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Brasilicardin A, a Natural Immunosuppressant, Targets Amino Acid Transport System L

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Summary

Lymphocytes in T cell activation require extracellular nutrients to provide energy for cellular proliferation and effector functions. Therefore, inhibitors of nutrient transporters are expected to be a new class of immunosuppressant. Here, we report that the molecular target of brasilicardin A (BraA), an immunosuppressive compound, is the amino acid transporter system L. BraA inhibited the cell-cycle progression of murine T cell lymphocyte CTLL-2 cells in G1 phase, and potently inhibited the uptake of amino acids that are substrates for amino acid transport system L. Moreover, BraA stimulated the GCN2 activation and, subsequently, the phosphorylation of eIF2α. These results suggest that the immunosuppressive activity of BraA is induced by amino acid deprivation via the inhibition of system L and that the amino acid transporter is a target for immunosuppressant.

Introduction

The use of cyclosporin and FK-506 has improved graft survival rates in cases of organ transplantation. It is well known that the immunosuppressive effects of both drugs depend on the inhibition of calcineurin [1]. However, these beneficial effects of immunosuppression have remained limited, because both drugs can induce nephrotoxicity and arterial hypertension [2, 3]. These side effects are also dependent on the inhibition of calcineurin. Rapamycin, another immunosuppressive drug, targets a different stage of the immune response from that of cyclosporin and FK-506 [4]. Studies of its mechanism of action have led to the discovery of the mTOR pathway, an evolutionarily conserved signaling network that plays critical roles in eukaryotic cell growth and cell-cycle progression [5]. mTOR participates in nutrient-sensitive signaling, and rapamycin mimics a signal generated by amino acid starvation. Interestingly, lymphocytes are more dependent than other cell types on amino acids. Glutamine is required for biosynthesis and energy production for lymphocytes, and an inadequate concentration of glutamine compromises lymphocyte proliferation [6, 7]. Cells of the innate immune system can also control the T cell response by regulating the extracellular concentration of tryptophan [8, 9]. Recently, the monocarboxylate transporter, MCT1, was identified as the target molecule of another type of immunosuppressive compound, the pyrrolo[3,4-*d*]pyrimidine-2,4-diones derivatives [10, 11]. Studies of these compounds have shown that they inhibited increases in the rate of glycolysis, which is required for the rapid phase of cell division in T cell activation, via an inhibition of lactate efflux. These results strongly suggest that high energy levels and nutrients are required for T cell activation [12], and inhibitors of nutrient transporters might be a novel type of immunomodulator.

Organic nutrients, such as sugars and amino acids, are provided to cells via transporters situated on the plasma membrane. Amino acid transporters are classified based on their substrate selectivity and Na^+ dependency [13]. The transport of large neutral amino acids with branched or aromatic side chains is mediated by Na^+-independent amino acid transport system L [13, 14]. At the molecular level, system L is identical to 4F2 antigen (CD98), which consists of two subunits, 4F2hc/SLC3A2, a type II membrane protein (heavy chain), and a membrane protein LAT1/2 (L-type amino acid transporter 1 or 2; light chain). The 4F2 antigen was originally identified as a cell-surface antigen associated with lymphocyte activation [15, 16]. The 4F2 antigen is involved in a variety of cellular activities, such as cell activation, growth, and adhesion [15–18]. To function as an amino acid transporter, 4F2hc is necessary for the trafficking of the light chain to the plasma membrane, whereas the light chains are thought to determine the transport characteristics. LAT1 requires a free carboxyl and an amino group for an aromatic amino acid to be used as a substrate, and the hydrophobic interaction between the substrate side chain and the substrate binding site of LAT1 appears to be crucial for substrate binding [19].

Brasilicardin A (BraA; Figure 1), a natural product isolated from the cultured broth of *Nocardia brasiliensis* IFM0406, consists of an amino acid moiety and an anti/syn/anti-perhydrophenanthrene skeleton with a sugar moiety (a rhamnose, an *N*-acetylglucosamine [GlcNAc], and 3-hydroxybenzoate) [20, 21]. This compound has been shown to exhibit immunosuppressive activity in a mouse mixed-lymphocyte reaction assay system, with an IC_{50} of 0.057 μg/ml, but it did not exert any inhibitory activity against interleukin (IL) 2 production, suggesting that the inhibition point of BraA might differ from that of other immunosuppressive drugs, such as cyclosporin, FK-506, and rapamycin. Here, we report that BraA arrested cell-cycle progression at G1 phase without inhibiting the IL-2 signaling involving Jak3/STAT5, the Ras-MAK kinase cascade, and the phosphatidylinositol 3-kinase (PI3K) pathway. The present structure-activity relationship (SAR) analysis suggests that BraA disturbs amino acid metabolism.

