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GPNA inhibits the sodium-independent transport system I for neutral amino acids

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Amino Acids

GPNA inhibits the sodium-independent transport system L for neutral amino acids

--Manuscript Draft--

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Abstract:	<p>L-γ-glutamyl-p-nitroanilide (GPNA) is widely used to inhibit the glutamine transporter ASCT2, although it is known that it also inhibits other sodium-dependent amino acid transporters. In a panel of human cancer cell lines, which express the system L transporters LAT1 and LAT2, GPNA inhibits the sodium-independent influx of leucine and glutamine. The kinetics of the effect suggests that GPNA is a low affinity, competitive inhibitor of system L transporters. In Hs683 human oligodendroglioma cells, the incubation in the presence of GPNA, but not ASCT2 silencing, lowers the cell content of leucine. Under the same conditions the activity of mTORC1 is inhibited. Decreased cell content of branched chain amino acids and mTORC1 inhibition are observed in most of the other cell lines upon incubation with GPNA. It is concluded that GPNA hinders the uptake of essential amino acids through system L transporters and lowers their cell content.</p>
Response to Reviewers:	<p>Referee 1 Reviewer's comment: Overall, the authors have addressed each of the questions from my initial review, however this reviewer maintains that the manuscript would be far more beneficial to the research community with the simple addition of oocyte experiments to identify the transporter(s) involved. As it currently stands, the following line should be altered on page 3, since there are no data showing specific inhibition of LAT1 and LAT2: "Here we show that GPNA inhibits the sodium-independent influx of leucine and lowers its cell content, indicating that the inhibitor hinders the activity of system L transporters LAT1 and LAT2." Reply: We agree with the Reviewer but we feel that the identification of the sensitivity</p>

of each L transporter to GPNA, although very interesting, goes far beyond the main aim of our work and would significantly delay the publication of our results. We also agree that the sentence, in its present form, is somewhat overreaching and have modified it.

"Here we show that GPNA inhibits the sodium-independent influx of leucine and lowers its cell content, indicating that the inhibitor hinders the activity of system L."

Reviewer's comment: Glutamine uptake inhibition by siASCT2 should also be shown in Figure 6.

Reply: Glutamine uptake inhibition by siASCT2 is now shown in Figure 6, c. The results indicate that ASCT2 silencing significantly inhibits glutamine uptake and that the inhibition is exclusively attributable to the decrease of the sodium-dependent transport.

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4 **GPNA inhibits the sodium-independent transport system L for neutral amino acids**
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Abstract

1 L- γ -glutamyl-p-nitroanilide (GPNA) is widely used to inhibit the glutamine transporter ASCT2, although it is known
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3 that it also inhibits other sodium-dependent amino acid transporters. In a panel of human cancer cell lines, which
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5 express the system L transporters LAT1 and LAT2, GPNA inhibits the sodium-independent influx of leucine and
6
7 glutamine. The kinetics of the effect suggests that GPNA is a low affinity, competitive inhibitor of system L
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9 transporters. In Hs683 human oligodendroglioma cells, the incubation in the presence of GPNA, but not ASCT2
10
11 silencing, lowers the cell content of leucine. Under the same conditions the activity of mTORC1 is inhibited. Decreased
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13 cell content of branched chain amino acids and mTORC1 inhibition are observed in most of the other cell lines upon
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15 incubation with GPNA. It is concluded that GPNA hinders the uptake of essential amino acids through system L
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17 transporters and lowers their cell content.
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21 Keywords: GPNA – Leucine – System L – LAT1 –ASCT2– Glutamine
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Introduction

