J. Biol. Chem.*, 1999, **274**, 19745.

33. K. Kizhatil and L. M. Albritton, *Am. J. Physiol. Cell. Physiol*., 2002, **283**, C1784.

34. S. Broer, *Physiol. Rev*., 2008, **88**, 249.

35. Y. Kajiwara, A. Yasutake, T. Adachi and K. Hirayama, *Arch. Toxicol*., 1996, **70**, 310.

36. W. Wang, T. W. Clarkson and N. Ballatori, *Toxicol. Appl. Pharmacol*., 2000, **168**, 72.

37. V. Vázquez, M. Calatayud, D. Vélez and V. Devesa. *Toxicology*, **2013**, 311, 147.

38. R. K. Zalups and S. Ahmad, *J. Am. Soc. Nephrol*., 2004, **15**, 2023.

FIGURE CAPTIONS

Figure 1. Expression of transporters in gene silenced Caco-2 cells versus non-silenced controls (in log scale base 2, $\log_2$). Values expressed as mean ± standard deviation (n=6). Statistically significant differences versus the controls are marked by an asterisk ($p<0.05$).

Figure 2. Relative expression of transporters in Caco-2 cells exposed to Hg(II)-Cys and CH_3Hg-Cys during 24 hours (in log scale base 2, $\log_2$). Values expressed as mean ± standard deviation (n=8). Statistically significant differences versus the controls not exposed to Hg-Cys are marked by an asterisk ($p<0.05$).

Table 1. Sequences and efficacies of the oligonucleotides used in qPCR for the determination of the transporters expression levels

Gene	GenBank no.	Sequence of primers 5'-3'	Amplicon (bp)	Efficacy
System $b^{0,+}$ *SL7A9*	NM_001126335,	F: GGCCTGACGATTCTAGGACTCA R: GGAGCCAGAACCAAAAACACA	125	2.03 ± 0.01
System $B^{0,+}$ *SLC6A14*	NM_007231	F: ACCGTGGTAACTGGTCCAAAA R: CGCCTCCACCATTGCTGTAG	114	1.98 ± 0.15
LAT1 *SLC7A5*	NM_003486.5	F: CACAGAGGAAATGATCAACCCCT R: TGATAGTTCCCGAAGTCCACGG	168	2.09 ± 0.08
y^+*LAT1* *SLC7A7*	NM_001126105	F: GATCCATGTTGAGCGGTTCAC R: CCACGCACAAGTAGATCAATGC	77	2.10 ± 0.05
LAT2 *SLC7A8*	NM_182728.1	F: TGAGGAGCTTGTTGATCCCTACA R: GCGACATTGGCAAAGACATACA	93	2.00 ± 0.09
y^+*LAT2* *SLC7A6*	NM_001076785.1	F: CCCACACCCACCTACCATCT R: CTGAGCCGATCATGTTGCC	160	2.06 ± 0.07

Table 2. Sequence of the small interfering RNA (siRNA) used in the gene silencing experiments.

siRNA	Gene	Sequence 5´-3
Hs_SLC7A9_3	System $b^{0,+}$ *SLC7A9*	F: UGUUCAUCUCGACAGUGAATT R: UUCACUGUCGAGAUGAACAAG
Hs_SLC7A9_7	System $b^{0,+}$ *SLC7A9*	FCGUGAUGACUGCCACCGAATT R: UUCGGUGGCAGUCAUCACGGT
Hs_SLC6A14_3	System $B^{0,+}$ *SLC6A14*	F: CAAUAGUAACUCACUGUAATT R: UUACAGUGAGUUACUAUUGGT
s22220	System $B^{0,+}$ *SLC6A14*	F: GGGACAAUUUGCUAGCUUATT R: UAAGCUAGCAAAUUGUCCCAG

Table 3. Mercury content in Caco-2 transfected with siRNA and in control cells treated with Hg(II)-Cys and CH_3Hg-Cys during one hour. Values expressed as ng Hg/10^6 cells (mean ± standard deviation, n=6). Statistically significant differences versus the non-transfected controls are marked by an asterisk ($p<0.05$).

	System $b^{0,+}$		**System $B^{0,+}$**	
	Control	**siRNA transfected**	**Control**	**siRNA transfected**
Hg(II)-Cys	55.0 ± 0.8	35.5 ± 0.8*	50.7 ± 3.8	51.4 ± 3.3
CH_3Hg-Cys	66.5 ± 1.0	64.8 ± 1.1	68.2 ± 0.2	56.1 ± 2.6*

Figure 1

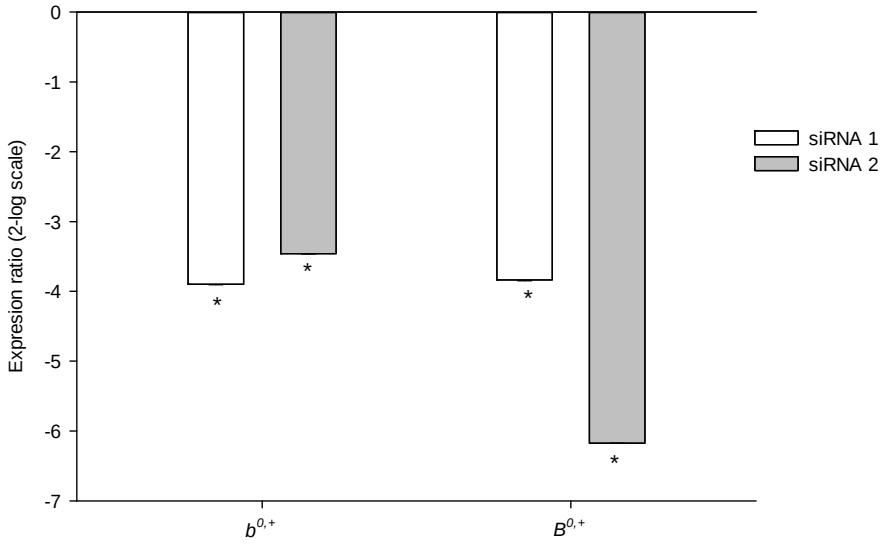

Figure 2