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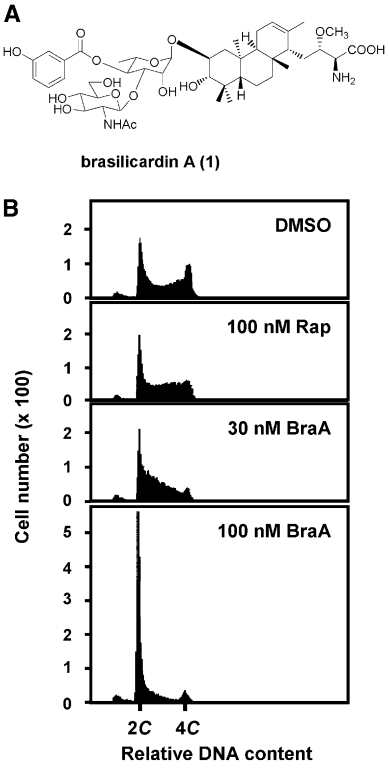

Figure 1. Structure and Effects of BraA on Cell-Cycle Progression

(A) Structure of BraA.

(B) Effects of various drugs on the cell-cycle progression of CTLL-2 cells. Asynchronous CTLL-2 cells were treated with Rap (100 nM) and BraA (30 and 100 nM) for 18 hr.

Ultimately, we characterized BraA as a novel and potent inhibitor of amino acid transporter system L. These results suggest that amino acid transport system L is, therefore, a novel target for immunosuppressants.

Results

BraA Inhibits Cell-Cycle Progression at G1 Phase without Interfering with IL-2 Signaling

BraA (Figure 1A) was isolated as a potent immunosuppressive compound; however, the mechanism of suppression remained unclear. To reveal this inhibition mechanism, we examined the effects of the compound on the cell-cycle progression of CTLL-2 cells, a cytotoxic T cell line dependent on IL-2 for growth. CTLL-2 cells were treated with DMSO, rapamycin, or BraA for 18 hr. Rapamycin showed some accumulation in G1 cells (Figure 1B). On the other hand, BraA inhibited cell-cycle progression at G1 phase in a dose-dependent manner.

Next, we examined whether or not BraA arrests cell-cycle progression at G1 phase by inhibiting IL-2 signaling. The propagation of IL-2-mediated signal transduction occurs following the binding of the ligand to a high-affinity IL-2 receptor complex, and at least three distinct pathways are activated: JAK-STATs, the Ras-MAP kinase cascade, and the PI3K pathway [22–24]. These signaling proteins regulate gene transcription controlling cell growth, differentiation, and immune responsiveness. To test the effects of BraA on IL-2 signaling, we employed an IL-2 deprivation and restimulation assay of CTLL-2 cells. Six hours of IL-2 deprivation resulted in the inactivation of most signaling pathways, based on the phosphorylation levels of STAT5 (Figure 2A), ERK1/2, mTOR, $p70^{S6k}$, and S6 protein (Figure 2B). Jak3 kinase was activated and phosphorylated the Tyr694 of STAT5, a substrate of Jak3, 30 min after the addition of IL-2 (Figure 2A). This phosphorylation was completely abolished by pretreatment with 200 nM Jak inhibitor I [25]. However, the STAT5 phosphorylation levels in the presence of rapamycin (100 nM) or with a high concentration of BraA (3 μM) were similar to those of the DMSO-treated cells, indicating that BraA interferes neither with IL-2 binding to its receptor nor with the subsequent Jak3 kinase activation (Figure 2A). Next, we investigated the effects of BraA on both the Ras-MAP kinase cascade and the PI3K pathway (Figure 2B). Activation of the Ras-MAP kinase cascade and the PI3K pathway was detected by the phosphorylation of ERK1/2 and mTOR, respectively. The phosphorylation of $p70^{S6k}$ and its substrate, S6 ribosomal protein, was also investigated, because $p70^{S6k}$ is a meeting point of the Ras-MAP kinase cascade and the PI3K pathway, and $p70^{S6k}$ regulates the efficiency of protein synthesis via the phosphorylation of S6 ribosomal protein. Both ERK1/2 and mTOR were phosphorylated by IL-2 stimulation, irrespective of the presence of rapamycin. However, the phosphorylation of $p70^{S6k}$ and S6 was completely blocked by rapamycin, thus indicating that 100 nM rapamycin inhibited the kinase activity, but not the activation of mTOR. On the other hand, there was either no change, or only a slight decrease in the phosphorylation levels of ERK1/2, mTOR, $p70^{S6k}$, and S6 by treatment with 100 nM BraA. These results suggest that BraA inhibits cell-cycle progression at G1 phase without interfering with IL-2 signaling.

