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Reanalysis of the involvement of γ-glutamyl transpeptidase in the cell activation process

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Abstract The inhibitor of γ-glutamyl transpeptidase (γ-GT) acivicin modulates cellular responses including growth, myeloid maturation and apoptosis. Whether these effects result from the inhibition of γ-GT enzyme activity remains unclear. We compared the cellular effects of acivicin against a more potent and specific inhibitor of γ-GT (L-2-amino-4-boronobutanoic acid (L-ABBA)) in γ-GT-negative (B lymphoblastoid Ramos) and γ-GT-positive (myelomonocytic HL-60, γ-GT-transfected Ramos) cell lines. Under non-oxidative stress conditions, acivicin-induced cell growth arrest, apoptosis and macrophage maturation occurred independent of γ-GT while L-ABBA did not influence any of these processes. Acivicin triggered tyrosine phosphorylation and increased nuclear factor κB activity. Further insight into the role of γ-GT in cellular processes is needed.

Key words: Acivicin; Apoptosis; Differentiation; Ectoprotease; Proliferation; Signaling

1. Introduction

Cell surface peptidases (ectopeptidases) are transmembrane enzymes present in a wide variety of tissues and cell types [1]. Dysregulated expression of some of them in human diseases triggers research on their value as disease markers and on their role in pathophysiology [1]. In vitro and in vivo use of specific inhibitors of ectopeptidases has revealed that some of these enzymes may influence major cell functions such as metabolic regulation, growth, apoptosis, and motility [1,2]. γ-glutamyl transpeptidase (γ-GT) (EC 2.3.2.2; CD224) is a widespread ectopeptidase that catalyzes the transfer of the γ-glutamyl moiety of γ-glutamyl peptides to other amino acid and peptide acceptors as well as the hydrolytic cleavage of the γ-glutamyl group of donor peptides [3]. Oxidative stress due to elevated amounts of reactive oxygen species (ROS) can lead to damage or destruction of cellular macromolecules such as lipids, proteins, and nucleic acids. Among enzymatic antioxidants capable to prevent ROS formation or scavenge radical species, γ-GT in vivo counteracts oxidative stress by breaking down extracellular glutathione and making its component amino acids available to the cells [4]. In vitro, cell surface γ-GT protects Ramos B cells from oxidation-induced cell death [5].

Acivicin is an irreversible inhibitor of γ-GT commonly used to study its action. Acivicin interacts with the enzyme close to the site at which γ-glutamylation occurs [6]. Acivicin blocks cell proliferation in various cell types including leukocytes, lung, colon, breast, renal and melanoma cells [1,2]. In human myeloid cell lines and blood monocytes, acivicin-mediated cell growth arrest is associated with an induction of maturation toward macrophages [1,2]. Growth arrest by acivicin also correlates with altered production of proinflammatory cytokines (interleukin-1β, TGF-β) [1,2]. Finally, acivicin may induce apoptosis of T and myeloid cell lines [1,7,8]. In phase I/II trials of acivicin infusion in a large variety of solid tumors and acute leukemias, tumor growth stabilization was associated with myelosuppression, neurotoxicity and anemia [1,2]. Therefore, limitations to the use of acivicin in vivo appear to include the toxicity and the low specificity of acivicin which is known to inhibit several glutamine-dependent amidotransferases and enzymes involved in purine and pyrimidine biosynthesis [9]. Therefore, new information on the functions of γ-GT may help unravel the physiological role of this ectoenzyme.

Recently, a potent and selective γ-GT inhibitor, L-2-amino-4-boronobutanoic acid (L-ABBA) [10], was described. In the present study, we used acivicin, L-ABBA and a cell system in which the parental γ-GT-negative B lymphoblastoid Ramos cell line was stably transfected with human γ-GT cDNA [11] to investigate the role of γ-GT in cell activation and apoptosis. We report here that in the absence of oxidative stress, certain effects induced by acivicin in B and myeloid lineage cells (growth, maturation and apoptosis) are independent of γ-GT expression.

