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SHORT COMMUNICATION

The non-proteinogenic amino acids L-methionine sulfoximine and DL-phosphinothricin activate mTOR

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Abstract L-Methionine sulfoximine (MSO) and DL-Phosphinothricin (PPT), two non-proteinogenic amino acids known as inhibitors of Glutamine Synthetase, cause a dose-dependent increase in the phosphorylation of the mTOR substrate S6 kinase 1. The effect is particularly evident in glutamine-depleted cells, where mTOR activity is very low, but is detectable for PPT also in the presence of glutamine. The stimulation of mTOR activity by either MSO or PPT is strongly synergized by essential amino acids. Thus, the non-proteinogenic amino acids MSO and PPT are mTOR activators.

Keywords Glutamine · Glutamine synthetase · Methionine sulfoximine · Phosphinothricin · mTOR

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Introduction

Integrating signals from nutrients, growth factors, energy and stress, the kinase mTOR is an important controller of protein synthesis and its activity markedly influences cell size and proliferation (Zoncu et al. 2011). mTOR operates through two macromolecular complexes, mTORC1 and mTORC2, the former accounting for mTOR-mediated control of protein synthesis by the phosphorylation of the S6 kinase 1 (S6K1) and the Eukaryotic initiation factor 4E Binding Protein 1 (4EBP1).

Due to mTOR importance in protein synthesis regulation, it is hardly surprising that amino acid availability is required for its activation. However, not all the natural amino acids are equally involved in mTOR regulation, with glutamine (Gln) and essential amino acids, playing the most important roles. In particular, it has been consistently observed that leucine is a powerful mTOR activator and also leucine analogs have been reported to stimulate mTORC1 activity, although with a smaller potency (Lynch et al. 2000, 2002). On the other hand, glutamine appears to exert inhibitory or stimulatory effects in different experimental settings and has been described to counteract leucine-induced mTOR stimulation (Nakajo et al. 2005) or, conversely, to stimulate mTOR through the increase in leucine uptake (Nicklin et al. 2009). Recently, studying the inhibition of mTOR consequent to glutamine depletion in intestinal cells, Boukhettala et al. (2010) have described a seemingly paradoxical effect of a well known inhibitor of Glutamine Synthetase (GS), the amino acid methionine-L-sulfoximine (MSO), which produced an apparent stimulation of mTORC1 activity, while further depleting cell glutamine.

Prompted by these results, we have decided to re-evaluate the effects on mTORC1-activity of MSO and

phosphinothricin (PPT), another non-proteinogenic amino acid that inhibits Glutamine Synthetase.

Materials and methods

Cells, treatments, materials

The hepatocellular carcinoma cell line HepG2 (a gift by prof. Giovanni Raimondo, Department of Internal Medicine, University of Messina, Italy) was grown in low glucose (1 g/l) Dulbecco's modified Eagle's medium (DMEM, cat. n° ECM0749L, Euroclone). This formula contains 1 mM pyruvate. For cell culture, medium was supplemented with 10% fetal bovine serum (FBS, Lonza, Basel, Switzerland), 4 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37°C in an atmosphere of 5% CO_2 in air, pH 7.4. After thawing, cultures were not used for more than 9 passages.

For the experiments, cells were seeded on 6-well plates at a density of 2×10^5 cells/cm^2 in normal growth medium. For glutamine depletion, cells were rapidly washed with Earle's Balanced Salt Solution (EBSS, composition in mM: NaCl 116, KCl 5.3, $CaCl_2$ 1.8, $MgSO_4 \cdot 7H_2O$ 0.81, $NaH_2PO_4 \cdot H_2O$ 0.9, $NaHCO_3$ 26, glucose 5.5, and supplemented with 0.02% Phenol Red) and then incubated in DMEM without FBS and Gln for 6 h. Alternatively, in the experiment shown in Fig. 2, cells were washed and incubated for 6 h in EBSS without FBS. Essential Amino Acids (EAA) were added (1x) from the 50× MEM Essential Amino Acid solution (Invitrogen cat. N° 11130-036) while L-Methionine sulfoximine (MSO, Sigma cat. n° M5379) and DL-Phosphinothricin (PPT, Glufosinate ammonium, Sigma cat. n° 45520) supplementations were obtained from 200 mM stock solutions in H_2O. Sigma (Milan, Italy) was the source of all the others chemicals, whenever not indicated otherwise.

