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# Anticancer Agent E7070 Inhibits Amino Acid and Uracil Transport in Fission Yeast

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## ABSTRACT

E7070 is a novel sulfonamide anticancer agent that inhibits cell cycle progression in $G_1$ in mammalian cells, but its action targets are not known. We recently employed the genetically amenable fission yeast *Schizosaccharomyces pombe* as a model organism to search for its targets. Here, we show that E7070 inhibits imports of amino acid and uracil into *S. pombe* cells. Unlike their prototrophic counterparts, leucine- and uracil-auxotrophic strains are sensitive to E7070 and are unable to proliferate with a delayed $G_1$-S transition in low-glucose yeast extract-polypeptone medium containing this drug because this chemical markedly inhibits the uptake of leucine and uracil in low glucose medium. Furthermore, addition of leucine or uracil to the culture medium or overexpression of genes encoding an amino acid or uracil transporter suppresses the E7070-imposed growth inhibition of these auxotrophic strains. Thus, some of the molecular targets for E7070 action in *S. pombe* are likely to be leucine and uracil transporters.

We identified recently the novel sulfonamide antitumor agent E7070 [ER-35744, *N*-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide] in screening for low-molecular-weight compounds targeting the $G_1$ phase of the cell cycle. E7070 was shown to arrest P388 murine leukemia cells in the $G_1$ phase and to delay the $G_1$/S progression of HCT116 human colorectal cancer cells in a dose-dependent manner. In in vivo tumor transplantation models, E7070 not only suppressed tumor growth but also reduced the tumor size of murine and human colon cancers (Owa et al., 1999). The unique end phenotype ($G_1$ arrest) of E7070-treated cancer cells and its tumor type selectivity of efficacy suggest that this drug may target a molecule(s) that differs from those for widely used anticancer drugs. However, its precise mode of action for antitumor effect is unknown.

The two different yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have successfully been used as valuable tools for understanding the mechanisms of action of certain drugs and identifying the targets of these drugs and their unique actions in mammals (Cardenas et al., 1999). For example, from genetic studies in *S. cerevisiae*, the TOR1 and TOR2 gene products were found to be targets of rapamycin (Heitman et al., 1991), and from *S. pombe* studies, the cellular target of leptomycin B was identified to be CRM1 (Nishi et al., 1994). Thus, the use of such an approach, which has been called *chemical genetics*, has led to the elucidation of action targets for chemicals with a variety of effects (Crews and Splittgerber, 1999).

*S. pombe* notably resembles higher eukaryotes in the mechanisms of gene expression, signal transduction, and cell cycle machinery (Okazaki et al., 1990), and several human genes involved in cell cycle control have been isolated by complementation of *S. pombe* mutants (Lee and Nurse, 1987; Igarashi et al., 1991; Nagata et al., 1991; Yamamoto et al., 1999). Accordingly, use of *S. pombe* would be relevant to understanding the action mechanism of certain bioactive compounds, such as anticancer agents. We used *S. pombe* as a model organism to search for targets for the action of E7070 and found that amino acid and uracil transporters are among the action targets of this novel drug in this organism. We present experimental data and discuss its relevance to the antitumor action of E7070.

## Materials and Methods

**Chemicals.** E7070 [*N*-(3-chloro-7-indol-yl)-1,4-benzenedisulfonamide (Owa et al., 1999), was synthesized in our laboratory (Eisai Co. Ltd., Tsukuba, Japan). A stock solution of E7070 was prepared by dissolving this compound in dimethyl sulfoxide at a concentration of 100 mg/ml and stored at 4°C. Radiolabeled amino acids and uracil were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

**Strains, Media, and Genetic Methods.** The *S. pombe* strains used in this study are L972 ($h^-$ prototroph), EV3A ($h^-$ *leu1-32*), EV4A ($h^{+S}$ *leu1-32*), EV5A ($h^-$ *ura4-D18*), EV7A ($h^-$ *leu1-32 ura4-D18*), EV9A ($h^-$ *ade6-M210*), EV17A ($h^-$ *ade6-M216*), and CH863 ($h^-$ *his1-102*). Media were as described elsewhere (Sharman et al.,