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2 Glutamine uptake through the transporter ASCT2 has been found stimulated in many cancer models (Fuchs and Bode
3 2005). This metabolic feature has prompted various attempts to inhibit glutamine entry as a device to hinder cancer cell
4 proliferation. L- γ -Glutamyl-*p*-nitroanilide (GPNA) was proposed several years ago as an ASCT2 inhibitor (Esslinger et
5 al. 2005) and has been subsequently widely used to this purpose (Hassanein et al. 2015; Ren et al. 2015; Wang et al.
6 2015; Bolzoni et al. 2016; van Geldermalsen et al. 2016). However, GPNA selectivity for ASCT2 had never been
7 assessed in depth until, most recently, Broer et al. (Broer et al. 2016) definitely demonstrated that the compound
8 inhibits, besides ASCT2, other Na⁺-dependent carriers, such as several members of the SNAT family.
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10 Besides ASCT2, also other transporters have been found overexpressed in human cancer cells *in vitro*, as well as in
11 primary and metastatic human tumors *in vivo*. In particular, two of these carriers, LAT1 (coded by *SLC7A5*) and LAT2
12 (coded by *SLC7A8*) are highly expressed in a wide array of human tumors (Fuchs and Bode 2005; Kaira et al. 2008;
13 Wang and Holst 2015; Barollo et al. 2016). LAT1 and LAT2, once complexed with the chaperone 4F2hc, account in
14 many tissues for the activity of System L, a sodium-independent, non electrogenic, exchange transport mechanism that
15 operates the transmembrane fluxes of most essential amino acids. LAT1 and LAT2 have comparable operational
16 features, both efficiently transport leucine, although LAT2 is endowed with a lower affinity for substrates (del Amo et
17 al. 2008), and, through leucine transport, have an important regulatory role in the stimulation of mTORC1 activity
18 (Nicklin et al. 2009; Chen et al. 2014; Milkereit et al. 2015).
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20 Here we show that GPNA inhibits the sodium-independent influx of leucine and lowers its cell content, indicating that
21 the inhibitor hinders the activity of system L.
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Materials and methods