Amino Acid Moiety of BraA Is Important for G1 Arrest of CTLL-2 Cells

To help identify the molecular target of BraA, we next investigated the relationship between cell-cycle arrest activities and BraA structure. The structures and the effects of BraA natural derivatives BraC (**2**) and semisynthetic analogs (**3–7**) on the cell-cycle progression of CTLL-2 cells are shown in Figures 3A and 3B, respectively. All compounds reduced G1 arrest activity, as compared with the results obtained with BraA (Figure 3B; compound **1**, 300 and 100 nM). BraC, a BraA derivative lacking both an *N*-acetylglucosamine (GlcNAc) and a 3-hydroxybenzoate, also accumulated in the G1 cells at a dose of 10 μM, suggesting that a GlcNAc and/or a 3-hydroxybenzoate are important, though not necessary, for cell-cycle inhibition. Compound **3** inhibited cell-cycle progression at G1 phase at concentrations above 1 μM, but did not at 300 nM (Figure 3B, data not shown). However, since compound **3** is a methyl-ester derivative, there remained the possibility that compound **3** was converted to the original compound **1** by cellular esterase, and then exhibited cell-cycle arrest activity. In contrast, all other derivatives containing a modified amino acid moiety (**4–6**) showed no cell-cycle inhibitory activity. Thus, these results suggest that the amino acid moiety of BraA is important for the inhibition of cell-cycle progression.

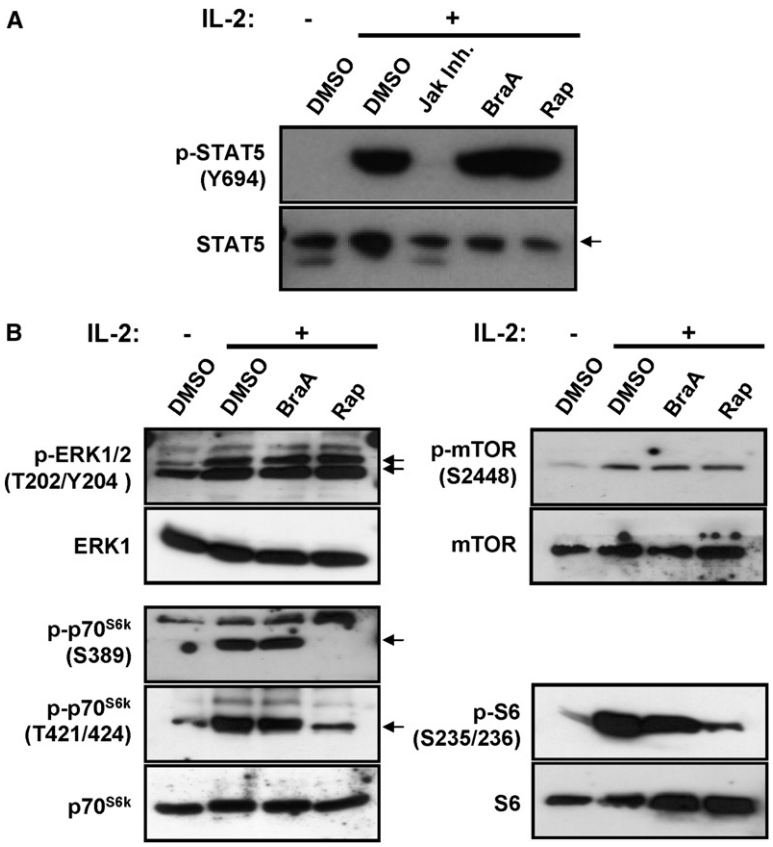

Figure 2. Effects of BraA on IL-2 Signaling

CTLL-2 cells were deprived of IL-2 for 6 hr, and the cells were incubated with various drugs for the final 30 min. IL-2 was added after a 6 hr deprivation period, and incubation was continued for an additional 30 min.

(A) Effects of BraA on the phosphorylation of STAT5 induced by IL-2. IL-2-deprived CTLL-2 cells were treated with Jak kinase inhibitor (Jak inh., 200 nM), BraA (3 mM), and Rap (100 nM) for 30 min.

(B) Effects of BraA on Ras-MAP kinase and PI3K pathways. IL-2-deprived CTLL-2 cells were treated with BraA (100 nM) and rapamycin (Rap, 100 nM) for 30 min.

