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Glycerophosphoinositols inhibit the ability of tumour cells to invade the extracellular matrix

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Abstract

The naturally occurring phosphoinositide metabolite, glycerophosphoinositol 4-phosphate, has recently been shown to induce rearrangements in the actin cytoskeleton through modulation of the small GTPases, Rac and Rho. Since this is directly linked to cell spreading and remodelling, we have evaluated the potential role of glycerophosphoinositol 4-phosphate and related metabolites in tumour cell invasion. The biological effects of these compounds were tested in a number of cellular activities related to cell spreading, including cell migration and cell invasion. We find that unlike other inositol-containing molecules, such as the inositol phosphates, glycerophosphoinositol and glycerophosphoinositol 4-phosphate prevent the invasion of epithelium-derived MDA-MB-231 breast carcinoma and A375MM melanoma cell lines through the extracellular matrix; this is due to a decreased ability to degrade matrix components. These data identify a specific activity of the glycerophosphoinositols that can be exploited for their development as novel anti-invasive drugs.

Keywords: Glycerophosphoinositols; Metastasis; Actin cytoskeleton; MDA-MB-231 cells; A375MM melanoma cells

1. Introduction

Inositol-containing molecules regulate several cell processes, acting either as second messengers or as interactors of lipid-binding modules, thus affecting events ranging from cellular signalling to cytoskeleton organisation and cell motility [1]. Among these compounds, we have focused our attention on the glycerophosphoinositols, naturally occurring water-soluble metabolites that derive from the membrane phosphoinositides by two phospholipase-A-dependent deacylation steps (for a review see [2]). The glycerophosphoinositols are found in virtually all cell types; glycerophosphoinositol (GroPIns), in particular, was originally associated with Ras-transformation, since its level was found to be increased in a number of Ras-transformed cell lines [2–5]. More recently, it has been shown that the endogenous levels of GroPIns cannot be clearly correlated to the invasive potential of a number of transformed cell lines [6]. In several cell types, the formation of the glycerophosphoinositols is modulated by receptors and calcium ionophores [2,3,7], while recent evidence suggests that their catabolism can also be regulated by G-protein-coupled receptors [8].

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When added to cells in culture, the glycerophosphoinositols can rapidly equilibrate across the plasma membrane, allowing for the exogenous control of their cytosolic concentrations [2,5,9]. This feature has been instrumental in determining the cellular functions of these compounds. Indeed, glycerophosphoinositol 4-phosphate (GroPIns4P) inhibits cyclic AMP levels in several cell lines, including epithelial thyroid, smooth muscle and pituitary cells, and pneumocytes [10], Swiss 3T3 fibroblasts and RBL cells [7], and also affects specific functions regulated by cyclic AMP in differentiated FRTL5 thyroid cells and in fibroblasts [10,11]. These GroPIns4P-induced effects have been related to an action on the heterotrimeric Gs protein [10]. Other G-protein-modulated enzymes can also be affected by the glycerophosphoinositols. Indeed, in epithelial thyroid cells, both GroPIns and GroPIns4P partially inhibit G-protein-mediated activation of phospholipase A_2, and thus also the resulting arachidonic acid release, whereas they do not affect Gi-protein-mediated inhibition of adenylyl cyclase [11]. Hence, these results have pointed to a role for the glycerophosphoinositols in the regulation of specific GTPases.

As a consequence, other cellular GTPases have been examined, and it has been shown that in fibroblasts, exogenously added GroPIns4P rapidly activates the Rac and Rho GTPases, and the consequent formation of actin ruffles and stress fibres [12]. These GTPases are known to regulate tumour cell motility and spreading through the control of actin dynamics [13]. Interestingly, all of these effects associated with GroPIns and GroPIns4P are highly specific, as indicated by the lack of activity of other phosphoinositide metabolites that can derive from the cellular processing of these glycerophosphoinositols, such as the inositol phosphates, lysophosphatidylinositol and lysophosphatidic acid [2,12]. In addition, all of the reported effects of the glycerophosphoinositols have been observed at concentrations that are well within the cellular (thus non-toxic) levels of these compounds, which generally vary from the low micromolar (GroPIns4P) to 200–500 μM (GroPIns) range, according to the cell system used [2,6,14].