2. Materials and methods

2.1. Reagents

L-γ-Glutamyl-*para*-nitroaniline (γ-Glu-pNA), glycylglycine (GlyGly), acivicin ((α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), bisindolylmaleimide, genistein, tyrphostin 25, *ortho*-vanadate, wortmannin and 1-oleyl-*sn*-glycero-3-phosphate were provided by Sigma Chemical Co. (St. Louis, MO, USA). L-ABBA was characterized previously [10]. Phycoerythrin (PE)-conjugated Apo 2.7

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Abbreviations: γ-GT, γ-glutamyl transpeptidase; γ-Glu-pNA, L-γ-glutamyl-*para*-nitroaniline; GlyGly, glycylglycine; FCS, fetal calf serum; pNA, *para*-nitroaniline; L-ABBA, L-2-amino-4-boronobutanoic acid; APN, aminopeptidase N; MAPK, mitogen-activated protein kinase

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(2.7A6A3, mIgG1), PE-conjugated mouse IgG1, goat F(ab′)$_2$ fragment anti-mouse fluorescein-conjugated Ig, monoclonal antibodies (mAbs) specific for CD11b (SP2, mIgG1), CD71 (YDJ.1.2.2, mIgG1) and control isotypes were obtained from Coulter Immunotech (Coultronics, France).

2.2. Cell lines and culture conditions

HL-60, Ramos and γ-GT-transfected Ramos cell lines [11] were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Parsley, UK, LPS levels <0.1 ng/ml), 2 mM L-glutamine, 1 mM sodium pyruvate and 40 μg/ml gentamicin (Flow Laboratories, Rockwell, MD, USA) in a 5% CO_2 humidified atmosphere at 37°C. γ-GT-transfected Ramos cell line was selected in culture medium with 0.25 mg/ml G418 antibiotic [10]. Thereafter, cells ($1–10\times10^5$ cells/ml) were resuspended in fresh medium containing FCS and 10 mM HEPES in tissue culture flasks, and were grown for various periods of time, in the absence or in the presence of different concentrations of acivicin and L-ABBA. After various periods of incubation, cells were collected, washed twice, counted with a Coulter Counter ZM equipped with a Coultronic 256 channelizer and their viability was determined by trypan blue exclusion.

2.3. Assessment of cell proliferation and differentiation

Cell number was assessed using a Coulter Counter ZM equipped with a Coultronic 256 channelizer. To quantify DNA synthesis, the cultures (100 μl) were pulsed for an additional 18 h with 1 μCi/well [^{3}H]methylthymidine (NEN-DuPont de Nemours, Paris, France). After cell harvesting, incorporated radioactivity was measured using a β-scintillation counter. HL-60 cell maturation was determined according to morphologic and phenotypic changes. For cell morphology, cells were resuspended in phosphate-buffered saline (4×10^4 cells/100 μl) and centrifuged at $50\times g$ for 5 min in a cytospin apparatus. Slides were stained with the Hemacolor kit from Merck and subsequent light microscope examination.

2.4. Flow cytometry analysis

Cells were immunostained as previously described [12]. Analysis was performed on a FACS flow cytometer analyzer (Becton Dickinson, Mountain View, CA, USA); 10 000 events were recorded and analyzed using the Lysys software (Becton Dickinson). Fluorescence data were expressed in relative fluorescence intensity (%) and antigen relative density per cell was obtained by substracting the peak channel number of the negative control from the peak channel number of the corresponding experimental sample. Induction of maturation was assayed by measuring CD11b and CD71 antigens [12]. Apoptosis was assessed by measuring the appearance of Apo 2.7 antigen, a mitochondrial membrane protein lately exposed on the surface of cells undergoing programmed cell death [13].

2.5. γ-GT assay

γ-GT activity at the surface of intact cells was assayed as previously described [14] using γ-Glu-pNA as a substrate of γ-GT hydrolytic activity and GlyGly as glutamate acceptor for the transpeptidation reaction. Results were expressed as nmol of pNA formed per 10^5 cells at 37°C.

3. Results

3.1. Effects of acivicin and L-ABBA on γ-GT activity at the surface of HL-60 and γ-GT-transfected Ramos cells

As shown in Fig. 1, HL-60 and γ-GT-transfected Ramos cells expressed a γ-GT activity associated with the surface of cells (Fig. 1A,B). L-ABBA and acivicin induced a dose-dependent inhibitory effect on γ-GT activity (Fig. 1). On both cell lines, L-ABBA was found to be a more potent inhibitor of γ-GT (with IC_{50} 30 nM) than acivicin (with IC_{50} 30 μM). Under identical conditions, the IC_{50} values for the inhibition of purified γ-GT were 45 μM for acivicin and 50 nM for L-ABBA. No γ-Glu-pNA hydrolyzing activity was detected at the surface of the parental Ramos cell line (data not shown).

