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# Specificity of Inhibitors of Serine Palmitoyltransferase (SPT), a Key Enzyme in Sphingolipid Biosynthesis, in Intact Cells

## A NOVEL EVALUATION SYSTEM USING AN SPT-DEFECTIVE MAMMALIAN CELL MUTANT

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**ABSTRACT.** In the present study, we demonstrate a model cell system for evaluating the specificity of inhibitors of serine palmitoyltransferase (SPT), the enzyme that catalyzes the first step of sphingolipid biosynthesis. The LY-B strain is a Chinese hamster ovary (CHO) cell mutant defective in SPT, and the LY-B/cLCB1 strain is a genetically corrected revertant of the mutant. Although LY-B cells grew only slightly in sphingolipid-deficient medium, their growth was restored to the level of LY-B/cLCB1 cells under sphingosine-supplied conditions, indicating that, in CHO cells, the growth inhibition caused by SPT inactivation was rescued almost fully by the metabolic complementation of sphingolipids. Cultivation of LY-B/cLCB1 cells in sphingolipid-deficient medium in the presence of 10 μM sphingofungin B and ISP-1 (myriocin, thermozymocidin), potent inhibitors of SPT activity, caused severe growth inhibition with ~95% inhibition of *de novo* sphingolipid synthesis. The growth inhibition by sphingofungin B and ISP-1 was rescued substantially by exogenous sphingosine, whereas the cytotoxicity of two other types of SPT inhibitor, L-cycloserine and β-chloro-L-alanine, was hardly rescued. Similar cytotoxic patterns of these inhibitors also were observed on the growth of SPT-defective LY-B cells cultured under sphingosine-supplied conditions. The SPT inhibitors did not affect metabolic conversion of exogenous [$^3$H]sphingosine to complex sphingolipids. Thus, the cytotoxicity of sphingofungin B and ISP-1, but not L-cycloserine or β-chloro-L-alanine, is due largely to inhibition of sphingolipid synthesis by inhibiting the SPT activity. BIOCHEM PHARMACOL **59**;10:1211–1216, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** sphingolipid synthesis; sphingofungin B; ISP-1; myriocin; L-cycloserine; β-chloro-L-alanine

Chemical compounds that inhibit specific cellular functions are useful tools for biological research and may also be pro-drugs leading to invaluable clinical medicines. However, it is often difficult to determine the target specificity of an inhibitor in intact cells, even when the primary target of the inhibitor is known. A cell mutant specifically defective in the target of the inhibitor can be a useful tool for resolving this problem. If an inhibitor is highly specific to a target, cellular responses to the drug should be the same as those caused by the genetic inactivation of the target, whereas cellular responses to a drug distinct from the responses to the genetic inactivation would reveal that the drug has another effect(s) in addition to inhibiting the specific target.

Sphingolipids are ubiquitous membrane components in eukaryotic cells. Various sphingolipid metabolites appear to modulate various cellular events including proliferation, differentiation, and apoptosis [1, 2], and, therefore, inhibitors of sphingolipid metabolism serve as useful pharmacological tools. The first step involved in sphingolipid biosynthesis is the condensation of serine and palmitoyl CoA, a reaction catalyzed by SPT‖ to produce 3-ketodihydrosphingosine [3]. Various natural compounds that potently inhibit SPT activity have been discovered in this decade [4–7]. However, the findings that potent SPT inhibitors have a common structural feature related to sphingosine (Fig. 1) and that sphingosine modulates various cell functions [1, 2] raise the possibility that SPT inhibitors have yet-unknown biological activities in addition to inhibiting *de novo* synthesis of sphingolipids. Moreover, other types of drugs, including L-cycloserine and β-chloro-L-alanine, which have also been used to inhibit

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‖ *Abbreviations*: SPT, serine palmitoyltransferase; CHO, Chinese hamster ovary; and SFB, sphingofungin B.