Cell invasion through the extracellular matrix (ECM) is a complex process that occurs during morphogenesis, differentiation, cell migration, apoptosis and tumour invasion. It involves a number of specialised cellular functions, including cell attachment to and interaction with the ECM, degradation and penetration of the ECM, and the ability to actively move towards the target tissue. Hence, since the glycerophosphoinositols can modulate the activity of both small and heterotrimeric G-proteins and actin dynamics, we have analysed the possible role of these compounds in controlling the motility and invasion of metastatic tumour cells.

2. Materials and methods

2.1. Reagents

All chemical reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, USA) except for Triton-X-100 (Fluka, Switzerland). The glycerophosphoinositols were either obtained from Sigma or by a standard deacylation procedure of the parent phosphoinositides (obtained from the Sigma Chemical Co) or donated by Euticals S.p.A.

2.2. Cell culture and transfection

A375MM cells (a highly metastatic variant of the A375 melanoma cell line [15]) were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 nutrient mixture (Life Technologies Italia, S. Giuliano Milanese, Italy). Medium was supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Breast carcinoma MDA-MB-231 cells were cultured in DMEM supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. ECM degradation assay

Fluorescent-matrix-coated coverslips were prepared and the assay carried out as previously described [16,17]. Briefly, thin layers of fluorescein-, rhodamine B- (Sigma, WI, USA) or Alexa 546- (Molecular Probes, OR, USA) conjugated gelatin (Sigma) were placed on coverslips, cross-linked with 0.5% glutaraldehyde for 15 min at 4 °C, and incubated for 3 min at room temperature with 5 mg/ml $NaBH_4$. After a wash and a short 10 min incubation in 70% ethanol, the coverslips were quenched with DMEM/F12 nutrient mixture or DMEM (depending on the cell line) containing 10% FCS for 1 h a 37 °C before cell plating. The cells were then cultured on the gelatin-coated coverslips in the absence or presence of the glycerophosphoinositols, and after incubation, they were fixed and processed for immunofluorescence. All ECM degradation experiments were analysed with a Zeiss LSM 510 laser scanning confocal microscope. Immunofluorescence images were acquired at high confocality (pin-hole = 1 Airy unit) to achieve the thinnest possible optical slices at the substrate–cell interface. The areas of degradation were determined as previously described in [16]. Briefly, we considered 100 random fields (containing at least 50 transfected cells) at a 63× magnification, and the area of each degradation patch was measured using the LSM 510 software together with an electronic spreadsheet. The total area for each condition was then normalised for cell number and expressed as a percentage of the control.

2.4. Immunofluorescence

After treatment, the cells were fixed in 4% paraformaldehyde for 15 min, permeabilised in PBS containing 0.02% saponin, 0.2% bovine serum albumin (BSA) and 50 mM NH_4Cl, incubated with primary antibodies of interest or fluorophore-conjugated phalloidin for 1 h, and then incubated with fluorophore-conjugated secondary antibodies (Molecular Probes) for 45 min. Finally, the coverslips were mounted in the SlowFade (Molecular Probes) antifade reagent.

2.5. Chemotaxis and chemoinvasion

Chemotaxis and chemoinvasion assays were carried out as previously described in [18] using modified Boyden chambers (Neuro Probe, Gaithersburg, MD, USA). Briefly, the attractant (serum-free 3T3 fibroblast-conditioned medium) was added to the lower compartment of the chamber and separated from the cells by a gelatin-coated, 8 μm pore-size, polycarbonate polyvinylpyrrolidone-free filter (Neuro Probe). The cells were detached, resuspended in medium containing 1% BSA at 2×10^6 ml^{-1}, and added to the upper compartment in the absence or presence of the glycerophosphoinositols. After a 4-h incubation at 37 °C, the filters were processed for staining with Diff-Quick (Dade Behring, Düdingen, Switzerland). Migrated cells adhering to the lower face of the filter were counted in 10 separate fields. The chemoinvasion assay was conducted in the same way, except that the upper side of the filters was coated with a layer of Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) (25 μg/filter) and the incubation time was 6 h.