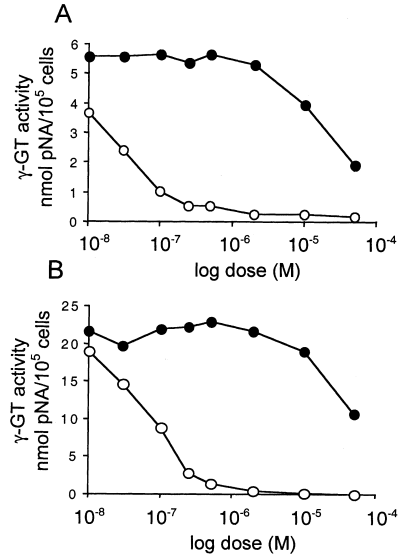

Fig. 1. Inhibition of cell surface γ-GT activity on HL-60 and γ-GT-transfected Ramos cells. Cells were incubated with γ-Glu-pNA and GlyGly in the absence or presence of increasing concentrations ($10^{-8}–10^{-4}$ M) of acivicin (●) or L-ABBA (○) for 60 min at 37°C as described in Section 2. A: HL-60 cells; B: γ-GT-transfected Ramos cells.

3.2. Divergent effects of acivicin and L-ABBA on HL-60 and Ramos cell proliferation

HL-60, parental and γ-GT-transfected Ramos cells were cultured for 3 days in the presence of a range of concentrations (from 10^{-7} to 10^{-4} M) of acivicin or L-ABBA. At day 3, the dose-dependent growth inhibition of two γ-GT-positive cell lines by acivicin (Fig. 2A) was accompanied by an inhibition of their surface γ-GT activity (Fig. 2B). Acivicin also induced a growth arrest in γ-GT-negative Ramos cells (Fig. 2A). The inhibitory growth effect of acivicin was already observed after 1 day of treatment in the same range of concentrations (data not shown). By contrast, L-ABBA, which was a more potent inhibitor of cell surface γ-GT activity than acivicin (Fig. 2D), did not affect the growth of any cell line (Fig. 2C).

3.3. Acivicin, but not L-ABBA, induces macrophage maturation

On day 3 of culture, treatment of HL-60 cells with acivicin (10^{-5} M) resulted in induction of macrophage maturation shown by increased CD11b and CD71 densities (Fig. 3A) and morphologic changes characteristic of macrophages (Fig. 3B, compare a and b). L-ABBA at the same concentration neither affected the levels of CD11b and CD71 nor cell morphology when compared to untreated HL-60 cells (Fig. 3, compare a and c).

3.4. Acivicin promotes apoptosis of γ-GT-negative and γ-GT-positive cells

To assess whether acivicin and L-ABBA are capable of either enhancing or down-regulating apoptosis, we measured the appearance of Apo 2.7 antigen by means of fluorescence flow cytometry. HL-60, parental and transfected γ-GT Ramos cells were cultured for 3 days in the absence or presence of 10^{-5} M inhibitor. As shown in representative cytograms (Fig. 4A, control), Apo 2.7 antigen was expressed on less than 10% of untreated cells. The levels of Apo 2.7 on L-ABBA-treated