Western Blot

Cells were lysed in a buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 2 mM imidazole and a cocktail of protease inhibitors (Complete, Mini, EDTA-free, Roche). Lysates were transferred in Eppendorf tubes, sonicated for 5 s and centrifuged at 12,000*g* for 10 min at 4°C. After quantification with the Bio-Rad protein assay, aliquots of 30 μg of proteins were mixed with Laemmli buffer 4× (250 mM Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, and 0.4 M DTT), warmed at 95°C for 5 min and loaded on a 10% gel for SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (Millipore—Immobilon-P). Non-specific binding sites were blocked with an incubation of 2 h at room temperature in 5% non-fat dried milk (AppliChem GmbH, Germany) in TBS-Tween. The blots were then exposed at 4°C overnight to anti-total p70S6K or anti-phospho-p70S6K (T389, T421/S424) antibodies (rabbit polyclonal, 1:1000, Cell Signaling Technology), diluted in a 5% BSA TBS-Tween solution. After washing, the blots were exposed for 1 h at room temperature to HRP-conjugated anti-rabbit peptide (Cell Signaling Technology) diluted 1:10000 in 2–5% non fat dried milk. Immunoreactivity was visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Cell content of amino acids, protein synthesis and cell number

The intracellular content of amino acids was determined as previously described (Dall'Asta et al. 1999). Briefly, cell monolayers were washed twice with ice-cold PBS, extracted in a 6.6 mM solution of acetic acid in ethanol and processed for protein determination with Lowry method. After lyophilization and reconstitution in LiOH buffer (pH 2.2), the intracellular content of the single amino acids was determined by HPLC analysis with a Biochrom 20 amino acid analyzer (Amersham Pharmacia Biotech), employing a high-resolution column (Bio 20 Peek Lithium) and the physiological fluid chemical kit (Amersham Pharmacia Biotech) for elution. The column effluent was mixed with ninhydrin reagent, passed through the high-temperature reaction coil and read by the photometer unit. The cell content of MSO and PPT was determined in the same extracts after the determination of their retention times with appropriate standards. Data are expressed as nmol/mg of protein.

For protein synthesis, L-[4,5-^{3}H]leucine (10 μCi/ml, Amersham Biosciences) was added to the incubation medium during the last 45 min of incubation. At the end of the incubation, cells were washed with PBS and extracted with a 6.6 mM solution of acetic acid in ethanol. Proteins were suspended in 150 μl of 5% sodium-deoxycholate in 1 N NaOH. While 50 μl were used for the determination of total proteins with Lowry method, scintillation fluid was added to the remaining aliquot and the incorporated radioactivity counted with a scintillation spectrometer (Wallac Microbeta Trilux counter). Data were expressed as CPM/mg prot/min. Cell number was assessed in parallel with a Coulter Z1 particle counter. For protein synthesis and cell number, two-tailed Student's *t* test for unpaired samples was used for statistical analysis.

Results

The effects of MSO and PPT, two non-proteinogenic amino acids widely used as GS inhibitors (see the