**ABBREVIATIONS:** YP, yeast extract-polypeptone; PM, pombe minimal; YPD, yeast extract-polypeptone-dextrose.

1986; Alfa et al., 1993; Tsukahara et al., 1998). Transformations were performed according to the lithium acetate procedure as described previously (Okazaki et al., 1990). The transducing vectors pALSK and its *URA3* version pAUSK, the latter of which has the *S. cerevisiae URA3* marker instead of *LEU2* in pALSK, were as described elsewhere (Tanaka et al., 2000). E7070-containing agar plates were prepared by adding E7070 into yeast extract-polypeptone (YP)-agar medium just after sterilization by autoclaving. The *aap1*$^+$ gene was kindly provided by K. Okazaki (Kazusa DNA Research Institute, Kisarazu, Japan).

**E7070 Sensitivity.** Cells were suspended in distilled water at a concentration of $10^7$ cells/ml and 10 $\mu$l of suspensions were spotted onto YP-agar plates containing 2% glucose and 0.5% glucose in the presence or absence of E7070 at indicated doses. The plates were incubated at 30°C for 3 days.

**Flow Cytometry.** Exponentially growing cells ($1 \times 10^8$) were arrested in $G_1$ by culturing at 30°C for 24 h in 100 ml of PM(-N) medium containing 50 $\mu$g/ml of leucine. The $G_1$-arrested cells were then stimulated to start cell cycling by reinoculating in 50 ml of 30°C YP medium containing 2 or 0.5% glucose in the presence or absence of 100 $\mu$g/ml of E7070, sampled at the indicated times and analyzed for their DNA content by Epics (Beckman-Coulter, Fullerton, CA).

**Amino Acid Transport Assay.** Exponentially growing cells ($9 \times 10^6$) were inoculated in 3 ml of YP medium containing 2 or 0.5% glucose in the presence or absence of E7070 at 30°C for the indicated times. The cells were then recovered by centrifugation, washed with PM medium containing 2% glucose and 0.5% bovine serum albumin, and resuspended in 1.5 ml of the same medium. Before incubation for 10 min, 1.5 $\mu$Ci of L-[4,5-$^3$H]leucine (specific activity, 152 Ci/mmol), 0.15 $\mu$Ci of [5,6-$^3$H]uracil (48 Ci/mmol), or 0.375 $\mu$Ci of L-[U-$^{14}$C]histidine (286 mCi/mmol) was added to the cell suspension. Aliquots (400 $\mu$l) of the labeled cell suspension were then placed in triplicate onto a centrifugal filter unit with sucking (Ultrafree-MC; Millipore, Bedford, MA) and washed twice with PM medium containing 2% glucose and 0.5% bovine serum albumin and resuspended in 200 $\mu$l of Dulbecco's phosphate-buffered saline. The radioactivity taken up by cells was quantified by scintillation counting.

In the direct inhibition assay, the cells were resuspended in 1.5 ml of PM medium containing 2% glucose and 135 $\mu$g/ml of the polypeptone that was dialyzed against 3 liters of distilled water three times at 4°C. L-[4,5-$^3$H]leucine (7.5 $\mu$Ci; specific activity, 152 Ci/mmol) was added to the cell suspension followed by incubation for 15 min in the presence or absence of E7070, and the radioactivity taken up by cells was quantified by scintillation counting.

**Isolation of E7070 Resistance-Conferring Genes.** An *S. pombe* genomic library was constructed by inserting completely *Hin*dIII-digested DNA of L972 into the pALSK vector (Tsukahara et al., 1998). The EV7A (*h*$^-$ *ura4-D18 leu1-32*) cells were transformed with the library. The leu$^+$ transformants produced were collected and reinoculated onto YP-agar plates containing 0.5% glucose and 200 $\mu$g/ml of E7070 and incubated for 3 days at 30°C. To exclude the plasmid containing the *ura4*$^+$ gene itself, E7070-resistant colonies picked up were streaked onto minimal medium agar plates. Colonies that could not grow on the plates were isolated, and plasmid clones contained therein were sequenced.