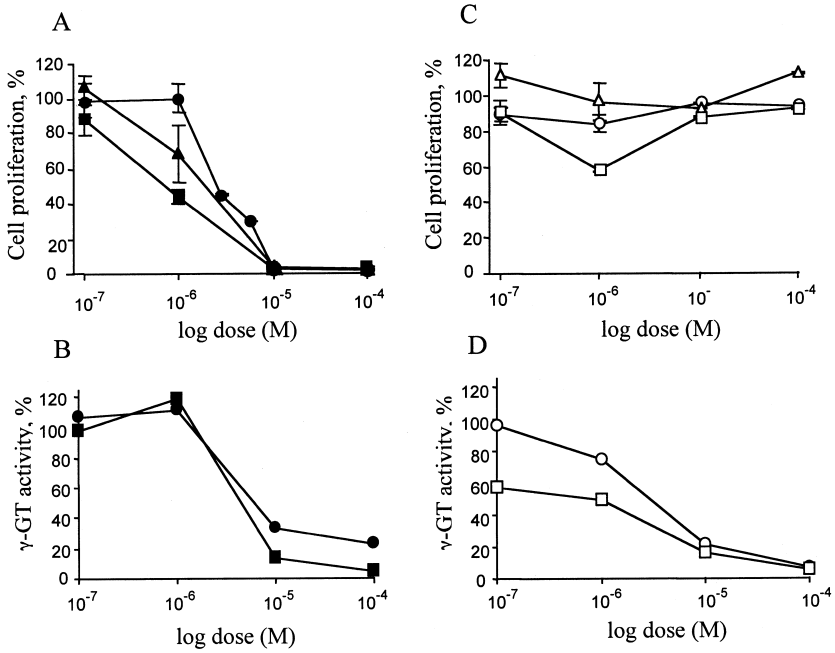

Fig. 2. Dose–response curves of the effect of acivicin or L-ABBA on cell growth and cell surface γ-GT activity. HL-60, parental and γ-GT-transfected Ramos cells were cultured in the absence or presence of increasing concentrations of acivicin (A and B) or L-ABBA (C and D) (from 10^{-7} to 10^{-4} M). At 3 days of incubation, cells were assayed for cell growth (A and C) and cell surface γ-GT (B and D) as described in Section 2. Cell proliferation as determined by [^{3}H]thymidine incorporation is expressed as the percentage of growth of untreated cells, each determination representing the mean of triplicates. γ-GT activity is expressed as the percentage of activity of unstimulated cells. Effects of acivicin (black symbols) or L-ABBA (white symbols) were assayed on HL-60 (●, ○), parental Ramos (▲, △) or γ-GT-transfected Ramos (■, □) cell lines.

cells remained close to that of untreated cells (Fig. 4A, +L-ABBA). In contrast, acivicin induced a marked increase of Apo 2.7 on HL-60 and γ-GT-transfected Ramos cells but also on γ-GT-negative Ramos cells (Fig. 4A, +acivicin). Acivicin consistently induced apoptosis at doses of 10^{-5} M for HL-60 cells (Fig. 4B) as well as for Ramos cells (data not shown).

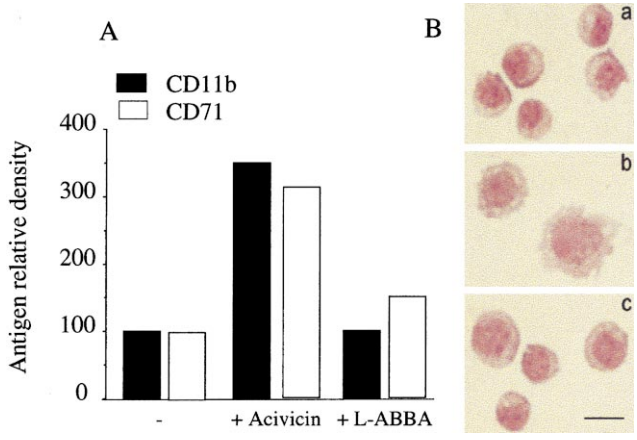

Fig. 3. Effects of acivicin and L-ABBA on morphologic and phenotypic changes of HL-60 cells. HL-60 cells were cultured in the absence or presence of acivicin or L-ABBA (10^{-5} M) for 3 days. A: Cells were assayed for expression and relative densities of CD11b and CD71. B: Light microscopy morphology of unstimulated cells (a) or cells exposed to acivicin (b) or to L-ABBA (c). Hematocolor staining. Scale bar, 10 μM.

3.5. *Inhibitors of tyrosine phosphorylation inhibit acivicin-mediated growth arrest in HL-60 cells*

In an attempt to determine which signaling pathways were involved in the HL-60 growth arrest mediated by acivicin (5×10^{-6} M), we examined the effects of various agents known to alter intracellular signaling (Table 1). Pretreatment of HL-60 cells with the two tyrosine phosphorylation inhibitors genistein or tyrphostin 25 reversed the acivicin-mediated growth arrest (Table 1). In contrast, the other agents were without effect (Table 1). However genistein did not protect HL-60 cells from apoptosis induced by doses of acivicin of 10^{-5} M (data not shown).