BraA Is a Novel Inhibitor of Amino Acid Transport

It is well known that lymphocytes are sensitive to amino acid starvation. Cells of the innate immune system can also control the T cell response by regulating the extracellular concentration of tryptophan by indoleamine 2,3-dioxygenase. Since the SAR analysis suggested that the amino acid moiety of BraA is important for cell-cycle inhibition, we suspected that BraA disturbs amino acid metabolism in the cells. Because cycloheximide, a potent protein synthesis inhibitor, strongly inhibited the cell-cycle progression of CTLL-2 cells at G1 phase (data not shown), it was considered possible that BraA is a protein synthesis inhibitor. However, BraA did not exhibit any inhibitory activity against in vitro protein synthesis when reticulocyte lysate was used (data not shown). We therefore speculated that BraA targets the molecule(s) involved in amino acid metabolism upstream of protein synthesis. One of the most attractive explanations for the results is that BraA targets the system for the transport of amino acid(s) required for T cell proliferation. Thus, we subsequently investigated the uptake of several amino acids using HeLa cells and found that 100 nM BraA strongly inhibited the uptake of amino acids containing a large neutral side chain (Ile, Leu, Met, Phe, Trp, and Val), but not that of amino acids containing acidic (Glu), basic (Lys), or small side chains (Gly, Pro, Ser) (Figure 4A). This inhibitory spectrum is identical to the substrate specificity of amino acid transport system L. System L transports several amino acids containing a large neutral side chain in a Na^+-independent manner, and this system is sensitive to 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), a system L-specific inhibitor. As expected, the inhibitory spectrum of BCH (2 mM) was completely identical to that of BraA, which strongly suggested that BraA targets system L. The mode of inhibition of BraA on the uptake of Met was competitive with the K_i of 12.2 nM (Figure 4B). The IC_{50} values of BraA derivatives listed in Table 1 showed a good correlation with the concentrations required for cell-cycle inhibition, with the exception of those for compound **3**. Thus, the present results suggest that system L inhibition by BraA induces G1 arrest.

BraA Induces Integrated Stress Responses In Situ

There are two signaling pathways to protect cells against amino acid starvation: one is the nutrient-sensitive mTOR pathway, and the other is the GCN2 pathway. Thus, we next examined the effects of BraA on the activity of both of these pathways. IL-2-stimulated CTLL-2 cells were cultured in the presence of 100 nM BraA or rapamycin for 18 hr, and the phosphorylation of the substrates of mTOR ($p70^{S6k}$ on Ser389) and GCN2 (eIF2α on Ser51) was investigated. As shown in Figure 5A, phosphorylation on Ser389 of $p70^{S6k}$ was completely inhibited by rapamycin. BraA treatment also reduced the phosphorylation of $p70^{S6k}$, but this decrease was not as marked as that obtained with rapamycin treatment, suggesting that the G1 arrest activity of BraA (Figure 1B) is not dependent on the downregulation of the mTOR pathway. On the other hand, the phosphorylation on Ser51 of eIF2α was stimulated by BraA treatment. It has already been established that there are at least four eIF2α kinases that are activated by distinct