## Results

**Leucine- or Uracil-Auxotrophic Strains Are Sensitive to E7070 in Low-Glucose Medium.** An effective approach to identify the target molecule for E7070 is the isolation of drug-specific resistant genes. The wild-type strain was, however, capable of forming colonies on YPD medium even containing 200 $\mu$g/ml of E7070, which is the maximum dose of dissolution of this compound. In search for the conditions under which cells show E7070-sensitive growth, we found that the growth of the *leu1-32* and *ura4-D18* auxotrophic, but not their prototrophic strains was completely inhibited when the glucose concentration in YPD medium was lowered from 2 to 0.5%. The *h*$^-$, *h*$^-$ *leu1-32*, *h*$^+$ *leu1-32*, *and h*$^-$ *ura4-D18* strains were spotted onto complete YPD (2% glucose) or low-glucose YPD (0.5% glucose) in the presence or absence of 200 $\mu$g/ml of E7070. On complete YPD plates, all the strains were able to grow irrespective of the presence or absence of the drug (Fig. 1, a and b). By contrast, these auxotrophic strains could not grow on low glucose-YPD plates at day 3 (Fig. 1, c and d). More quantitative analysis of cell proliferation and viability was performed with the leucine auxotrophic strain in a 12-h liquid culture. In high-glucose YP medium, the cells proliferated 37-fold in the absence of the drug and 7-fold in the presence of the drug (Fig. 2a) with no obvious decrease in viability (Fig. 2b). However, in low-glucose YP medium, the cells proliferated 14-fold in the absence and 4.6-fold in the presence of the drug but with a marked reduction in viability when the drug was present. These results indicate that E7070 is not only cytostatic but also cytocidal to the yeast.

For mammalian cells, E7070 causes $G_1$ arrest or a delayed $G_1$-S progression (Owa et al., 1999). For the fission yeast, it seems to impart a similar effect. The *leu1-32* cells were synchronized to $G_1$ by nitrogen starvation and released to start the cell cycle in YP medium containing high (2%) or low (0.5%) glucose in the absence or presence of E7070. In the absence of the drug, they progressed into S phase with a rapid reduction in the $G_1$ cell population, which disappeared at 8 h in both 2 and 0.5% glucose medium. On the other hand, in the presence of E7070, their entry into S phase was sig-

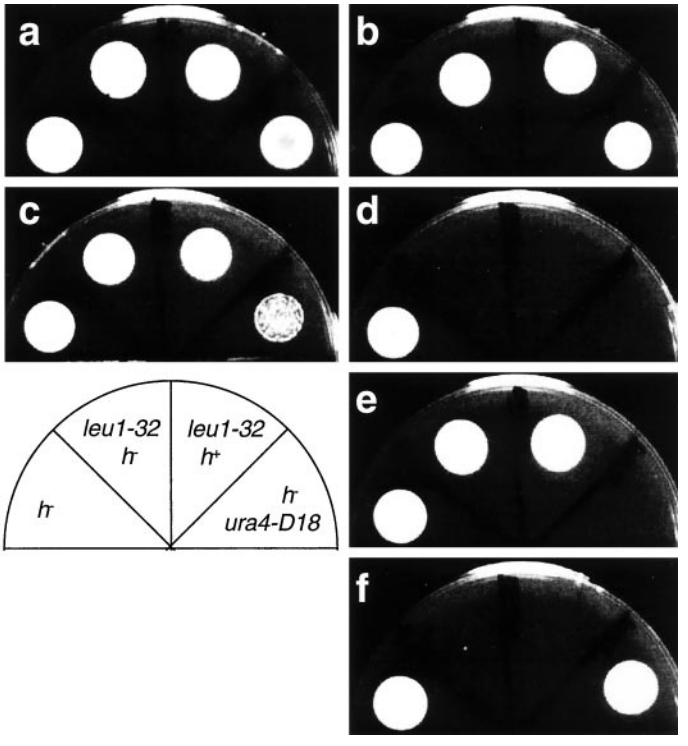


**Fig. 1.** Leucine- and uracil-auxotrophic strains are sensitive to E7070 under low-glucose condition. $10^5$ cells of *h*$^-$, *h*$^-$ *leu1-32*, *h*$^{+S}$ *leu1-32*, and *h*$^-$ *ura4-D18* strains were spotted onto YP agar plates containing 2% glucose (a and b), 0.5% glucose (c, d, e, and f) in the presence (b, d, e, and f) or absence (a and c) of 200 $\mu$g/ml of E7070. Five-fold excess amounts (250 $\mu$g/ml of final concentrations) of leucine (e) or uracil (f) were exogenously added to the plates. The plates were incubated at 30°C for 3 days.