2.6. Actin cytoskeleton analysis

Cells were cultured overnight on either glass coverslips or gelatin-coated coverslips (as described above) at 37 °C. Before treatment, the cells were maintained for a further 24 h in serum-starvation medium (DMEM containing 0.1% BSA). To visualise filamentous actin, the cells were fixed and permeabilised as described above, and incubated with 0.1 mg/ml rhodamine-labelled phalloidin for 40 min. Finally, the coverslips were mounted in Mowiol and analysed with a Zeiss Axiophot microscope (100× magnification). In some experiments, fluorescence was acquired by wide-field fluorescence microscopy on a Delta Vision Restoration Microscope System (Delta Vision, Oregon, USA). To remove the blurring caused by the objective lens and reduce the background caused by fluorescence from out-of-focus regions, the images were subjected to mathematical deconvolution with an acquired point spread function (Delta Vision).

Double-blind scoring for ruffle and stress fibre formation of more than 100 cells from at least two independent experiments was carried out as previously described in [12]. Briefly, a score was assigned to each individual cell, depending on the level of response, ranging from zero (absence of the feature) to two (full response).

3. Results

The ability of the glycerophosphoinositols to modulate the invasive potential of the breast carcinoma cell line MDA-MB-231 (hereafter referred to as MDA) was evaluated in a modified Boyden chamber chemoinvasion assay (see Section 2). Treatment with GroPIns or GroPIns4P at the maximal effective dose known to activate the Rho GTPases (50 μM) inhibited the migration of MDA cells through the ECM (48% and 50% inhibition, respectively; Fig. 1(a)). As a negative control, the bis-phosphorylated form of GroPIns, GroPIns 4,5-bisphosphate, was ineffective in all of the assays (Fig. 1(a)).

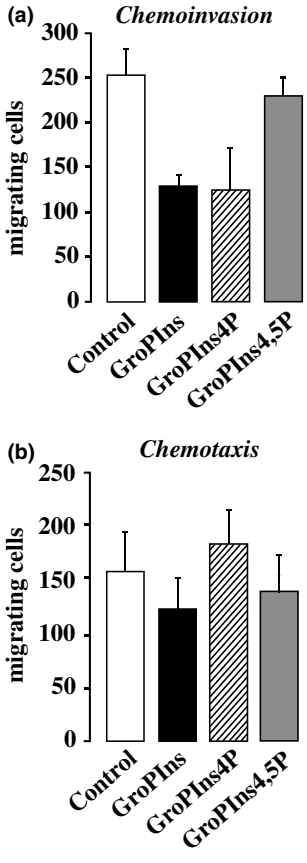

Fig. 1. Effects of GroPIns and GroPIns4P on cell invasion and migration. MDA-MB-231 cells were seeded in modified Boyden chambers and treated without or with 50 μM GroPIns or GroPIns4P for 6 h in the chemoinvasion (a) and for 4 h in the chemotaxis (b) assays. The data presented are means ± standard deviation (SD) of at least three independent experiments, each performed in triplicate.

Cell invasion through the ECM is a multistep process that involves a number of specialised cellular functions, among which there are cell interactions with and degradation of the ECM and cell motility. To determine which of these steps is affected by GroPIns and GroPIns4P, we performed assays designed to discriminate between ECM degradation and cell motility.

The ability of MDA cells to move up a chemotactic gradient was assayed in a modified Boyden chamber chemotaxis assay, using serum-free 3T3-cell-conditioned medium as a chemoattractant. Under these conditions, GroPIns and GroPIns4P did not significantly affect the migration of MDA cells (Fig. 1(b)). This suggests that the inhibition in the chemoinvasion assay was not due to reduced cell motility.

Next, we examined the effects of the different glycerophosphoinositols on MDA cell ECM degradation. This requires the direct participation of released and exposed