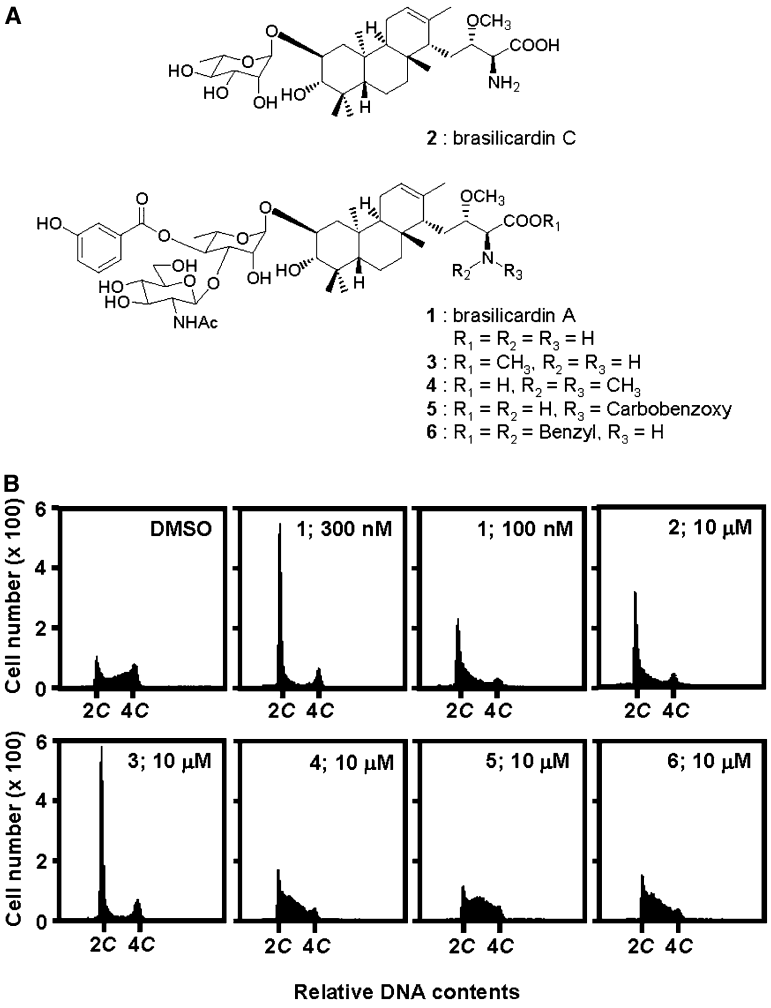

Figure 3. Structure and Effects of BraA Derivatives on Cell-Cycle Progression

(A) Structure of BraA derivatives.

(B) Effects of BraA (1) and its derivatives on the cell-cycle progression of CTLL-2 cells. Asynchronous CTLL-2 cells were treated with BraA derivatives for 18 hr.

upstream stress signals [26]. To confirm GCN2 activation by BraA treatment, we assessed the phosphorylation on The898 of GCN2, which is among the autophosphorylation sites located in the activation loop. It was revealed that GCN2 phosphorylation also occurred with 18 hr of BraA treatment (Figure 5A). Activation of GCN2 and subsequent eIF2α phosphorylation were induced within 1 hr after the addition of BraA (Figure 5B). Finally, we examined the effects of amino acid deprivation on cell-cycle progression. CTLL-2 was cultured in medium lacking the amino acids that are substrates for system L (His, Ile, Leu, Met, Phe, Trp, Tyr, and Val) for 18 hr. As shown in Figure 5C, amino acid deprivation induced cell-cycle arrest at G1 phase, as was also the case with BraA treatment in the presence of amino acids (Figure 1B). These results strongly suggest that BraA evokes integrated stress responses via decreases in the intracellular amino acid pool, and thus inhibits cell-cycle progression at G1 phase.

Discussion

BraA Is a Potent Inhibitor of Amino Acid Transport System L

In the current study, we investigated the effects and molecular target of an immunosuppressive compound, BraA, and demonstrated that BraA is a potent inhibitor of amino acid transport system L. System L transports large neutral amino acids in an Na^+-independent manner and is sensitive to a system L-specific inhibitor, BCH [13, 14]. There are two types of system L: (1) a heterodimeric transporter composed of 4F2 antigen (CD98; heavy chain) and LAT1/2 (light chain); and (2) a monomeric transporter, LAT3/4. A heterodimeric transporter was originally identified as a cell-surface antigen associated with lymphocyte activation [15, 16], suggesting that an extraordinary uptake of amino acids is required for T cell activation.

We revealed that BraA strongly inhibits the uptake of Ile, Leu, Met, Phe, Trp, and Val, but not that of Glu, Lys, Gly, Pro, or Ser (Figure 4A). The inhibitory spectrum of BraA was completely identical to that of BCH and reflected the substrate specificity of amino acid transport system L. Furthermore, the IC_{50} values of BraA and BCH were 14.5 nM and 52.8 μM, respectively, indicating that BraA is approximately 3000-fold more potent an inhibitor than BCH (Table 1). These results clearly indicate that BraA is a novel and potent inhibitor of system L.

Based on the SAR of BraA, it was revealed that: (1) the sugar moiety is important, but not necessary; and (2) a free amino group is necessary for the inhibition of both cell-cycle progression and system L (Figure 3

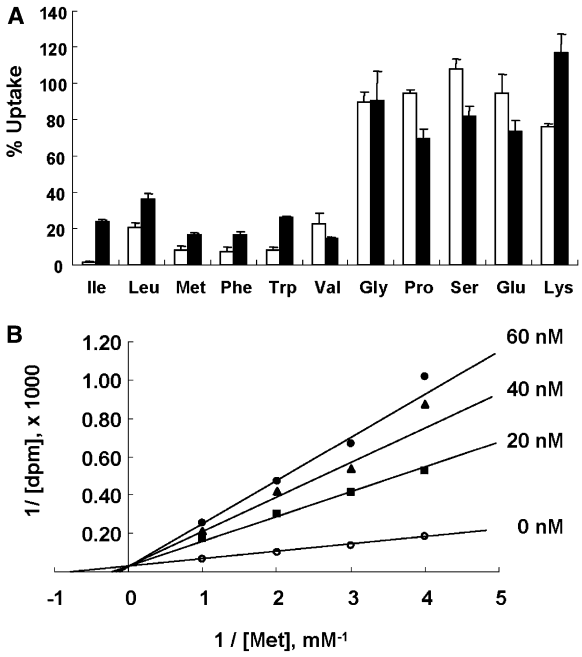

Figure 4. Effects of BraA on the Uptake of Amino Acids

(A) The uptake of tritium-labeled amino acids into HeLa cells was tested in the presence of 100 nM BraA (open bars) or 2 mM BCH (closed bars). Bars represent mean + SD obtained from three independent experiments.