nificantly inhibited, with a persistent presence of the $G_1$ cell population even at 12 h in low-glucose medium (Fig. 2c).

As expected, the high drug susceptibility of these yeast strains were completely suppressed by the supplementation of leucine or uracil in the culture medium (Fig. 1, e and f) or introduction of the pcL expressing *S. cerevisiae* Leu2 protein that corresponds to *S. pombe* Leu1 protein (Fig. 3a). However, not all the auxotrophic mutants are susceptible to E7070. At least adenine-auxotrophic strains were resistant to this drug (Fig. 3b). These results suggest that E7070 specifically inhibits the import of at least leucine and uracil into the cells, thereby exerting a killing effect.

**E7070 Inhibits Leucine and Uracil Uptakes by Cells.** To extend our findings, we directly assayed the effect of E7070 on leucine and uracil uptakes by the corresponding auxotrophic mutants. E7070 is hydrophobic and consequently difficult to dissolve in water in the absence of bipolar compounds or macromolecules such as detergents or proteins (Owa et al., 1999). Accordingly, unlike soluble compounds, such a hydrophobic compound is generally slow in both being taken up and diffusing away from the cells. The polypeptone in YP medium could serve as bipolar macromolecules that carry this chemical. Therefore, we pretreated the *leu1-32* and *ura4-D18* strains with YP medium containing the indicated concentration of E7070 and then assayed for the uptake of [$^3$H]leucine and [$^3$H]uracil, respectively. As shown in Fig. 4a, E7070 inhibited leucine uptake in both 2 and 0.5% glucose-containing YP medium. However, in 0.5% glucose, inhibition was more evident and became markedly severe as cells were treated longer. Thus, E7070 actually inhibited leucine uptake, but this inhibition was largely suppressed by a high concentration of glucose. The inhibition of uptake was observed in as little as 15 min of exposure, and 60-min treatment was enough to obtain a maximum inhibition (Fig. 4b). The time-dependent inhibition seems to have resulted from E7070's slow diffusion because of its hydrophobicity.

To examine whether E7070 can inhibit transport without pretreatment or not, we performed a direct transport inhibition assay with the drug. As shown in Fig. 4c, the uptake of leucine was moderately inhibited by E7070 in the transport medium. These results suggest that E7070 directly inhibits leucine transporter molecules. In addition, consistent with the growth inhibition of the uracil-auxotrophic mutant by this chemical, uracil uptake was also inhibited by E7070 (Fig. 4c).

**Specificity of E7070 to Amino Acid Transporters.** To examine the specificity of E7070 to transporters, we compared the inhibitory activities of E7070 to leucine and histidine transports in leucine- and histidine-auxotrophic strains, respectively. E7070 inhibited leucine uptake in *leu1-32* cells in a dose-dependent manner and the level of inhibition reached 72% by treatment with 200 $\mu$g/ml of E7070 (Fig. 5a). By contrast, histidine uptake in *his1-102* cells was inhibited