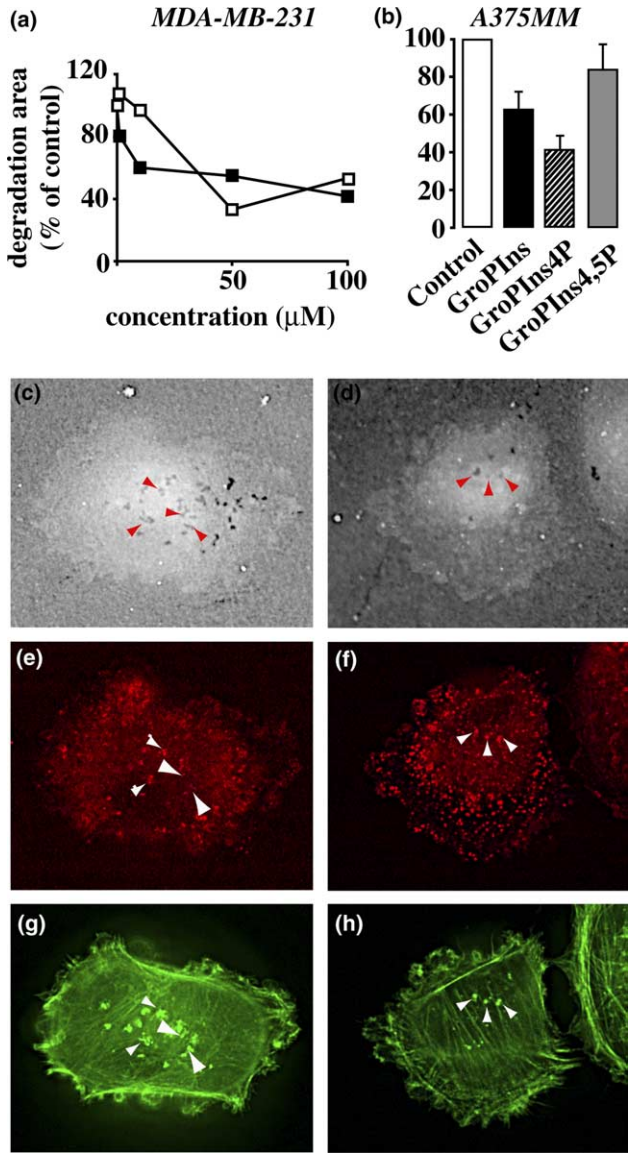

Fig. 2. Effects of GroPIns and GroPIns4P on degradation of the ECM and on the organisation of degradation structures. ECM degradation: MDA-MB-231 (a) or A375MM (b) cells were plated onto fluorophore-conjugated gelatin for 15 h in the absence or presence of GroPIns (black squares) or GroPIns4P (white squares), fixed, stained with Alexa Fluor® 488-phalloidin, and analysed using the confocal microscope. For MDA-MB-231 cells, increasing concentrations of both compounds were tested ranging from 1 to 100 μM, whereas A375MM were incubated in the presence of 50 μM GroPIns or GroPIns4P. In both cases, the percentages of the areas of degradation were quantified (see Section 2). The data presented are means ± SD of at least three independent experiments analysing at least 50 cells/experiment. Matrix degradation structures: MDA-MB-231 cells were plated onto cross-linked fluorophore-conjugated gelatin and incubated for 3 h in the presence of 50 μM GroPIns or GroPIns4P, and then fixed, stained with an anti-cortactin antibody and Alexa Fluor® 488-phalloidin, and analysed on the Delta Vision restoration microscope system. Control cells were similarly processed, after incubation in the absence of GroPIns or GroPIns4P. The panels show representative cells for control (c, e and g) and treated (d, f and h) samples. Fluorescent gelatin degradation patches (c and d, arrows) clearly co-localized with both cortactin (e and f) and actin (g and h), without or with GroPIns or GroPIns4P treatment. Images are representative of three independent experiments.

proteases, among which there are the matrix metalloproteases (MMPs, [19]). We thus performed an assay based on the degradation of a cross-linked fluorophore-conjugated gelatin matrix as a model for the ECM [16,17]. This gelatin layer is quite stable, and is insoluble and resistant to bland proteolysis, such that only invasive protease-secreting cells are able to attack it, causing the formation of dark (i.e. non-fluorescent) areas of degradation that can be counted and quantified. In a typical experiment, up to 60% of all of the cells efficiently degraded this matrix. In doing so, they displayed a characteristic pattern of degradation, which consisted of clusters of degradation patches that depended on the times of incubation. Typically, cells feature a strong co-localisation of actin and cortactin at sites of degradation. With increasing incubation times, the degradation patches tend to become larger, coalescing as degradation progresses. Degradation of the ECM under these conditions is strictly metalloprotease-dependent, as it is completely inhibited by the broad-range metalloprotease inhibitor, BB94 [18,20], but not by other protease inhibitors [16]. Quantification of the extent and number of degradation events indicated that in MDA cells both GroPIns and GroPIns4P inhibited the total area of degradation of the matrix from 10–20% (1 μM) to 60% (100 μM) (Fig. 2(a)). A possible effect of these compounds on cell adhesion to the substrates can be excluded since the number of adhering cells (relative to the control) was not affected whether the compounds were added before or after plating (data not shown). Taken together, these results indicate that GroPIns and GroPIns4P reduce the ability of the cells to degrade the ECM.