(B) Double reciprocal plot analyses of the inhibitory effects of BraA on [^{3}H]methionine uptake. The uptake of [^{3}H]methionine (250, 333, 500, and 1000 μM) was measured in the presence of BraA (closed squares, 20 nM; closed triangles, 40 nM; closed circles, 60 nM) as well as without BraA (open circles).

and Table 1). It is of potential interest that BraC, a natural BraA derivative lacking both a GlcNAc and a 3-hydroxybenzoate, showed only 2-fold weaker inhibitory activity with respect to system L than did BraA, but its activity was approximately 30-fold weaker in terms of cell-cycle inhibition. Furthermore, BCH could not inhibit the cell-cycle progression even at 2 mM. Although it is difficult to estimate the concentration of amino acids required for cell-cycle progression, we assume that complete inhibition of amino acid transport is required for cell-cycle arrest. The amino acids required to promote the cell cycle might be supplied by the degradation of cellular proteins, a stress response triggered by amino acid deprivation. It also appears to be noteworthy that compound **3**, a methylester derivative of BraA at the carboxylate moiety, showed cell-cycle inhibition, but not system L inhibition (Figure 3B and Table 1). It is possible that compound **3** converted to active compound **1** by cellular esterase, as seen in our previous SAR analysis of reveromycin analogs [27]. The 10 min incubation in our system L assay might not have been sufficient to convert compound **3** to compound **1**; therefore, no inhibition was observed in the amino acid transport assay. The other derivatives modified by an amino acid moiety (**4**–**6**) showed no cell-cycle inhibitory activity, and only weak inhibition was observed against system L (Figure 3B and Table 1). These results suggest that at least a free amino group is necessary for the inhibition of both cell-cycle progression and system L by BraA. The requirement of a free amino group is coincident with the structural features of system L substrates [19]. Taken together with the kinetic data showing that BraA competitively inhibits Met uptake (Figure 4B), these results strongly suggest that the amino acid moiety of BraA binds on the substrate binding site of system L.

Table 1. IC_{50} Values of BraA Derivatives on Met Uptake

Compound	IC_{50} (nM)
1 (BraA)	14.5 ± 1.3
2 (BraC)	25.3 ± 5.0
3	3,500 ± 100
4	1,700 ± 100
5	6,200 ± 300
6	1,800 ± 100
BCH	52,800 ± 400

HeLa cells were preincubated for 5 min in the presence of the drugs, after which the medium was replaced by uptake solution containing tritium-labeled methionine; the cells were then incubated for an additional 15 min. Means ± standard deviation obtained from three independent experiments are shown.

BraA Induces Integrated Stress Responses in CTLL-2 Cells

It should be noted that BraA exerted only a slight influence on IL-2 signaling involved in the Jak3-STAT5, Ras-MAP kinase, and PI3K pathways, and BraA strongly inhibited the cell-cycle progression at G1 phase (Figures 1 and 2). The question remains regarding how the inhibition of amino acid transport by BraA results in cell-cycle arrest at G1 phase. It is assumed that the BraA-treated cells were unable to take up large, neutral amino acids, and that they would undergo amino acid deprivation as a result. It is known that such amino acid-starved conditions lead to the accumulation of uncharged tRNA and then evoke the GCN2 kinase-mediated integrated stress response [28]. GCN2 is the primary sensor of amino acid starvation, and activated GCN2 kinase phosphorylates the eIF2α on Ser51 (Figures 5A and 5B). The phosphorylation of eIF2α converts eIF2 from a substrate to an inhibitor of guanine nucleotide exchange factor eIF2B by stabilizing the eIF2α-GDP-eIF2B interaction. Since eIF2 is present at higher levels than eIF2B, the phosphorylation of only a fraction of eIF2 can lead to significant eIF2B sequestration and a resultant dramatic inhibition of translation. Therefore, we assume that BraA treatment evokes the blockage of protein translation in situ. Although we noted previously here that BraA had no effect on in vitro protein synthesis (data not shown), it is thought that externally supplied amino acids suppressed the integrated stress response system in vitro. Furthermore, not only a reduction in protein synthesis, but also eIF2α phosphorylation plays a pivotal role in cell-cycle arrest at G1 in the case of clorimazole-induced Ca^{2+} deprivation [29] and of tunicamycin-induced ER stress [30, 31]. Recent evidence has indicated that ER stress-induced G1 arrest resulted from the specific loss of cyclin D1 protein via an inhibition of the translation of cyclin D1 [32]. The results of previous reports, when taken together with our findings, strongly