4. Discussion

γ-GT is an ectoenzyme expressed in different cell types including hematopoietic cells. Thus far, knowledge about the involvement of γ-GT in cell physiology comes mainly from the inhibition of its catalytic activity by acivicin [1,2]. Acivicin blocks cell proliferation in various cell types including leukocytes, lung, colon, breast, renal, and melanoma cells [1,2]. The inhibitor-mediated cell growth arrest is associated with an induction of maturation of myeloid precursors toward macrophages, and apoptosis of T and myeloid cell lines [1,2]. Acivicin inhibits several glutamine-dependent amidotransferases and enzymes involved in purine and pyrimidine biosynthesis. In the present study, we sought to reevaluate the acivicin-associated apoptosis and the antiproliferative/differentiative action of acivicin. Using γ-GT-negative (Ramos) and γ-GT-positive (HL-60 and γ-GT-transfected Ramos) cell lines as well as a more potent and selective inhibitor of γ-GT (L-ABBA) recently developed [10], we report here that, in the

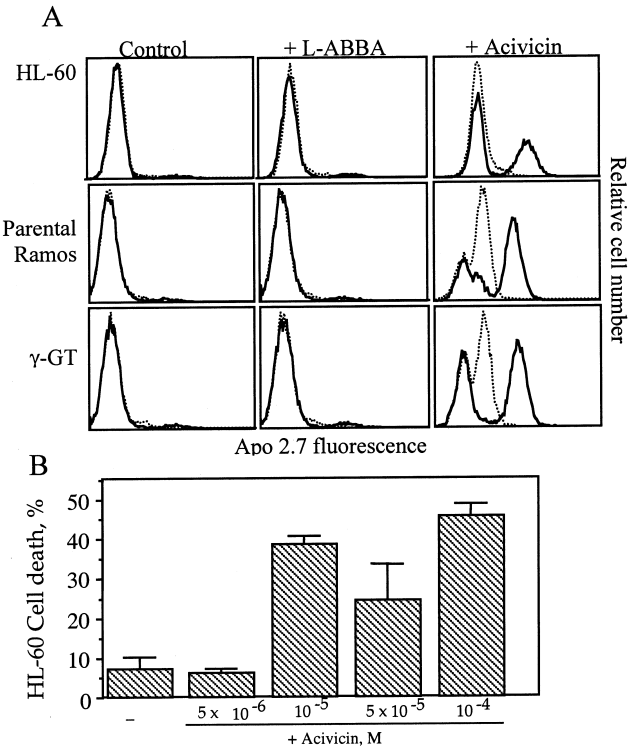

Fig. 4. Effects of acivicin and L-ABBA on the levels of apoptosis in HL-60, parental and γ-GT-transfected Ramos cells. A: HL-60, parental and γ-GT-transfected Ramos cells cultured for 3 days in the absence or presence of acivicin or L-ABBA (10^{-5} M) were assayed for expression of Apo 2.7 as described in Section 2. FACS profiles from a representative experiment are shown. The dotted histograms represent background fluorescence obtained with the isotype control and the solid histograms represent specific fluorescence obtained with Apo 2.7 mAb. B: HL-60 cells cultured for 3 days in the absence or presence of increasing concentrations of acivicin (5×10^{-6}–10^{-4} M) were assayed for apoptosis ($n=8$ for control, $n=4$ for 5×10^{-6} M, $n=3$ for 10^{-5} and 5×10^{-5} M, $n=2$ for 10^{-4} M).

absence of oxidative stress, the induction of growth arrest, differentiation and apoptosis in target cells by acivicin is not mediated through inhibition of γ-GT.

The boronate compound L-ABBA is a potent inhibitor of purified γ-GT [10] designed as a specific transition state analog of the natural substrate. When the inhibitory potency of acivicin and L-ABBA on HL-60 and γ-GT-transfected Ramos γ-GT surface activity was investigated, we observed that L-ABBA was a stronger inhibitor (IC_{50} 30 nM) than acivicin (IC_{50} 30 μM). In the concentration range of 10^{-7}–10^{-4} M, L-ABBA did not exhibit any growth-inhibiting properties on γ-GT-positive as well as γ-GT-negative cell lines. In contrast, acivicin not only inhibited growth of γ-GT-positive cells as previously shown [1,2] but also the γ-GT-negative Ramos cell line.