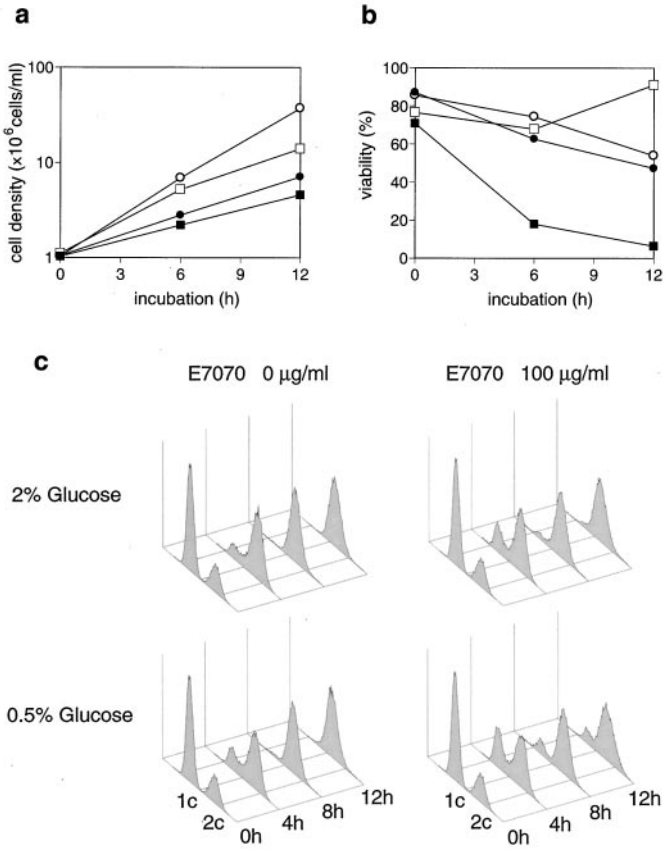


**Fig. 2.** a, E7070 inhibits the proliferation of the leucine auxotrophic strain in liquid culture. $h^-$ *leu1-32* cells were cultured in YP medium containing 2% (circles) or 0.5% (squares) glucose in the presence (open symbols) or absence (filled symbols) of 200 $\mu$g/ml of E7070 for the indicated times. The number of cells was counted under the microscope. b, E7070 reduces cell viability. The same cultures as in a were diluted 500-fold, and 100-$\mu$l aliquots were plated and colonies were counted as the number of viable cells. The percentage viability of cells was calculated by dividing the number of viable cells by the number of the cells counted in a. c, E7070 delays the $G_1$-S progression. $h^-$ *leu1-32* cells were synchronized to $G_1$ by nitrogen starvation and then released to start the cell cycle in YP medium containing 2 or 0.5% glucose in the absence or presence of 100 $\mu$g/ml of E7070. The cells were harvested at indicated times and their DNA content was analyzed by flow cytometry for monitoring cell cycle progression.

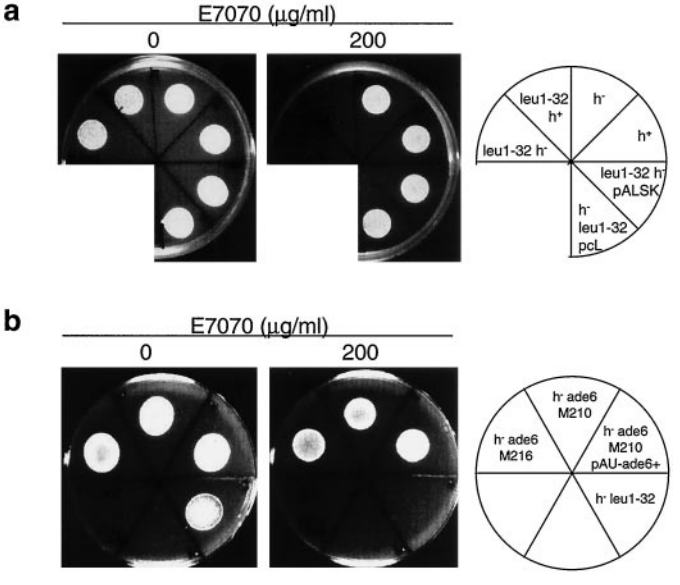


**Fig. 3.** a, plasmids expressing Leu2 protein confer E7070 resistance. $h^-$ *leu1-32* cells were transformed with empty pALSK or pcL vectors and spotted onto low-glucose YPD agar plates containing 0 or 200 $\mu$g/ml of E7070 with various reference strains and incubated for 3 days at 30°C. b, adenine-auxotrophic strains do not show E7070-sensitivity. $h^-$ *ade6-M216*, $h^-$ *ade6-M210*, and $h^-$ *ade6-M210* expressing pAU-*ade6*$^+$, and $h^-$ *leu1-32* cells were spotted onto YP plates containing 0.5% glucose in the presence or absence of E7070 and incubated at 30°C for 3 days.

only 43% by a maximum dose of E7070 (Fig. 5b). Consistent with these observations, the *his1-102* strain showed mild inhibition, growing slowly on low-glucose YPD plates containing E7070 (Fig. 5c). These results suggest that E7070 inhibits both neutral and cationic amino acid transporters with slightly different specificity.