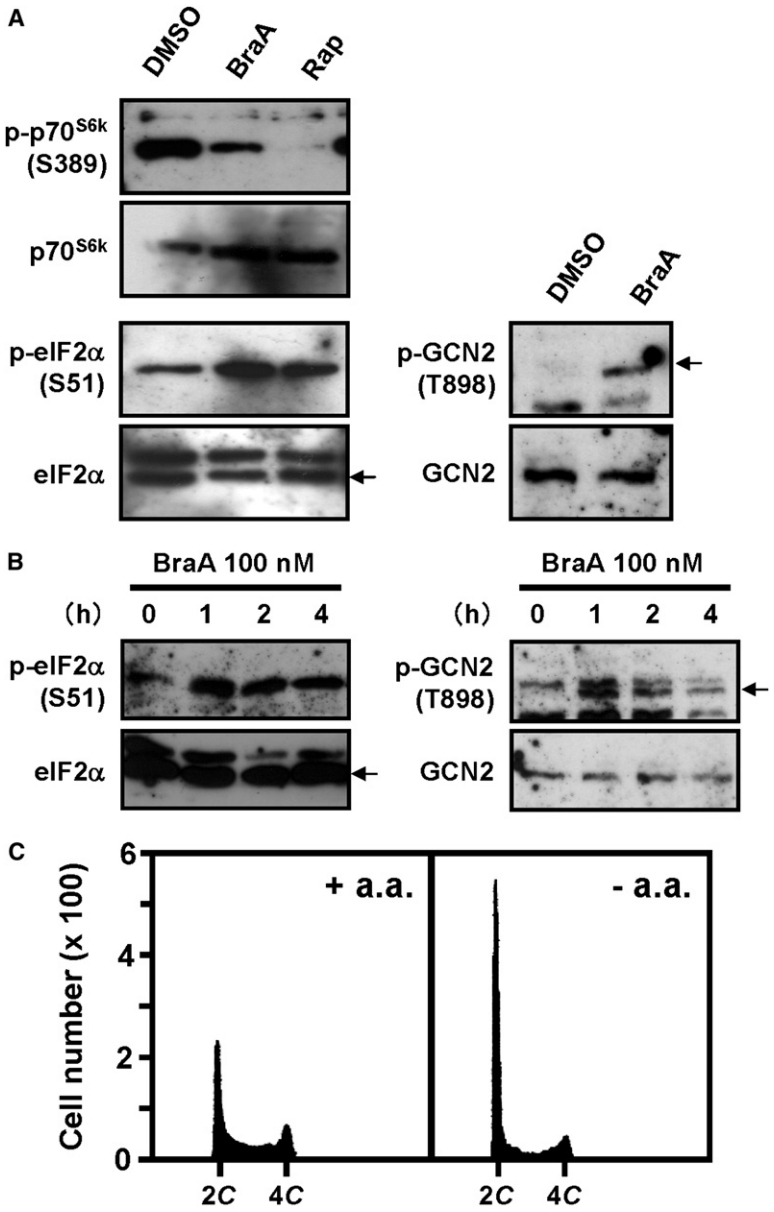

Figure 5. BraA and Amino Acid Deprivation Induce Integrated Stress Responses

(A) Effects of BraA on the phosphorylation of eIF2α, p70^{S6k}, and GCN2. CTLL-2 cells cultivated in the presence of IL-2 were treated with BraA (100 nM) and rapamycin (Rap, 100 nM) for 18 hr.

(B) The phosphorylation of GCN2 and eIF2α was rapidly induced by BraA treatment. CTLL-2 cells cultivated in the presence of IL-2 were treated with 100 nM BraA for 0, 1, 2, and 4 hr.

(C) Amino acid deprivation inhibited the cell-cycle progression at G1 phase. CTLL-2 cells were cultivated for 18 hr in medium containing IL-2 but lacking His, Ile, Leu, Met, Phe, Trp, Tyr, and Val.

suggest that eIF2α phosphorylation induced by BraA treatment is responsible for G1 arrest.