Comparative experiments with L-ABBA showed that, in addition to cell proliferation, acivicin also affected myeloid differentiation and apoptosis independently of its inhibition of γ-GT. The γ-GT-independent effect of acivicin on apoptosis has recently been observed in V79 Chinese hamster cells transfected with human γ-GT [15]. The molecular mechanisms behind all forms of acivicin-induced growth arrest/apoptosis are not known. Acivicin causes cell cycle arrest due to the inhibition of de novo purine and pyrimidine synthesis. Culture of acivicin-treated cells in nucleoside-supplemented medium bypasses this block in DNA synthesis (D. Karp, unpublished data). In Ramos cells transfected with γ-GT cDNA, short-term incubation with acivicin (10^{-5} M) under conditions of oxidative stress induces apoptosis that can be reversed with thiol supplementation [5]. This is consistent with the role that γ-GT plays in the recapture of cysteine from extracellular glutathione. Other transformed cells are not as sensitive to oxidative stress as Ramos (D. Karp, unpublished data).

Induction and inhibition of growth arrest/apoptosis are both associated with signaling through protein phosphorylation [16–19]. Protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs) (extracellular-regulated kinase (ERK), c-Jun N-terminal kinase and p38) are implicated in the regulation of many cellular processes [16–19]. There is evidence that tyrosine phosphorylation steps are involved in ectopeptidase signaling [1,20–26]. CD26/dipeptidyl peptidase IV inhibitors activate MAPK-p38 and inhibit MEK1 and ERK1/2 in CD3 antibody-mediated activated T cells [1,20,23,25]. Intracellular calcium flux and the MAPK phosphorylation are implicated in the activation of monocytes and T cells through CD13/aminopeptidase N (APN) [1,21,22,24–26]. Monocytic-induced differentiation of HL-60 cells is associated with changes in the patterns of protein phosphorylation [27]. Here, we provided evidence that the acivicin-induced growth arrest signaling pathway in HL-60 cells involves tyrosine phosphorylation. Moreover, cellular stimulation with acivicin coincided with a higher degree of nuclear factor κB (NF-κB) activation (data not shown). NF-κB plays a critical role in proliferation and cell survival and is stimulated by a variety of agents including tumor necrosis factor-α (TNF-α) [28]. However, untreated as well as acivicin-treated Ramos and HL-60 cells produced no detectable amounts of TNF-α (data not

Table 1
Acivicin-mediated growth arrest response of HL-60 cells upon treatment with agents of signaling pathways

Pretreatment	Effect	Residual cell growth (%)		
		Exp 1	Exp 2	Exp 3
None	control	18 ± 3	8 ± 1	24 ± 2
Bisindolylmaleimide	PKC inhibitor	16 ± 3		
1-Oleyl-*sn*-glycero-3-phosphate	activator of G proteins	16 ± 3		18 ± 1
Vanadate	tyrosinephosphatase inhibitor	17 ± 1		
Wortmannin	phosphatidylinositol-3-kinase inhibitor	23 ± 2		
Tyrphostin 25	tyrosine kinase inhibitor	74 ± 13		
Genistein	tyrosine kinase inhibitor	102 ± 15	97 ± 6	77 ± 5

Cells were pretreated with different agents (bisindolylmaleimide and 1-oleyl-*sn*-glycero-3-phosphate 4 μM; wortmannin 5 μM; genistein 7.5 μM; tyrphostin 25 and vanadate 10 μM) for 1 day prior to exposure to acivicin (5×10^{-6} M). In the absence of acivicin, the agents were without any toxic effect on growth. After 2 days, cell proliferation was determined by [^{3}H]thymidine incorporation. Results are expressed as the percentage of growth of untreated cells (± S.D., $n=3$).

shown) excluding its participation in the effects produced by acivicin. Details of the mechanisms of acivicin-induced cell activation are currently under investigation.

In conclusion, growth inhibition, monocyte maturation and apoptosis induced by acivicin can be mediated by mechanisms other than inhibition of γ-GT. Other investigators have recently shown that the antiproliferative effects of actinonin, an inhibitor of the ectopeptidase CD13/APN involved in tumor invasion [24], are independent of CD13/APN [29]. L-ABBA was a potent inhibitor of γ-GT, yet did not cause growth arrest or apoptosis in our cellular models. Thus, γ-GT activity is dispensable for cells cultured with typical levels of sulfur-containing amino acids for the synthesis of glutathione, and without additional oxidative stress to deplete intracellular glutathione stores.

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