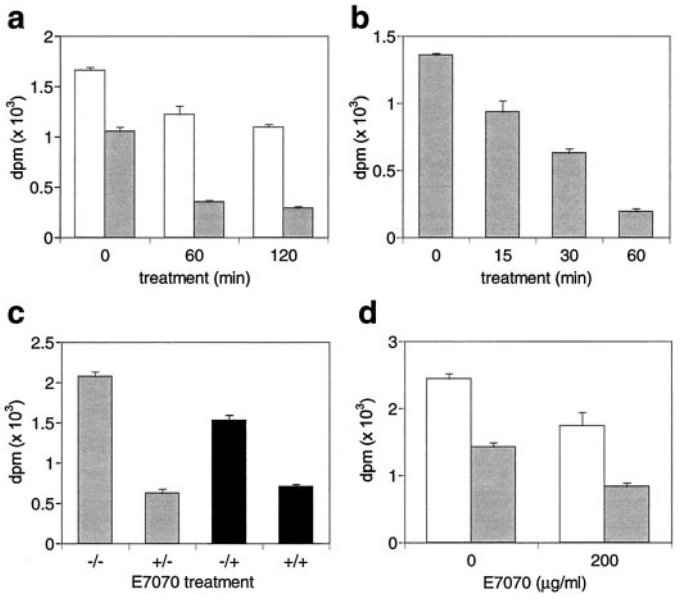


**Fig. 4.** E7070 inhibits leucine and uracil imports into cells. a, Effect of glucose concentration. *h⁻ leu1–32* cells were cultured in YP medium containing 2% (□) or 0.5% glucose (▨) in the presence or absence of 200 μg/ml of E7070 for the indicated times. After washing, the cells were labeled with [³H]leucine for 15 min and counted the incorporated radioactivity. Data were shown by the incorporated radioactivity per $10^6$ cells. b, inhibition of leucine uptake is time-dependent. *h⁻ leu1-32* cells were treated with 200 μg/ml of E7070 in YP medium containing 0.5% glucose for the indicated times and then examined for [³H]leucine uptake. c, direct inhibition of E7070 on leucine uptake. *h⁻ leu1-32* cells were treated with 0 or 200 μg/ml of E7070 in YP medium containing 0.5% glucose for 60 min and then examined for [³H]leucine uptake in the absence (▨) or presence (■) of 200 μg/ml of E7070. Labels of horizontal axis indicate the status of E7070 treatment (pretreatment/direct treatment). d, inhibition of uracil import. *h⁻ ura4-D18* cells were treated with 200 μg/ml of E7070 in YP medium containing 2 or 0.5% glucose for 60 min and examined for [³H]uracil uptake.