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42 Human oligodendroglioma Hs683 cells, provided by Prof. R. Kiss, University of Bruxelles, were grown in low-
43 glucose (1 g/L) Dulbecco's modified medium, DMEM (Euroclone), supplemented with 10 % FBS (Lonza, Basel,
44 Switzerland), 4 mM Gln, 25 mM HEPES, and antibiotics (100 U/ml penicillin, and 100 μ g/ml streptomycin). Human
45 cervix carcinoma HeLa cells, obtained from ATCC, human breast adenocarcinoma MCF7 cells, purchased from the
46 IZSLER Cell Bank (Brescia, Italy), and human hepatocellular carcinoma Huh7 cells, a gift of Prof. G. Raimondo,
47 University of Messina, were grown in high-glucose (4.5 g/L) DMEM supplemented with 10 % FBS, 4 mM Gln, and
48 antibiotics. Human lung alveolar carcinoma A549 cells, provided by Prof. L. Migliore, University of Pisa, were grown
49 in Ham's F12 medium supplemented with 10 % FBS, 1 mM Gln, and antibiotics. Cells were incubated at 37°C at 5%
50 CO₂; after thawing, all cells were used for less than 10 passages.
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1 Total cell RNA (1 µg) was isolated and reverse transcribed, and cDNA analyzed as previously described (Chiu et
2 al. 2014). Primers were: 5'-GTGGAC TTCGGGA ACTATCACC (*SLC7A5*, for), 5'-GAACAGGGACCCATTGACGG
3 (*SLC7A5*, rev); 5'-AGGCTGGA ACTTTCTGAAT (*SLC7A8*, for), 5'-ACATAAGCGACATTGGCAA (*SLC7A8* rev);
4 5'-TGGTCTCCTGGATCATGTGG (*SLC1A5* for), 5'-TTTGCGGGTGAAGAGGAAGT (*SLC1A5* rev); 5'-
5 CACCACAGGGAAGTTCGTATTC (*SLC38A1* for), 5'-CGTACCAGGCTGAAAATGTCTC (*SLC38A1* rev); 5'-
6 ATGAAGAAGGCCGAAATGGGA (*SLC38A2* for), 5'-TGCTTGGTGGGGTAGGAGTAG (*SLC38A2* rev); 5'-
7 GCAGCCATCAGGTAAGCCAAG (*RPL-15*, for), 5'-AGCGGACCCTCAGAAGAAAGC (*RPL-15*, rev). Data
8 analysis was made according to the Relative Standard Curve Method (Bustin 2000).
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16 Immunoblotting was performed as previously described (Chiu et al. 2012) using anti-LAT1 (rabbit, polyclonal,
17 1:1000, Cell Signaling Technology), anti-S6K1 phospho T389 (rabbit, monoclonal, 1:1000, Cell Signaling
18 Technology), anti-S6K1 total (rabbit, monoclonal, 1:1000, Cell Signaling Technology), anti-ASCT2 (rabbit,
19 monoclonal, 1:4000; Cell Signaling Technology) and anti-β-actin (rabbit, polyclonal, 1:1000, Sigma).
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24 Intracellular leucine, isoleucine and glutamine were extracted with ice-cold absolute ethanol and determined with
25 liquid chromatography coupled with mass spectrometry as previously described (Bolzoni et al. 2016).
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28 For ASCT2 gene silencing, Hs683 cells were transfected with a scrambled (ON-TARGETplus Non-targeting
29 Pool) or with a siRNA targeting ASCT2 (ON-TARGETplus SMARTpool, *SLC1A5*, Thermo Scientific DharmaFECT).
30 72 h after transfection, cells were rinsed in PBS and fresh medium was added. After 9 h, intracellular leucine was
31 extracted, and its content determined.
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36 For transport experiments, cells were seeded on 96-well plates at a density of 15 x 10³ cells/well in normal growth
37 medium. The initial influx of L-[3,4-³H]-glutamine (Amersham Biosciences) and L-[4,5-³H]-leucine (Amersham
38 Biosciences) was measured following the method previously described (Bianchi et al. 2012). For L-glutamine transport,
39 cells were rapidly washed with an Earle's Balanced Salt Solution (EBSS, composition in mM: NaCl 117, KCl 5.3,
40 CaCl₂ 1.8, MgSO₄·7H₂O 0.81, choline phosphate 0.9, glucose 5.5, supplemented with 0.02 % Phenol Red, kept at pH
41 7.4 with 26 mM Tris-HCl) and transport assay (30 s) was performed in the same solution. For L-leucine , before
42 transport determination, Na⁺-free EBSS, where N-methyl-D-glucamine chloride was used to replace NaCl, was used
43 for the washing and the transport assay
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52 The non-saturable component of leucine influx was estimated measuring leucine uptake in the presence of 2 mM
53 leucine.
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56 For the kinetic analysis, L-leucine influx data, obtained at different concentrations of the amino acid, were fit to

57 the equation: $v = \frac{v_{\max} \cdot [\text{Leu}]}{K_m + [\text{Leu}]} + K_D \cdot [\text{Leu}]$ (Eq. 1)
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1 For the kinetic analysis of GPNA inhibition activity, L-leucine influx data, obtained at different concentrations of
2 the inhibitor, were fit to the equation for competitive inhibition: $v = v_0 - \frac{I_{\max} \cdot [\text{GPNA}]}{[I]_{0.5} + [\text{GPNA}]}$ (Eq. 2)
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4 where v_0 is the influx in the absence of inhibitor, I_{\max} the maximal inhibition and $[I]_{0.5}$ the GPNA concentration at
5 which the inhibition is half-maximal.
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8 GraphPad Prism 5.0™ was used for all the statistical analyses, and p values <0.05 were considered statistically
9 significant. Unless otherwise stated, Sigma-Aldrich was the source of all the chemicals, included GPNA.
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11