To verify whether these inhibitory effects of GroPIns and GroPIns4P on MDA cell ECM degradation can be extended to other cell models, we also examined A375MM cells, a highly metastatic variant of the human melanoma A375 cell line that we have previously shown to degrade the ECM *in vitro* via MMPs [16]. As seen with the MDA cells, at 50 μM, both GroPIns and GroPIns4P inhibited the A375MM cell matrix degradation (40% and 60%, respectively; Fig. 2(b)).

In addition, the specificity of these effects was verified by testing several free inositol phosphates, the potential products of phospholipase C action on the glycerophosphoinositols; none of these compounds had any effects on ECM degradation, even at a 100 μM concentrations (data not shown), indicating that the inhibition by the glycerophosphoinositols cannot be due to the inositol moiety *per se*, but rather that it requires the whole molecule.

Degradation of the ECM occurs at specialised plasma membrane structures, the invadopodia [21], that are enriched in structural and signalling proteins, including the integrins, actin, the actin-binding proteins, the tyrosine kinases and the MMPs. We investigated whether the glycerophosphoinositols can directly affect invadopodia structure/function. To this end, MDA cells cultured on cross-linked fluorescent gelatin (as described above) were labelled with anti-cortactin antibodies and fluorophore-conjugated phalloidin (to label actin). The distribution of these markers was examined by immuno-fluorescence using a confocal microscope (Fig. 2(c), (e) and (g)). As previously reported in [22], and in agreement with our previous studies with A375MM cells [16], colocalization of actin and cortactin was detected at sites of matrix degradation, consistent with the formation of invadopodial structures. Although treatment with either GroPIns or GroPIns4P decreased the number of degradation events, and, correspondingly, of invadopodial structures, their organisation did not appear to be affected (Fig. 2(d), (f) and (h)). This would imply that once established, invadopodial structures are not affected by these compounds; rather, it is some triggering event that must be inhibited.

Next, we analysed the acute effects of the glycerophosphoinositols on actin cytoskeleton organisation in MDA cells, both under conditions comparable to those reported previously for the effects of GroPIns4P in Swiss 3T3 fibroblasts [12], and under the experimental condi-

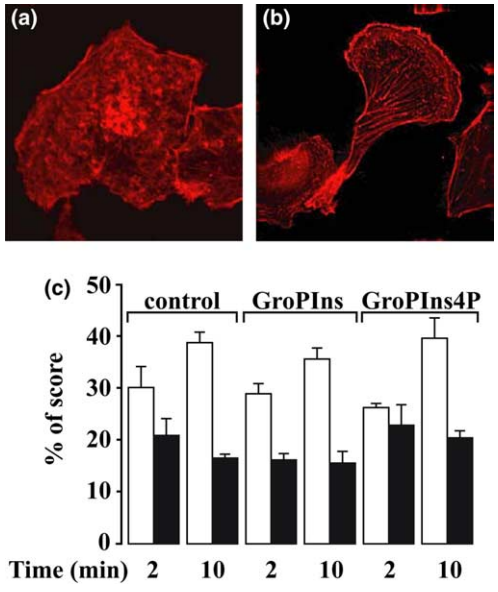

Fig. 3. Actin cytoskeleton organisation in MDA-MB-231 cells. Cells were plated either directly onto glass coverslips (a) or onto gelatin-coated coverslips (b). After serum starvation, the cells were fixed and stained with rhodamine-labelled phalloidin (see Section 2). The cells shown are representative of each condition. (c) Effects of GroPIns and GroPIns4P on the actin cytoskeleton organization of MDA-MB-231 cells. Serum-starved MDA-MB-231 cells plated on gelatin-coated coverslips were treated with either GroPIns or GroPIns4P (50 μM). The cells were fixed and stained with rhodamine-labelled phalloidin and double-blind morphological scoring was performed (see Section 2). The percentage of score represents the score for each morphological feature (ruffles, white bars; stress fibres, grey bars) expressed as a percentage of the potential maximum score (2 × cell number). Data are means ± standard error of the mean (SEM) ($n = 4$) of two independent experiments.