Immunosuppressive and Antitumor Activity of BraA

Previously, we reported that BraA exhibits immunosuppressive activity in a mouse mixed-lymphocyte reaction assay [20, 21, 33, 34]; we have also reported cytotoxic activity against several tumor cell lines in vitro [34, 35]. Both activated lymphocytes and tumor cells require that metabolic substances be provided from the extracellular environment for the support of their extraordinary proliferation. To acquire amino acids, it has been reported that tumor cells highly express the system L component 4F2hc/LAT1 heterodimer [13, 15, 16]. Therefore, inhibitors of nutrient transporters might provide a novel class of immunosuppressants or antitumor agents. In this context, BraA appears to be a good candidate agent. These results suggest that amino acid transporters are novel targets for immunosuppressive and antitumor therapies.

Significance

Both activated lymphocytes and tumor cells require metabolic substances, such as sugars and amino acids, for the support of their extraordinary proliferation. Since organic nutrients are provided to cells via transporters situated on the plasma membrane, inhibitors of nutrient transporters might provide a novel class of immunosuppressants or antitumor agents. The result that a natural immunosuppressive and antitumor product, BraA, targets the amino acid transporter system L is in accord with this idea. Inhibition of amino acid uptake by BraA decreases the amino acid pool within the cells and causes the GCN2-dependent integrated stress responses. Activation of

integrated stress responses results in the inhibition or retardation of cell proliferation. Because immunosuppression by tryptophan deprivation is suggested to be a function of physiological immunoregulation, these results suggest that amino acid transporters are novel targets for immunosuppressant.

Experimental Procedures

Materials

BraA and its related compounds were purified as described previously [20, 33, 34]. Unless otherwise specified, all other materials used were obtained from Sigma (Kent, UK). Jak inhibitor I [25] was purchased from Merck Biosciences (Darmstadt, Germany). Anti-ERK1, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-Stat5, and anti-phospho-Stat5 (Tyr694) antibodies were obtained from BD Biosciences (Franklin Lakes, NJ). Anti-mTOR, anti-phospho-mTOR (Ser2448), anti-p70 S6 kinase, anti-phospho-p70 S6 kinase (Thr389), anti-phospho-p70 S6 kinase (Thr421/Ser424), anti-S6 ribosomal protein, anti-phospho-S6 ribosomal protein (Ser235/236), anti-eIF2α, and anti-phospho-eIF2α (Ser51) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Tritium-labeled amino acids were obtained from GE Healthcare Bio-Science Corporation (Piscataway, NJ). Recombinant human IL-2 was prepared as described previously [36], with the expression plasmid kindly provided by Dr. Fukushima at the Sasaki Institute.

Cell Culture and Flow Cytometry

A mouse T cell lymphoma cell line, CTLL-2, was obtained from RIKEN Cell Bank (Ibaraki, Japan) and was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 100 U/ml of rhIL-2 at 37°C with 5% CO_2. HeLa cells were cultured in DMEM containing 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 mM sodium pyruvate. Flow cytometry was performed as described previously [37].

Immunoblot Analysis

Cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris-HCl [pH7.4], 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4, and 1% Triton X-100), containing protease inhibitors (Complete; Roche Applied Science, Indianapolis, IN), by brief sonication on ice. The total soluble proteins were obtained by centrifugation (20,000 × g, 10 min, 4°C), and the proteins were separated by 10% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Billerica, MA), and were blocked with 5% nonfat skim milk. All immunoblots used the aforementioned antibodies, diluted 1/1000 in Can Get Signal (Toyobo, Osaka, Japan). The protein bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Measurement of Amino Acid Uptake

The measurement of amino acid uptake was performed as previously described [38], with slight modifications. Briefly, HeLa cells were seeded on 24-well plates (1×10^5 cells/well) in fresh growth medium. After cultivation for 24 hr, the cells were washed three times with standard uptake buffer (125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 1.2 mM KH_2PO_4, and 5.6 mM glucose [pH 7.4]), and were preincubated for 5 min in the presence of drugs. The medium was then replaced by uptake solution containing tritium-labeled amino acids. Uptake was terminated by removal of the uptake solution followed by washing the cells three times with ice-cold uptake solution; cells were then solubilized with 0.25 N NaOH. Radioactivity was measured by a liquid scintillation counter (LS6500 Multi-Purpose Scintillation Counter; Beckman Coulter). For the measurement of the uptake of [^{3}H]amino acids, three wells of HeLa cells were used for each data point. To confirm the reproducibility of the results, three or four separate experiments were performed for each measurement.

To determine the K_i values, the uptake rate of [^{3}H]methionine were measured for 5 min at 250, 333, 500, and 1000 μM, with or without BraA. The K_i value was determined by double-reciprocal plot analysis in which 1/uptake rate of [^{3}H]methionine was plotted against 1/methionine concentration.

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