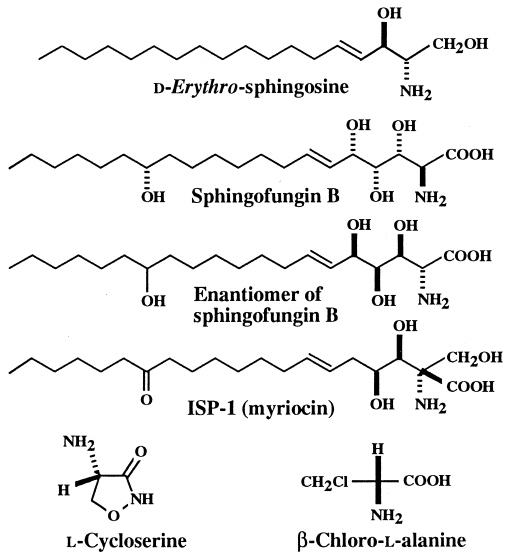


**FIG. 1. Structures of D-*erythro*-sphingosine and various SPT inhibitors. Sphingofungin B (SFB) and ISP-1/myriocin, potent SPT inhibitors, are structurally related to sphingosine. The enantiomer of SFB is inactive as an SPT inhibitor.**

the SPT reaction in intact cells [8, 9], appear to inhibit various enzymes in addition to SPT. Thus, evaluation of the specificity of these SPT inhibitors in intact cells is an urgent issue to be addressed.

Genetic and biochemical studies have shown that the SPT enzyme comprises at least two subunits, the LCB1 and LCB2 proteins [10–14]. We recently have isolated CHO cell mutants (designated the LY-B strain) defective in SPT activity due to a lack of expression of the LCB1 subunit, and also have obtained the corrected revertant (designated LY-B/cLCB1) by stable transfection of the LY-B strain with the wild-type hamster *LCB1* cDNA [14]. These two strains have provided an ideal model cell system to explore the cellular responses specific to inactivation of SPT. In the present study, we report the specificity of various SPT inhibitors, having used the model cell system.

## MATERIALS AND METHODS

### *Chemicals*

SFB and its enantiomer were synthesized chemically [15], and ISP-1 was purified from the culture broth of the fungus *Isaria sinclairii* [16]. L-Cycloserine and β-chloro-L-alanine were purchased from the Sigma Chemical Co., and D-*erythro*-sphingosine was purchased from Matreya.

### *Cells*

The LY-B and LY-B/cLCB1 strains were established previously by us [14]. Cells were maintained routinely in Ham's F-12 medium supplemented with 10% newborn bovine serum and antibiotics at 33°. When necessary, Nutridoma-BO medium [Ham's F-12 medium containing 1% (v/v) Nutridoma-SP (Boehringer Mannheim), 10 μM sodium oleate (a complex with BSA), and 10 μg/mL of gentamicin] was used as a sphingolipid-deficient medium [14].

### *Metabolic Labeling of Lipids in CHO Cells*

Metabolic labeling of lipids with L-[U-$^{14}$C]serine was carried out as described previously [12, 17] with some modifications. Briefly, the subconfluent cell monolayer in a 60-mm dish was preincubated in 1.5 mL of Nutridoma-BO medium containing various drugs at 37° for 1 hr. The concentration of DMSO, the vehicle of the drug stock solutions, during preincubation was adjusted to 0.1% (w/v) in each dish. After the addition of 0.75 μCi of L-[U-$^{14}$C]serine (162 mCi/mmol, Amersham Pharmacia Biotech), the cells were incubated at 37° for 2 hr. TLC analysis of radioactive lipids extracted from the cells was carried out with a solvent of methyl acetate:*n*-propanol:chloroform:methanol:0.25% KCl (25:25:25:10:9, by vol.). Metabolic labeling of lipids with D-*erythro*-[3-$^{3}$H]sphingosine (20 Ci/mmol, American Radiolabeled Chemicals Inc.) was carried out as described previously [18] with some modifications for the preincubation of cells with various drugs as described above. A solvent of chloroform:methanol:water (65:25:4, by vol.) was used for TLC. Radioactive lipids separated on TLC plates were visualized and analyzed with either a BAS2000 or a BAS1800 image analyzer (Fuji Film Co.).

### *Determination of Cell Growth Yield*

Five thousand CHO cells were seeded in 2 mL of the normal medium in a 35-mm diameter petri dish, cultured at 37° for 3–6 hr, washed twice with 1 mL of PBS, and then cultured in 2 mL of Nutridoma-BO medium in the presence or absence of various drugs at 37° for 5 days. After harvest of the cell monolayer by trypsinization, the cell number was determined with a Coulter counter. For metabolic complementation experiments, 10 μL of 0.1 mM sphingosine associated with fatty acid-free BSA was supplied to Nutridoma-BO medium at the start, and 20 μL of 0.1 mM sphingosine was subsequently added to the dishes every 48 hr during culture. Statistical analysis was performed by Student's *t*-test.