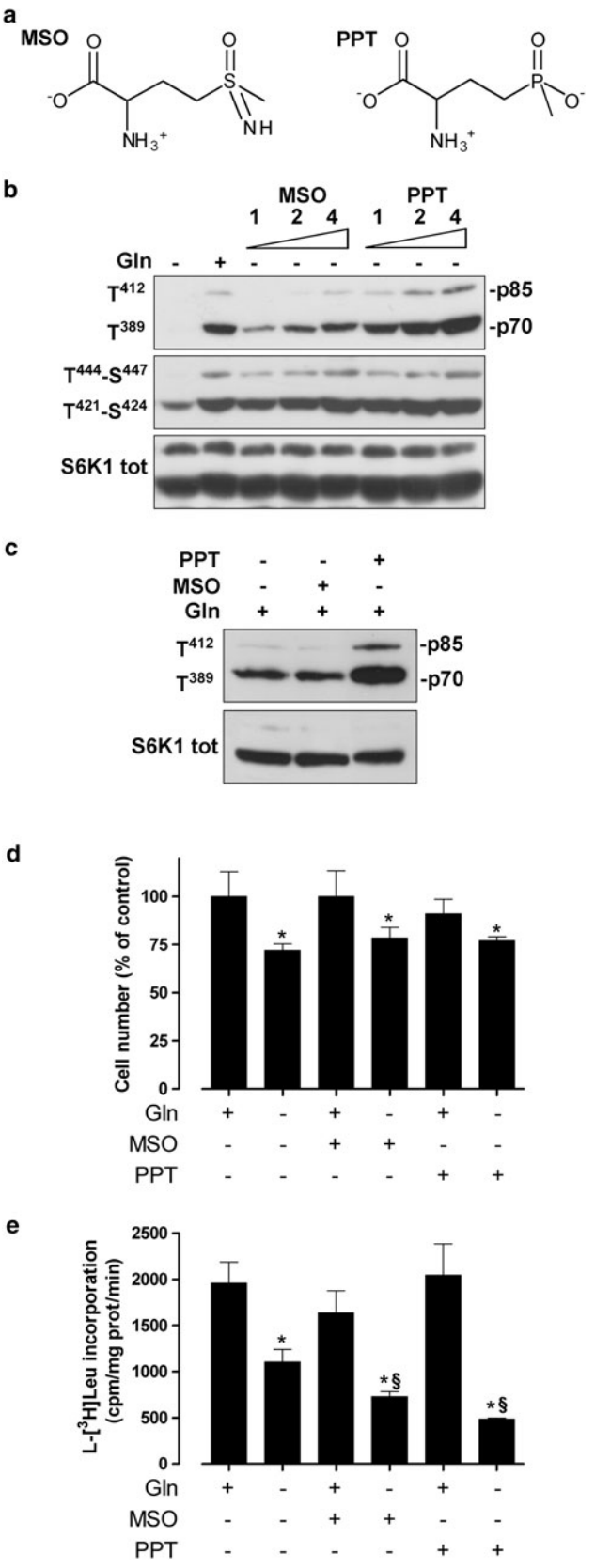

Fig. 1 The effect of MSO and PPT on mTOR activity. **a** The structure of MSO and PPT. **b** Cells were incubated for 6 h in DMEM without Gln in the presence of increasing concentrations (1, 2, 4 mM) of MSO or PPT, as indicated. Control cultures were maintained in the presence of 4 mM Gln (+). At the end of the incubations, the expression of total and phosphorylated S6K1 (both the p85 and the p70 isoforms are shown) was evaluated with Western Blot. **c** Cells were maintained for 6 h in DMEM (FBS absent) with 4 mM Gln in the presence or in the absence of 4 mM MSO or PPT, as indicated. **d, e** Cells were treated as described in **b**, **c**. Cell number (**d**) and the rate of protein synthesis (**e**) were determined at the end of the incubations, as described under "Materials and methods". For **b** and **c**, representative experiments, performed twice with comparable results, are shown. For **d** and **e**, data represent mean ± SD ($n = 3$). $^{*}p < 0.05$ versus control (+ Gln), $^{\S}p < 0.05$ versus cells incubated in the absence of Gln

structures in Fig. 1a), on the phosphorylation of the mTORC1 substrate S6K1 and their functional consequences are shown in Fig. 1b, c, d, e. The phosphorylated forms of S6K1 were markedly lower in HepG2 cells depleted of Gln for 6 h than in the control cells (Fig. 1b). The addition of either MSO or PPT to Gln-depleted cells caused a dose-dependent restoration of mTOR activity, well evident at the T389 phosphorylation site. At equimolar concentrations, PPT stimulated mTOR more than MSO. The abundance of total S6K1 did not significantly change under any experimental condition. When added to complete growth medium ([Gln] = 4 mM) at a concentration of 4 mM, PPT, but not MSO, further increased mTOR activity (Fig. 1c).