12 13 14 **Results**

15
16 In a panel of human cancer cell lines, L- γ -glutamyl-*p*-nitroanilide (GPNA), an inhibitor of the sodium-dependent carrier
17 ASCT2, inhibited most of glutamine influx in the presence of sodium (Fig. 1a). However, GPNA significantly inhibited
18 glutamine transport also in the absence of sodium (Fig. 1b). To identify the sodium-independent transport system
19 inhibited by GPNA, the saturable influx of leucine was determined in the same cell models in the absence of sodium.
20 Leucine influx was different in the lines tested, with HeLa cells exhibiting the fastest influx and Huh7 cells the slowest
21 (Fig. 1c). In all the cell lines GPNA significantly inhibited the sodium-independent leucine influx, with inhibitions
22 ranging from almost 40% for Huh7 cells to over the 50% for Hs683 and MCF7 cells.
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25 LAT1 and LAT2 system L transporters were consistently expressed, although at a variable degree, in all the cancer cell
26 lines (Fig. 2a, b). Human oligodendrogloma Hs683 cells showed the highest relative LAT1 and the lowest LAT2
27 mRNA expression, while MCF7 breast cancer cells had the highest LAT2 but relatively low LAT1 mRNA expression,
28 and hepatocellular carcinoma Huh7 cells had a low relative expression of both transporters.
29

30 The kinetic analysis of the Na⁺-independent Leu influx, performed in Hs683 cells (Fig. 3a), indicated that GPNA
31 increased the K_m for leucine from $56 \pm 2 \mu\text{M}$ to $104 \pm 15 \mu\text{M}$, while the V_{\max} was not significantly modified ($15.1 \pm$
32 2.10 nmol/mg/min , GPNA absent, versus $14.1 \pm 1.33 \text{ nmol/mg/min}$, GPNA present). The diffusion constant K_D was
33 also comparable in the absence and in the presence of GPNA ($49.5 \pm 2.42 \text{ min}^{-1}$, GPNA absent, versus $50.5 \pm 3.33 \text{ min}^{-1}$,
34 GPNA present). The inhibition pattern was satisfactorily fitted with an equation for a competitive inhibition (Fig. 3b).
35

36 At $10 \mu\text{M}$ [Leu], the maximal inhibition by GPNA was more than 65% of the uninhibited total influx with a half-
37 maximal inhibitory concentration of $807 \pm 70 \mu\text{M}$. The inhibitory effects on the sodium-independent leucine influx of
38 GPNA and of BCH, an amino acid analogue that preferentially inhibits system L, are compared in Fig. 3c. BCH
39 inhibited leucine influx by more than 80%, while GPNA-dependent inhibition was roughly 50% of the total leucine
40 uptake.
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42 We next investigated the intracellular content of the two system L substrates leucine and isoleucine upon 9 hours of
43 incubation with 3 mM GPNA. Under control conditions, the intracellular content of both leucine and isoleucine varied
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1 among the cell lines (Fig. 4a, b). Hs683 cells had the highest content of either leucine (14.4 ± 0.37 nmol/mg prot) or
2 isoleucine (14.3 ± 0.85 nmol/mg prot), while Huh7 cells had the lowest (Leu = 4.3 ± 0.09 nmol/mg prot; Ile = $3.9 \pm$
3 0.59 nmol/mg prot). In all but Huh7 cells, the intracellular content the two amino acids significantly decreased in the
4 presence of GPNA, with the highest inhibition (more than 40%) in Hs683 cells. The cell content of glutamine, measured
5 in the same cells (Fig. 4c) also varied among the various cell lines, with MCF7 showing the highest intracellular levels
6 and A549 cells the lowest. Only in Hs683 cells, GPNA significantly decreased intracellular glutamine.
7

8 Under the same conditions, the abundance of the phosphorylated form of S6K1, an indicator of the activity of the kinase
9 mTORC1, which is stimulated by intracellular leucine, was markedly lowered in all the cell lines (Fig. 5a). In all cells,
10 but Huh7, changes in pS6K1 were not paralleled by changes in total S6K1 and, therefore, could be attributed to an
11 effective decrease in mTORC1 activity. Interestingly, GPNA had inconsistent effects on LAT1 expression, with Hs683
12 and HeLa exhibiting sizable increases, while A549 and MCF7 showed a decreased expression of the transporter. As
13 expected, rapamycin suppressed S6K1 phosphorylation in all the cell lines, confirming its absolute dependence upon
14 mTORC1 activity (Fig. 4b).
15