## RESULTS

### *Effects of SFB on* De Novo *Sphingolipid Biosynthesis in CHO Cells*

Metabolic labeling of lipids with [$^{14}$C]serine showed that SFB inhibited *de novo* synthesis of sphingolipids in LY-B/cLCB1 cells in a concentration-dependent manner. Incorporation of radioactivity into the sphingolipids (ceramide, glucosylceramide, $G_{M3}$ ganglioside, and sphingomyelin) in the presence of SFB at 1, 5, and 10 μM were about 40, 10, and 5%, respectively, of the drug-minus control level (Fig. 2, lanes 2–5). SFB did not affect the labeling of phosphatidylserine and phosphatidylethanolamine, which are syn-

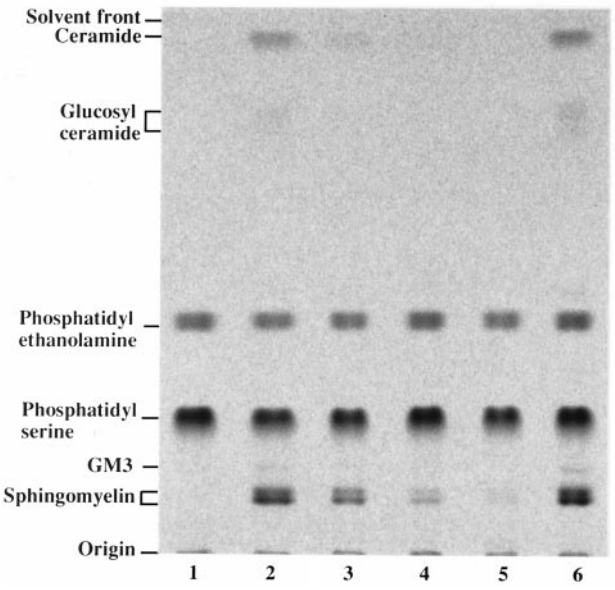


**FIG. 2. Effects of SFB on *de novo* sphingolipid synthesis in CHO cells. LY-B cells (lane 1) or LY-B/cLCB1 cells (lanes 2–6) were incubated with [$^{14}$C]serine in the presence or absence of SFB at the indicated concentrations at 37° for 2 hr. Lipids were extracted from the cells and separated by TLC. Radioactive images of lipids separated on a TLC plate are shown. Radioactivity of each lipid on the plate was measured by image analysis. Lanes 1 and 2, no drug; lane 3, 1 μM SFB; lane 4, 5 μM SFB; lane 5, 10 μM SFB; and lane 6, 10 μM SFB enantiomer.**

thesized via a different pathway from that for sphingolipids (Fig. 2). The enantiomer of SFB at 10 μM caused no inhibition of *de novo* sphingolipid synthesis (Fig. 2, lane 6), consistent with our previous study showing that this compound is inactive as an SPT inhibitor [15]. No labeling of sphingolipids was observed in LY-B cells (Fig. 2, lane 1), which express no appreciable activity of SPT [14].

### *Comparison of Cytotoxic Effects between Genetic Inactivation of SPT and Drug-Dependent Inhibition of SPT*

Cells at an initial density of 5000/dish were cultured in Nutridoma-BO, a sphingolipid-deficient medium, for 5 days, and the resultant cell number per dish was scored as the growth yield. LY-B cells grew only poorly in the sphingolipid-deficient medium, whereas LY-B/cLCB1 cells grew with a high yield. Under sphingosine-supplied conditions, the growth of LY-B cells was restored to ~100% of the growth level of LY-B/cLCB1 cells, whereas the growth of LY-B/cLCB1 cells was not affected by exogenous sphingosine under the experimental conditions used (Fig. 3), in agreement with our previous study [14]. SFB caused a concentration-dependent inhibition of the growth of LY-B/cLCB1 cells in the sphingolipid-deficient medium, and 10 μM SFB, which inhibited *de novo* sphingolipid synthesis by ~95% (Fig. 2, lane 5), reduced the growth yield to less than ~10% of the drug-minus control level (Fig. 3). The growth inhibition of LY-B/cLCB1 cells by SFB was rescued dramatically by the addition of sphingosine to the culture medium. This recovery, however, exceeded by 70% the level of the drug-minus control (Fig. 3).