The functional consequences of changes in mTOR activity have been assessed evaluating cell number (Fig. 1d) and the rate of protein synthesis (Fig. 1e). In the presence of Gln, MSO and PPT had no significant effects on cell number. Cell number was instead decreased after a 6-hour incubation in the absence of Gln but, also in this case, both MSO and PPT produced no further decrease, as demonstrated also by the microphotographs of the treated cultures (see Electronic Supplementary Material, Fig. S1). Moreover, protein synthesis was significantly hindered by Gln-free incubation but, in this case, MSO and PPT produced further significant decreases of leucine incorporation. On the contrary, neither compound had significant effects on protein synthesis in the presence of Gln.

To assess if mTOR stimulation by MSO or PPT was additive with the effect of essential amino acids, known to be powerful mTOR activators, we performed the experiment reported in Fig. 2. A stringent amino acid depletion of HepG2 cells was accomplished through an incubation in plain amino acid- and serum-free saline solution. Under these conditions, mTOR activity was severely hindered, as demonstrated by the absence of T389 and a very low level of T421/S424 phosphorylation of S6K1, compared to the high levels of p-S6K1 detected in the presence of Gln and EAA (Fig. 2a). When used alone, MSO and, at a lesser

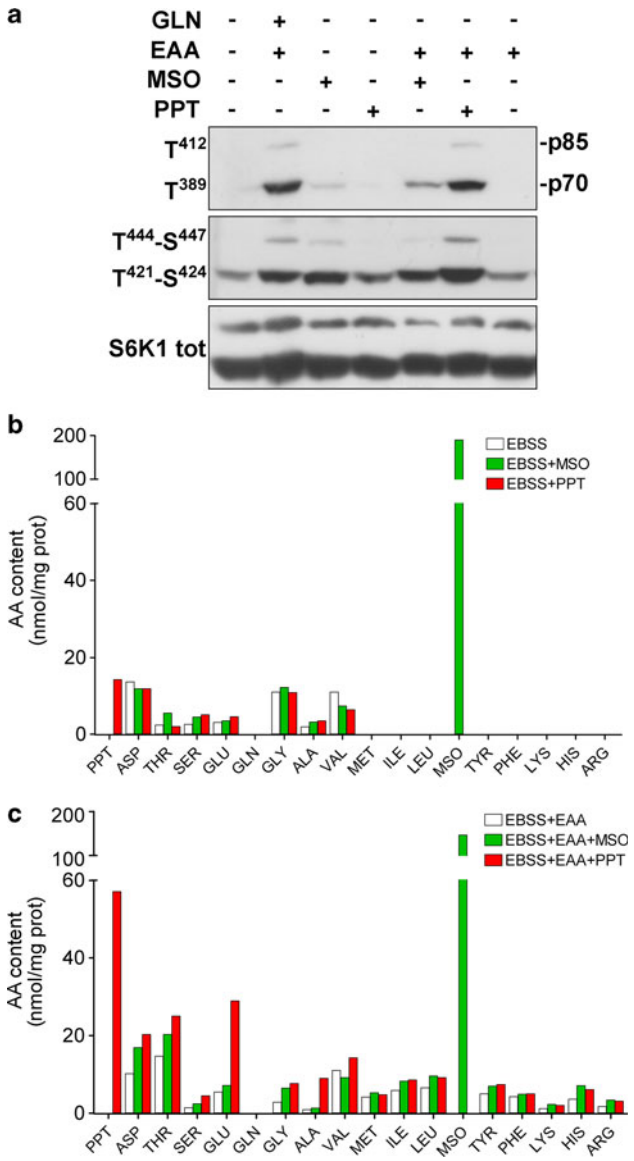

Fig. 2 The interactive effects of Gln, EAA, MSO and PPT on mTOR activity. **a** Cells were incubated for 6 h in plain EBSS in the absence or in the presence of Gln, MSO, PPT (each at 4 mM) and EAA (1×), as indicated. At the end of the incubations, the expression of total and phosphorylated S6K1 was evaluated with Western Blot. **b**, **c** Amino acid contents were evaluated in parallel cultures in the absence (**b**) or in the presence of EAA (**c**). Amino acids are reported in the order of chromatographic elution. A representative experiment, performed twice with comparable results, is shown