tions of the ECM degradation assay. The actin cytoskeleton of serum-starved MDA cells grown on glass appeared disorganised (Fig. 3(a)), and no effects of 2 or 10 min treatments with either GroPIns and GroPIns4P (50 μM) were detected. When grown on a gelatin matrix (in the absence or presence of serum), however, MDA cells displayed an extremely well-organised actin cytoskeleton, with a high percent (>80%) of cells displaying stress fibres and ruffles (Fig. 3(b)). Even under these conditions, the addition of either GroPIns or GroPIns4P for 2 or 10 min (50 μM) did not affect ruffle or stress fibre formation (Fig. 3(c)). Longer treatment times (3–4 h) also had no effects on the actin cytoskeleton (data not shown). Hence, the inhibition of matrix degradation by the glycerophosphoinositols does not appear to involve a direct action on the actin cytoskeleton. This is in accordance with the lack of effect of the glycerophosphoinositols on cell migration (Fig. 1(b)).

4. Discussion

In this study, we demonstrate that two of the naturally occurring glycerophosphoinositols (the non-phosphorylated and mono-phosphorylated forms: GroPIns and GroPIns4P) inhibit chemoinvasion through the ECM of the epithelium-derived MDA-MB-231 breast carcinoma and A375MM melanoma cell lines. This occurs at concentrations compatible with the physiological levels of these compounds [6], and it is a specific effect of the glycerophosphoinositols that is due to the decreased ability of these cells to degrade matrix components (see Section 3).

It has recently emerged that degradation of the ECM can occur at highly organised and specialised plasma-membrane structures, termed invadopodia [21]. A striking feature of invadopodia is their enrichment in integrins, actin and actin-binding proteins, tyrosine kinase signalling machinery and MMPs. This glycerophosphoinositol-induced inhibition of matrix degradation could be through a number of these regulatory mechanisms that affect the structure and/or the regulation of the ''degrading machinery''. Clearly, focalised delivery/exposure of MMPs is crucial in physiological ECM remodelling events and tumour cell invasive behaviour [23], and the actin cytoskeleton and its regulatory proteins are fundamental regulatory factors. Furthermore, to reach the plasma membrane, these enzymes must be transported and processed by the secretory pathway. However, evidence provided here makes it unlikely that the glycerophosphoinositols exert their inhibitory effects on ECM degradation by modifying the invadopodial structures themselves, or by acting on actin cytoskeleton remodelling. However, the observed decreases in degraded areas (Fig. 2) clearly indicate that the glycerophosphoinositols impair the machinery leading to ECM digestion. Indeed, by monitoring not only the total areas of degradation (Fig. 2), but also by analysing the average areas of the single events (in treated versus untreated cells), we show that GroPIns and GroPIns4P consistently decreased these single-event areas (by approximately 20%). This indicates that the invadopodial machinery regulating focalised degradation is less efficient in the presence of these compounds. We have previously shown that second messengers, signal-transducing enzymes and plasma-membrane receptors have profound and widespread effects on constitutive intracellular transport [24,25]. Hence, a possibility under investigation is that GroPIns and GroPIns4P could affect MMPs transport and targeting to (and/or activation at) sites of degradation. Preliminary results suggest that the transport and activation of MMP2 and membrane type 1 MMP (MT1-MMP) are not affected by GroPIns and GroPIns4P under our experimental conditions (data not shown). An alternative possibility is that the glycerophosphoinositols could act at the level of as yet-unidentified components of the invadopodial structure.

In summary, the information presented in this study demonstrates that the naturally occurring compounds, GroPIns and GroPIns4P, have the potential to interfere with the invasion of breast carcinoma and melanoma cells. Since the glycerophosphoinositols are also water-soluble, of low-molecular-weight and non-toxic (no effects on growth; no cell death) compounds, they are ideal candidates for *in vivo* studies, and thus for development as lead compounds in cancer therapy.

Conflict of interest statement

None declared.

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