We also examined the cytotoxic patterns of ISP-1 (myriocin, thermozymocidin), another SPT inhibitor that structurally resembles sphingosine [6] (Fig. 1). ISP-1 inhibited sphingolipid synthesis more efficiently than SFB, as 1 μM ISP-1 was enough to inhibit *de novo* sphingolipid synthesis by 95% in LY-B/cLCB1 cells (data not shown). Accordingly, ISP-1 inhibited the growth of LY-B/cLCB1 cells more efficiently than SFB (Fig. 3). When sphingosine was added to the medium, the cell growth in the presence of 10 μM ISP-1 was recovered to about 80% of the drug-minus control level (Fig. 3). Metabolic labeling of lipids in LY-B/cLCB1 cells with [$^{14}$C]serine showed that 10 μM

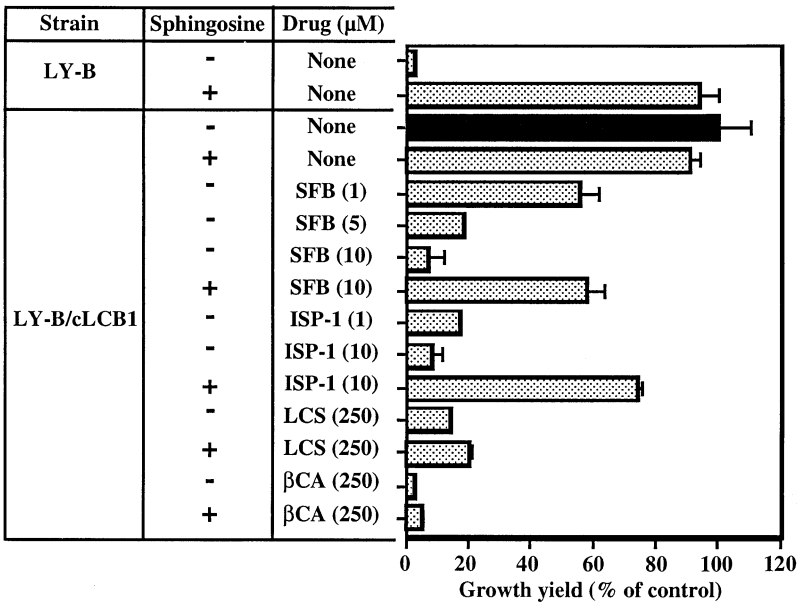


**FIG. 3. Cytotoxicity to CHO cells of the genetic or chemical inhibition of SPT, and suppression of the cytotoxicity by metabolic complementation with sphingosine. LY-B and LY-B/cLCB1 cells were seeded at 5000 cells per dish and cultured in Nutridoma-BO medium with or without various drugs at the indicated concentrations for 5 days. Sphingosine was supplied (+) or not supplied (−) to the medium as described under Materials and Methods. The data (means ± SD from 6 experiments) are shown as percentages of the mean yield (highlighted by a black bar; the actual number of cells = 4.1 × 10$^5$) of LY-B/cLCB1 cells cultured without sphingosine or drugs. LCS, L-cycloserine; βCA, β-chloro-L-alanine.**

**TABLE 1. Effects of SPT inhibitors on the growth of LY-B cells in Nutridoma-BO medium supplemented with sphingosine**

| Drug (μM) | Growth yield (% of drug-minus control) |
|---|---|
| None | 100 ± 7.0 |
| SFB (10) | 83.3 ± 4.0 |
| Enantiomer of SFB (10) | 73.2 ± 2.2 |
| ISP-1 (10) | 85.4 ± 3.7 |
| L-Cycloserine (250) | 21.9 ± 6.4 |
| β-Chloro-L-alanine (250) | 5.3 ± 1.3 |

Growth yields of LY-B cells in Nutridoma-BO medium supplemented with sphingosine and various drugs were determined as described under Materials and Methods. The data (mean values ± SD from 6 experiments) are shown as percentages of the mean yield of LY-B cells cultured without drugs. The initial cell density was 5000 cells per dish, and the mean yield of the drug-minus control was $3.8 \times 10^5$ cells per dish.

each of SFB and ISP-1 inhibited *de novo* sphingolipid synthesis by ~95% even in the presence of 1 μM sphingosine (data not shown), eliminating the possibility that inhibition of SPT activity by the drugs was weakened when sphingosine was supplemented.