degree, PPT produced a detectable increase in the phosphorylation of S6K1, particularly evident at the T421/S424 site. In the presence of EAA, the stimulation of mTOR by MSO and, much more evidently, PPT was further increased, as indicated by the higher levels of T389 p-S6K1. Without Gln, EAA alone produced a very small, if any, activation of mTOR.

Intracellular amino acids, measured in parallel cultures, are shown in Fig. 2b, c. As expected, the incubation in EBSS produced a complete depletion of cell Gln and of most EAA, with the exceptions of Thr and Val. Interestingly, when incubated for 6 h with 4 mM MSO or PPT, HepG2 cells accumulated much more MSO than PPT, both in the presence (Fig. 2c) and, more evidently, in the absence of EAA (Fig. 2b). PPT levels were more than fourfold increased if EAA were present. Conversely, MSO accumulation was lower in the presence of EAA (Fig. 2c) than in their absence (Fig. 2b, see also Electronic Supplementary Materials, Fig. S2). The intracellular content of Gln in cells incubated in EAA + 4 mM Gln was comparable to the intracellular content of MSO in cells incubated in EAA + 4 mM MSO (Electronic Supplementary Material, Fig. S2). While MSO and PPT did not change appreciably the cell content of other amino acids when added to plain EBSS (Fig. 2b), both MSO and, at a greater extent, PPT increased the content of most amino acids in the cells incubated in EBSS supplemented with EAA (Fig. 2c). The change concerned both EAA (in particular, Thr) and non essential amino acids, with Asp, Glu and Ala exhibiting the most marked increases.

Discussion

The results reported in this contribution demonstrate that MSO and PPT, two amino acids known to be inhibitors of Glutamine Synthetase, stimulate the activity of mTOR, evaluated from the phosphorylation of the mTORC1 substrate S6K1. The meaning of the two S6K1 phosphorylation sites assessed (T389 and T421-S424 of the p70 isoform) is different, since T389 is exclusively a substrate of mTORC1, while also CDC2 and several MAP kinases can phosphorylate T421-S424 (Fenton and Gout 2011). Thus, while the increase in the phosphorylated T389 is a reliable indicator of mTORC1 stimulation, the increases observed in the abundance of T421-S424 phosphorylation may indicate the intervention of other kinases. Therefore, the higher relative change in T389 phosphorylation as compared to T421-S424 phosphorylation observed in the cells treated with MSO and PPT (Fig. 1b) is a clear cut indicator of the activation of mTORC1 by the two amino acids.

MSO is a neutral amino acid that derives from methionine, which mimics leucine effects on mTOR (Lynch et al. 2000). However, MSO can be also considered a structural analog of glutamate since it is recognized and phosphorylated by Glutamine Synthetase (GS) resulting in the irreversible enzyme inhibition (Griffith and Meister 1978). The fact that MSO and Gln reach high, comparable intracellular levels suggests that the two compounds share the same high-capacity transport systems for neutral amino acids, such as those of the SLC38 (System A) and SLC1

(System ASC) families (Kanai and Hediger 2004; Mackenzie and Erickson 2004). Recently, Nicklin et al. (2009) have demonstrated that cellular uptake of L-glutamine through ASCT2, encoded by SLC1A5, and its subsequent rapid efflux in the presence of EAA are the rate-limiting steps that activate mTOR. If this mechanism occurs also in the HepG2 cell line used here, then MSO would stimulate mTOR behaving as an analogue of Gln, an hypothesis consistent with the higher levels of EAA detected in cells incubated in the presence of MSO (Fig. 2c).