16 To verify if the inhibition of ASCT2 by GPNA could be involved in the effects of the analog on the cell content of
17 leucine, SLC1A5 was silenced in Hs683 cells, causing an almost complete suppression of ASCT2 expression both at
18 mRNA (Fig. 6a) and protein level (Fig. 6b), and a significant inhibition of glutamine uptake that is exclusively detected
19 in the presence of sodium (Fig. 6c). No evidence of a compensatory increase in the expression of the other sodium-
20 dependent transporters SNAT1 (encoded by *SLC38A1*, Fig. 6e) or SNAT2 (encoded by *SLC38A2*, Fig. 6f) was detected.
21 However, ASCT2 silencing did not change the cell content of leucine (Fig. 6d).
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26 Discussion

27 This report demonstrates that GPNA is a competitive inhibitor of system L transport activity. This sodium-independent
28 transport agency accounts for the cell uptake of most essential amino acids and is stimulated in many tumors (Kanai et
29 al. 1998). Although the kinetics of the inhibitory effect on leucine influx have been studied in Hs683 cells, which are
30 endowed with the highest expression of the system L transporter LAT1 when compared to the other cell lines tested,
31 both LAT1 and LAT2 transporters are likely inhibited by GPNA. Indeed, the percentage inhibition of the Na⁺-
32 independent leucine influx is substantially comparable in Hs683 and MCF7 cells, although the two cell lines exhibit
33 specular differences in LAT1 and LAT2 mRNA expression. However, the approach used in this study is not sufficient
34 to definitely discriminate if LAT1 or LAT2 are equally sensitive to GPNA or to exclude a possible sensitivity of the
35 other system L transporters LAT3 and LAT4.
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1 In the past few years, GPNA has been used as an experimental device to inhibit glutamine transport through the Na⁺-
2 dependent transporter ASCT2 in several models of cancer cells (Hassanein et al. 2013; Indo et al. 2013; Ren et al. 2015;
3 Takahashi et al. 2015; Wang et al. 2015; Bolzoni et al. 2016; van Geldermalsen et al. 2016). Therefore, the inhibition of
4 cell growth by GPNA has been taken as an evidence for the essential metabolic role of the ASCT2 transporter in those
5 models. Inhibition by GPNA has been also used to demonstrate that glutamine analogues, synthesized as potential
6 probes of ASCT2 transport function for Positron Emission Tomography, effectively interact with the transporter
7 (Lieberman et al. 2011; Tang et al. 2016). At the light of the data presented here, these interpretations should be taken
8 with caution. In particular, system L inhibition may contribute to the suppression of glutamine uptake by GPNA.
9 Moreover, since the inhibition of system L by GPNA could directly hamper, besides glutamine uptake, the uptake of
10 essential amino acids needed for protein synthesis and cell growth, the effects of GPNA on cell growth cannot be
11 attributed to the sole inhibition of ASCT2. .

12 According to the model of Nicklin (Nicklin et al. 2009), leucine influx may be inhibited as an indirect effect of GPNA
13 inhibition of the glutamine influx mediated by the sodium-dependent ASCT2 transporter that would lower the amount
14 of intracellular glutamine available for promoting the influx of leucine through system L exchange transporters LAT1
15 and LAT2. The involvement of this mechanism in the GPNA-mediated inhibition of leucine influx is highly unlikely,
16 since, in the transport experiments, the inhibitor is only present during the assay (30s), which, moreover, occurs in the
17 absence of sodium. With the same argument, it is possible to exclude the involvement of other sodium-dependent
18 transport systems, such as SNAT1 and SNAT2, recently described to be sensitive to GPNA inhibition (Broer et al.
19 2016).