L-Cycloserine and β-chloro-L-alanine, which are structurally unrelated to sphingosine (Fig. 1), inhibit SPT activity by blocking the pyridoxal phosphate group in the enzyme [8, 9]. Metabolic labeling experiments with [$^{14}$C]serine in the presence of various concentrations of L-cycloserine or β-chloro-L-alanine showed that the concentration required in both drugs for ~95% inhibition of *de novo* sphingolipid synthesis was ~250 μM (data not shown), and L-cycloserine or β-chloro-L-alanine at 250 μM severely inhibited the growth of LY-B/cLCB1 cells in the sphingolipid-deficient medium (Fig. 3). The growth inhibition by L-cycloserine and β-chloro-L-alanine, however, was hardly rescued by exogenous sphingosine (Fig. 3). Since sphingoid base-1-phosphate lyase, which is involved in the degradation of sphingoid bases, is a pyridoxal phosphate-dependent enzyme [19], the poor rescue by sphingosine might be due to a shift of the optimum concentration of sphingosine for the growth of SPT-deficient cells in the presence of these drugs. However, this possibility was unlikely, because the addition of various concentrations (0.1, 0.25, 0.5, and 1 μM) of sphingosine to medium every 48 hr during culture was similarly ineffective in restoring growth in the presence of 250 μM L-cycloserine or β-chloro-L-alanine (data not shown).

### *Cytotoxicity of SPT Inhibitors to SPT-Defective Mutant Cells*

We also determined whether the SPT inhibitors were toxic to LY-B cells, which are genetically defective in SPT [14], under sphingosine-supplied conditions. As shown in Table 1, 10 μM SFB, its enantiomer, and ISP-1 each reduced the growth yield of LY-B cells by 15–30% ($P < 0.01$), compared with the drug-minus control level. In agreement with the observation that 250 μM L-cycloserine and β-chloro-L-alanine each showed severe cytotoxicity to LY-B/cLCB1 cells (Fig. 3) even under sphingosine-supplied conditions, these two drugs also reduced the growth yield of LY-B cells by 80% or more (Table 1). The cytotoxic effects of the drugs on the cells genetically defective in SPT represented compelling evidence that these drugs affected cellular functions in addition to SPT.

### *Effects of SPT Inhibitors on Sphingosine Anabolism*

To examine whether the SPT inhibitors affected metabolic utilization of exogenous sphingosine, we carried out metabolic labeling of sphingolipids with [$^3$H]sphingosine in the presence or absence of the drugs. SFB, ISP-1, L-cycloserine, and β-chloro-L-alanine did not affect or only slightly affected conversion of exogenous [$^3$H]sphingosine to complex sphingolipids. The metabolic conversion efficiency in these drug-treated LY-B/cLCB1 cells was similar to that in drug-untreated LY-B/cLCB1 or LY-B cells, although only a slight inhibition was observed in the SFB-treated cells (Fig. 4).

## DISCUSSION

The LY-B/cLCB1 strain is a genetically corrected revertant of the mutant LY-B strain defective in SPT activity [14]. Sphingosine, when externally supplied to culture medium, is utilized for formation of ceramide and complex sphingolipids in CHO cells, thereby bypassing the SPT reaction metabolically [14, 20]. Therefore, the observation that the growth of LY-B cells was restored to the level of LY-B/