PPT is an anionic amino acid at physiological pH and can be considered a glutamate analog that is also phosphorylated by GS (Berlicki 2008). Its negative charge on the side chain should hinder the interaction with the concentrative transport systems for neutral amino acids, restraining its entry routes mainly to the low-capacity transport systems for anionic amino acids of the SLC1 (EAAT transporters) and SLC7 (System x_C^-) families (Kanai and Hediger 2004; Verrey et al. 2004). These considerations would justify why, after 6 h of accumulation, the intracellular levels of PPT are far lower than those reached by MSO (Fig. 2b) or Gln (see Electronic Supplementary Material, Fig. S2). However, despite its negative charge, it is clear that PPT is also a substrate of exchange transporters for neutral amino acids, since it is accumulated at an higher level if EAA are present in the extracellular compartment and, conversely, cell levels of EAA are higher if cells are incubated in the presence of PPT (Fig. 2c). These results would point to the involvement of a transport mechanism able to exchange neutral and anionic amino acids. ASC transporters would be good candidates for this role, since they preferentially interact with neutral amino acids but, at low affinity, can transport also anionic amino acids (Dall'Asta et al. 1983; Kanai and Hediger 2004) and work as sodium-dependent amino acid exchange systems (Bussolati et al. 1992). Interestingly, one of these transporters, ASCT2, encoded by SLC1A5, has been repeatedly implied in mTOR stimulation by amino acids (Fuchs and Bode 2005; Nicklin et al. 2009).

The results presented in this contribution indicate that the stimulation of mTOR, previously described in Gln-depleted intestine cells incubated with MSO (Boukhettala et al. 2010), is not a specific property of this amino acid but is also shared by PPT, another non-proteinogenic amino acid inhibitor of GS. Despite being accumulated at lower levels than MSO, PPT is a more powerful mTOR activator than MSO. Moreover, 4 mM PPT, but not 4 mM MSO, further stimulates mTOR, when added to a complete medium containing 4 mM Gln (Fig. 1c), and 1 mM PPT yields a stimulation comparable to that observed with 4 mM Gln (Fig. 1b). The increase in mTOR activity observed in the cells treated with PPT in the presence of Gln seems to have no significant consequences on the rate of protein synthesis (Fig. 1e). On the contrary, in Gln-starved cells the rate of protein synthesis is significantly lowered by both MSO and PPT, a result that may be referred either to the complete glutamine depletion obtained through GS inhibition or to the suppression of adaptive mechanisms, such as autophagy, triggered by mTOR inhibition.

Several mechanisms may underlie mTOR stimulation by MSO and PPT. First, since both compounds inhibit GS, their effect on mTOR may be caused by complete Gln depletion, as previously suggested for MSO (Boukhettala et al. 2010). However, PPT is able to stimulate mTOR even in the presence of 4 mM Gln (Fig. 1c), indicating that the effect does not require Gln depletion. Moreover, given the low K_is of MSO and PPT for GS inhibition [K_i = 105 μM and 25 μM for brain sheep GS, respectively (Eisenberg et al. 2000)], even the lowest dose used (1 mM) should completely suppress GS activity and, indeed, the incubation with 1 mM MSO is sufficient to fully inhibit GS activity in a different human cell model (Rotoli et al. 2005). Nevertheless, with either MSO and PPT the stimulation observed at 4 mM is higher than that observed at 2 and 1 mM (Fig. 1b), pointing to the dissociation between mTOR stimulation and GS inhibition. Therefore, we consider unlikely that MSO and PPT stimulate mTOR through GS inhibition, although a definite conclusion would require the determination of GS activity or the complete silencing of GS expression.

Alternatively, MSO and PPT may stimulate mTOR independently of their inhibitory effect on GS activity. In this case, MSO and PPT may act increasing the intracellular levels of EAA, as indicated by the data reported in Fig. 2b, c, or may interact, possibly with different affinities, with one or more sensors upstream of mTOR, mimicking the effect of the proteinogenic amino acids that physiologically activate the kinase. In either case, the ability of MSO and PPT to stimulate mTOR may prove an useful tool to clarify the mechanisms through which amino acids control the activity of the kinase.

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