20 On the contrary, it is possible that the significant depletion of the intracellular pool of leucine and isoleucine, observed
21 in cells incubated for 9h with GPNA, may involve the decrease in cell glutamine attributable to ASCT2 inhibition.
22 Actually, in human oligodendroglioma Hs683 cells long term GPNA treatment significantly lowered also the cell
23 content of glutamine (Fig. 4c). However, GPNA decreased leucine influx and lowered the cell content of the essential
24 amino acid in all the cell lines tested, with the only exception of Huh7 cells. In three of these cell lines (HeLa, A549,
25 and MCF7) cell leucine decreased in the absence of a significant depletion of intracellular glutamine. Even in Hs683
26 cells a contribution of ASCT2 inhibition to leucine depletion seems unlikely, since ASCT2 silencing does not
27 significantly affect cell leucine while markedly inhibits total and sodium-dependent glutamine uptake (Fig. 6). Although
28 a role for other GPNA-sensitive sodium-dependent transporters, such as SNAT1 and SNAT2, cannot be excluded , it
29 should be noted that no compensatory induction of these transporters is detected in ASCT2-silenced Hs683 cells (Fig.
30 6d,e), at variance with the results reported in other cell models (Broer et al. 2016).

1 Interestingly, in all the cell lines tested, the long term incubation with GPNA markedly decreases, although at a variable
2 degree, mTORC1 activity. Under the same conditions, the expression of LAT1 exhibited inconsistent changes, being
3 increased in Hs683 and in HeLa cells and decreased in A549 and MCF7 lines. Therefore, the inhibition of mTORC1
4 was not associated d to changes in LAT1 expression and should be attributable to the inhibition of leucine influx by
5 GPNA and/or to the partial depletion of the intracellular amino acid. Consistently, mTORC1 inhibition had been also
6 observed in cells incubated with the system L inhibitor BCH (Ishizuka et al. 2008). However, given that also glutamine
7 activates mTORC1 independently of leucine (Chiu et al. 2012; Jewell et al. 2015), GPNA-dependent inhibition of
8 ASCT2-mediated glutamine influx may also contribute to kinase inhibition.
9

10 In conclusion, this report demonstrates that the ASCT2-inhibitor GPNA also inhibits the influx of essential amino acids
11 through LAT1/2 and that this inhibition, depending on the expression level of LAT1/2, may affect the composition of
12 intracellular amino acid pool and the activity of mTORC1. The demonstration of the metabolic relevance of ASCT2 in a
13 cancer model should, therefore, rely on the genetic suppression of the transporter rather than on GPNA effects.
14
15

16 **Authors' contribution**

17 MC, CS, GT, and MGB performed the experiments. RA performed LC/MS-MS analysis. MC analyzed the data. MC
18 and OB designed the study and wrote the manuscript. NG discussed the results and revised the text. All Authors have
19 approved the final version.
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23 MC is supported by a research fellowship of the University of Parma.
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25

26 **Conflict of Interest**

27 The authors declare that they have no conflict of interest.
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30 **Research involving Human Participants and/or Animals**

31 This article does not contain any study with human participants or animals performed by any of the authors.
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Figure Legends

Fig. 1a,b L-[3,4-³H(N)]-Glutamine influx (25 μM; 5 μCi/mL; transport time, 30 s) was assayed in EBSS (a) or in Na⁺-free EBSS in the absence (Control) or in the presence of L-γ-glutamyl-*p*-nitroanilide (GPNA, 3 mM) in Hs683, HeLa, A549, MCF7 and Huh7 cells. **c** The determination of Na⁺-independent influx of L-[4,,5-³H(N)]-leucine (10 μM; 2 μCi/mL; transport time, 30 s) was performed in the same cells in the absence (Control) or in the presence of GPNA, 3 mM. The saturable influx was obtained subtracting the non-saturable influx (see Methods), determined in parallel under the same conditions, from the total influx. Data are expressed as nmol/mg prot/min. * *p* < 0.05, *** *p* < 0.001 versus control assessed with Student *t* test for unpaired data.