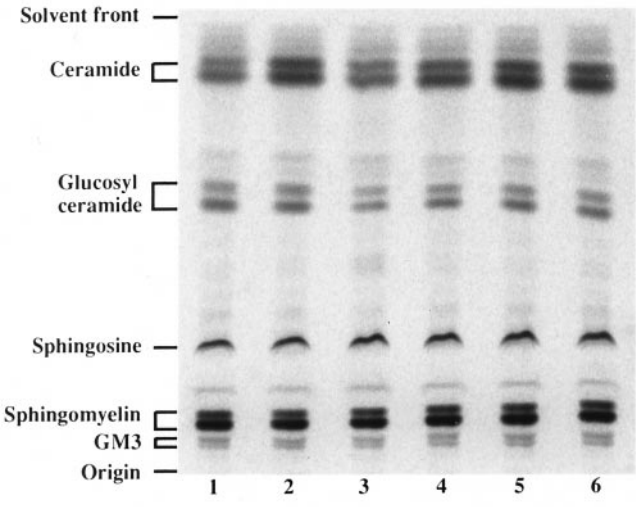


**FIG. 4. Effects of various SPT inhibitors on anabolism of [$^3$H]sphingosine. LY-B (lane 1) or LY-B/cLCB1 cells (lanes 2–6) were incubated with [$^3$H]sphingosine in the presence or absence of various SPT inhibitors at 37° for 2 hr. Then lipids were extracted from the cells and separated by TLC. Radioactive images of lipids separated on a TLC plate are shown. Radioactivity of each lipid on the plate was measured by image analysis. Lanes 1 and 2, no drug; lane 3, 10 μM SFB; lane 4, 10 μM ISP-1; lane 5, 250 μM L-cycloserine; and lane 6, 250 μM β-chloro-L-alanine.**

cLCB1 cells by the addition of sphingosine to the sphingolipid-deficient medium (Fig. 3) indicates that, in CHO cells, the growth inhibition caused by SPT inactivation is rescued almost fully by metabolic complementation with sphingosine.

When LY-B/cLCB1 cells were incubated with L-cycloserine or β-chloro-L-alanine at concentrations required for 90–95% inhibition of *de novo* sphingolipid synthesis, cell growth was inhibited almost completely in the sphingolipid-deficient medium (Fig. 3). This cytotoxicity, however, was poorly suppressed by exogenous sphingosine (Fig. 3), indicating that the toxicity of these drugs does not result primarily from an inhibition of SPT. L-Cycloserine and β-chloro-L-alanine probably affect various cell functions in addition to *de novo* sphingolipid synthesis, as these two drugs are wide-range inhibitors of pyridoxal phosphate-dependent enzymes [8, 9]. By contrast, the cytotoxicity of SFB and ISP-1 was dramatically, but never fully, recovered by exogenous sphingolipids (Fig. 3). Thus, inhibition of SPT activity was the major cause of SFB and ISP-1 cytotoxicity. A minor part of the toxicity of SFB and ISP-1 appeared to be independent of SPT inhibition, because 10 μM SFB and ISP-1 showed minor toxicity toward the growth of SPT-defective mutant LY-B cells under sphingosine-supplied conditions (Table 1). Furthermore, 10 μM SFB enantiomer, which is inactive as an SPT inhibitor [15], also exhibited similar minor toxicity (Table 1).

The failure of full growth recovery by exogenous sphingosine in the presence of the SPT inhibitors was not due to an inhibition of sphingosine anabolism by these drugs, because the metabolic conversion of exogenous sphingosine to complex sphingolipids was not affected or only slightly affected by SFB, ISP-1, L-cycloserine, and β-chloro-L-alanine (Fig. 4). Since some sphingosine-like compounds are known to inhibit sphingosine kinase [21, 22], one might imagine that SFB could also inhibit this enzyme. However, 10 μM SFB did not inhibit the sphingosine kinase activity of rat liver cytosol significantly (Kono K, Kobayashi S and Hanada K, unpublished observations). Very low concentrations (~50 nM) of ISP-1 are enough to induce apoptosis of mouse CTLL-2 cells and splenic lymphocytes, but not most other cell types [6, 23]. Interestingly, some compounds structurally related to ISP-1 are inactive as SPT inhibitors, but show a potent immunosuppressive activity similar to ISP-1, suggesting that the viability of the specific lymphoid cell types is strongly affected by ISP-1 and its related compounds through a mechanism other than the inhibition of SPT [24]. Although the mechanisms of the additional cytotoxic effects of SFB and ISP-1 remain unknown, the present study demonstrates that a phenotypic comparison among mutant cells specifically defective in a cellular function and wild-type cells exposed to chemical inhibitors of the function is a rational approach for evaluating the specificity of the inhibitors in intact cells.

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