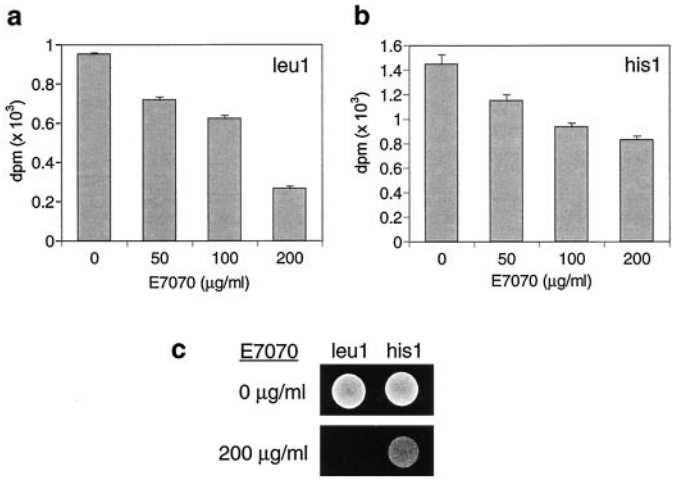


**Fig. 5.** Specificity of the E7070 action against amino acid transporters. a and b, dose-dependent inhibition of amino acid uptake by E7070. *h⁻ leu1-32* (a) and *h⁻ his1-102* (b) cells were treated with 0, 50, 100, or 200 μg/ml of E7070 in YP medium containing 0.5% glucose for 60 min and then examined for [³H]leucine (a) and [³H]histidine (b) uptakes, respectively. c, *h⁻ leu1-32* and *h⁻ his1-102* cells were spotted onto low-glucose YPD agar plates containing 0 or 200 μg/ml of E7070 and incubated for 2 days at 30°C.

**The Amino Acid Permease Gene *aap1*⁺ Confers E7070-Resistance to Cells.** All the results presented indicate that the action targets of E7070 include several nutrient transport systems. To confirm this, we examined whether the E7070 susceptibility was reversed by the overexpression of a relevant transporter gene. The *aap1*⁺ gene encodes an amino acid permease that facilitates several amino acids, including leucine (K. Okazaki and H. O., unpublished observations). The *h⁻ ura4-D18 leu1-32* strain was transfected with an empty vector or *aap1*⁺ inserted in the pAUSK vector that complements the *ura4-D18* mutation. As shown in Fig. 6a, the cells expressing an exogenous *aap1*⁺ gene from a multicopy vector became highly resistant to E7070 and formed colonies in low glucose YP-agar plates in the presence of the drug. These results suggest that the Aap1 amino acid permease itself is likely to be a direct target for this anticancer drug.

**Genes That Confer E7070 Resistance.** To further confirm the results, we searched for genes whose expression from a multicopy vector confers E7070 resistance to the cells. An *S. pombe* genomic library constructed with the pALSK vector that complements *leu1-32*, thereby enabling selection of transfected cells, was transfected into the *h⁻ ura4-D18 leu1-32* strain (see *Materials and Methods*). Two genes that confer E7070 resistance were identified. One was the *ura4*⁺ gene itself, and the other was *fur4*⁺, which encodes a uracil permease (de Montigny et al., 1998). As shown in Fig. 6b, expression of *fur4*⁺ from the multicopy vector conferred drug resistance to the *ura4-D18* cells. These results support the possibility that certain nutrient permeases themselves are likely to be targets for the action of E7070.

## Discussion

Identification of the molecular targets for the drugs developed solely on biological effectiveness is generally difficult. We employed the genetically amenable fission yeast *S. pombe* as a model organism to search for possible targets of the

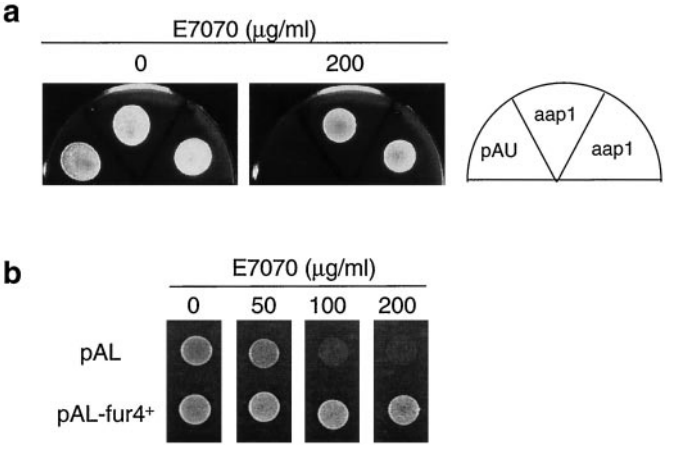


**Fig. 6.** Ectopic expression of leucine and uracil transporters confers resistance to E7070. *h⁻ leu1-32 ura4-D18* cells were transformed with pAUSK, pAUSK-*aap1*⁺ (a), pALSK, or pALSK-*fur4*⁺ (b). The transformed cells were spotted onto YP-agar plates containing 0.5% glucose in the presence of 0 or 200 (a), and 0, 50, 100, or 200 μg/ml of E7070 (b), respectively, and incubated for 2 days at 30°C.