Fig. 2a *SLC7A5*, encoding for LAT1 (left), and *SLC7A8*, encoding for LAT2 (right), expression was assessed by RT-PCR in the indicated cells incubated in growth medium. Data were normalized to the expression of RPL-15. **b** LAT1 protein expression was assessed by Western Blot in Hs683, HeLa, A549, MCF7 and Huh7 cells. β-actin was used for loading control. A representative experiment, performed twice with comparable results, is shown.

Fig. 3a Kinetic analysis of Na⁺-independent leucine uptake by Hs683 cells. Cells were incubated for 30 s in Na⁺-free EBSS with L-[3,4-³H(N)]-Leu (2 μCi/mL; 1, 5, 10, 25, 50, 500 μM, left panel) in the absence (Control) or in the presence of 3 mM GPNA. Grey lines represent the saturable component of leucine influx for Control (dashed line) and GPNA (dotted line). Lines represent the best fit to Eq. 1 (*R*² 0.999 for control, 0.994 for GPNA inhibited influx). **b** Analysis of Leu uptake inhibition by GPNA. Cells were incubated for 30 s in Na⁺-free EBSS with L-[3,4-³H(N)]-Leu (2 μCi/mL; 10 μM) in the absence or in the presence of GPNA (0.3, 1, 3 mM). The line represents the best fit to Eq. 2 (*R*² 0.980). **c** Inhibition of Na⁺-independent leucine (10 μM) uptake by GPNA (3 mM) or BCH (1 mM). For **a-c**, data represent means ± SD of five independent determinations each. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001 vs. control, assessed with Student *t* test for unpaired data.

Fig. 4 The cell content of Leu (**a**), Ile (**b**) and Gln (**c**) in Hs683, HeLa, A549, MCF7 and Huh7 cells incubated in growth medium without (Control) or with 3 mM GPNA for 9 h. Means ± SD of three experiments are shown. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 versus control; ns, not significant, as assessed with Student *t* test for unpaired data.

Fig. 5a Western blot of S6K1 (phosphoT389 and total) and LAT1 in Hs683, HeLa, A549, MCF7 and Huh7 cells incubated in growth medium (Control) or with 3 mM GPNA for 9 h. β-actin was used for loading control. **b** Cells were

1 incubated in the absence or in the presence of rapamycin (100 nm) for 9h. At the end of the incubation, the Western
2 Blot of S6K1 (phosphoT389 and total) was performed. Representative experiments, performed twice with comparable
3 results, are shown.
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8 **Fig. 6** The expression of *SLCIA5* (for ASCT2, **a**) *SLC38A1* (for SNAT1, **e**) and *SLC38A2* mRNA (for SNAT2, **f**) was
9 assessed by RT-PCR in scramble- or anti-ASCT2-siRNA-transfected Hs683 cells 72h after transfection. Data were
10 normalized to the expression of RPL-15. **b** Western blot of ASCT2 in scramble- and anti-ASCT2-siRNA-transfected
11 Hs683 cells. **c** 72h after transfection, L-glutamine uptake (25 μ M, 10 μ Ci/ml) was measured, in the presence and in the
12 absence of sodium, in Hs683 cells transfected with scramble- or anti-ASCT2-siRNA as described under Materials and
13 Methods. **d** 72h after transfection, Hs683 cells, transfected with scramble- or anti-ASCT2-siRNA, were incubated for 9
14 h in fresh growth medium, and intracellular leucine was determined at the end of the incubation. For **a**, **e** and **f**, n = 4;
15 for **c**, n = 5; for **d**, n = 3; for **b**, a representative experiment, performed twice, is shown. * p < 0.05, ns, not significant, as
16 assessed with a Student t test for unpaired data.
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