novel anticancer chemical E7070, and identified leucine and uracil transporters as likely targets in this organism. However, this drug seems to target not only fission yeast but also mammalian transporters, although we have no evidence that this drug inhibits mammalian transporters. We have recently identified the mammalian amino acid transporter LAT1/4F2Lc to bind this drug by affinity column chromatography (Y. Oda, T. Owa, N. Miyamoto, unpublished observations). LAT1/4F2Lc is the recently isolated $Na^+$-independent neutral amino acid transporter (Kanai et al., 1998; Mastroberardino et al., 1998; Nakamura et al., 1999) and its expression is up-regulated in some tumor cell lines and mitogen-stimulated lymphocytes. In addition, this drug imparts a phenotypically similar effect on both organisms: inhibition of the $G_1$-S transition (Fig. 2c; Owa et al., 1999).

As presented, the sensitivity of the uracil- or leucine-auxotrophic strains to E7070 is greatly enhanced by low glucose. This sensitivity enhancement by low glucose seems to be attributable at least partly to an intrinsic nature of the regulation of the transporters for the following reasons: 1) Sensitivity enhancement to the drug failed to be obtained by any of other growth-impairing conditions tested, such as treatment with cycloheximide, hygromycin, or tunicamycin; high- or low-temperature shift; and UV-irradiation (data not shown). 2) We observed the leucine-transport activity was reduced by a low-glucose shift even in the absence of E7070. In addition, there are several reports that suggest the dependence of amino acid permease activity on glucose. A study with *S. pombe* shows that lysine transport activity rapidly decays after removal of glucose (Sychrova et al., 1989). In *S. cerevisiae*, the general amino acid permease activity is stimulated by glucose in a dose-dependent manner (Iglesias et al., 1990).

The mechanism by which this drug inhibits the transport of at least leucine and uracil is not known, but all our data are consistent with the possibility of direct inhibition of the corresponding transporters. The immunosuppressant FK506 inhibits amino acid import in *Saccharomyces cerevisiae*, but this inhibition requires at least 5 h of preincubation of the cells with this agent, suggesting that FK506 might indirectly inhibit transporters by affecting their proper folding, assembly, or transport to the right destination (Heitman et al., 1993). By contrast, the inhibition by E7070 was observed in as little as 15 min of exposure, and 60-min treatment was sufficient to obtain a maximum inhibition. Furthermore, E7070 present in the leucine transport assay was effective. Considering the fact that E7070 is highly hydrophobic and accordingly requires bipolar molecules, such as proteins for its dissolution in an aqueous solution, the sufficiency of 60 min of preincubation to obtain the maximal inhibition suggests that, unlike FK506, this chemical is likely to directly inhibit mature transporter molecules.

E7070 inhibits both amino acid and uracil transports, and their inhibitions are reversed by overexpression of the amino acid permease *aap1*$^+$ and the uracil permease *fur4*$^+$, respectively. They are low in amino acid homology, but similar in size (594 amino acids in Aap1, 582 amino acids in Fur4) and both integral membrane proteins with putative 12 membrane-spanning domains (POMBASE, The Sanger Center). E7070 may recognize the common stereochemical structure in these proteins, especially their hydrophobic regions, and inhibit certain common regulatory mechanisms involved in the catalytic activity of both permeases.

Amino acid starvation is an effective strategy for cancer therapy. L-Asparaginase has been used for the treatment of leukemia (Abshire et al., 2000). Dietary methionine depletion causes the tumor regression in nude mice (Guo et al., 1993). B16 murine melanoma cells are induced apoptosis by phenylalanine and tyrosine starvation (Fu et al., 1999). Arginine deiminase arrests the cell cycle at early $G_1$ phase in mitogen-stimulated T lymphocytes (Sugimura et al., 1989) and at $G_1$/$G_2$ in A375 human melanoma cells (Sugimura et al., 1990), and shows in vivo antitumor activity (Takaku et al., 1992). Thus, nutritional starvation is a potential candidate for the target of anticancer drugs. Further investigation is necessary to elucidate the importance of these findings for E7070 anticancer activity.

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