BE-23372M, A NOVEL PROTEIN TYROSINE KINASE INHIBITOR

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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BE-23372M, a novel protein tyrosine kinase inhibitor, was isolated from the culture broth of a fungus. The producing strain, F23372, was identified as *Rhizoctonia solani*, based on the cultural and morphological characteristics. The active principle was extracted from the mycelium with acetone and purified by solvent extraction, silica gel column chromatography and Sephadex LH-20 column chromatography.

BE-23372M showed strong inhibitory activity against EGF receptor kinase with IC_{50} values of 0.02 and 0.03 μM on two different substrates, whereas IC_{50} values against protein kinase C and cAMP-dependent protein kinase were 4.5 and >20 μM, respectively. The compound inhibited the growth of A431 human epidermoid carcinoma and MKN-7 human stomach cancer cell lines with IC_{50} values of 8 and 24 μM, respectively.

Many ligand-activated growth factor receptors and a number of oncogene products are protein tyrosine kinases[1]. Their aberrant activation has been implicated in human clinical cancers[2]. Translocation of the *abl* gene was found in chronic myelogeneous leukemia[3]. Amplification of the EGF receptor gene is often found in squamous cell carcinoma[4]. Amplification of its homolog, *erb*B-2, has been detected in breast, ovary and stomach adenocarcinoma[5,6]. Expression of *erb*B-2 protein has been known to be an indicator of a poor prognosis in these cancers[6~9]. Activation of other protein tyrosine kinases such as *met*[10], k-*sam*[11] and so on in some human cancers has also been reported.

A specific inhibitor for a certain enzyme is generally valuable for its further study. For some protein tyrosine kinases found in human cancers, an inhibitor would be a useful tool to study the relationships between kinase activity and cancer cell proliferation. The growth inhibitory effects of some EGF receptor kinase inhibitors on cultured cell lines[12~14] suggested that the screening for protein tyrosine kinase inhibitors may lead to new, selective drugs for treating cancer.

From this point of view, we have screened protein tyrosine kinase inhibitors of microbial origin, using EGF receptor kinase and found a novel inhibitor, BE-23372M. In this publication, we describe the producing organism, fermentation, isolation and biological activities of BE-23372M. The physico-chemical properties, structure elucidation and synthetic studies of this compound will be reported in the following papers[15,16].

Materials and Methods

Microorganism

The producing organism, strain F23372, was isolated from the bark of *Ginkgo bioba* L. collected in

Ageo City, Saitama Prefecture, Japan. This strain has been deposited in the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Japan, under the accession No. FERM P-12077.

Fermentation

The culture F23372 from an agar slant was inoculated into four 500-ml conical flasks each containing 100 ml of the following seed medium (pH 6.0): Polypepton 0.3%, glucose 1%, wheat germ 1%, gluten meal 0.5%, meat extract 0.3%, maltose 3%, NaCl 0.2%, $NaNO_3$ 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.0002%, $CuCl_2 \cdot 2H_2O$ 0.00004%, $MnCl_2 \cdot 4H_2O$ 0.00004%, $CoCl_2 \cdot 6H_2O$ 0.00004%, $ZnSO_4 \cdot 7H_2O$ 0.00008%, $Na_2B_4O_7 \cdot 10H_2O$ 0.00008% and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.00024%. These flasks were then incubated on a rotary shaker (180 rpm) at 28°C for 72 hours. The seed culture (2 ml each) was transferred into 145 flasks of the same type containing 100 ml of the above medium and incubated on a rotary shaker (180 rpm) at 28°C for 72 hours.

Protein Kinase Assays

EGF receptor was partially purified from A431 cells by means of WGA agarose column chromatography as described by AKIYAMA *et al.*[17]. The enzyme assay was performed in 50 μl of reaction mixture containing 1 μunit (1~2 μg protein) EGF receptor kinase, 20 mM HEPES·Na (pH 7.4), 30 mM $MgCl_2$, 0.5 mM $MnCl_2$, 150 mM NaCl, 1 μg/ml recombinant human EGF (Wakunaga Pharmaceutical Co., Osaka, Japan), 20 μM [γ-^{32}P]ATP (9.25 kBq/assay, Amersham), 0.1% Triton X100, 10% glycerol, 1 mg/ml poly(Glu : Tyr) 4 : 1 (Sigma) and the test compound. The mixture was incubated at 30°C for 30 minutes. The reaction was terminated with 10 μl of 70% trichloroacetic acid, and radioactivity incorporated into the acid precitable fraction was collected on Multiscreen HA filter (Millipore) and counted by a liquid scintillation counter. When RR-SRC peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, 1 mg/ml, Peptide Institute Inc., Osaka, Japan) was used as a substrate, the reaction was terminated with 25 μl of 10% trichloroacetic acid, and the radioactivity incorporated was determined using P81 paper (Whatman) as described by GLASS *et al.*[18].

Crude lysate containing protein kinase C was prepared from mouse brains, and the kinase activity was determined as described by KIKKAWA *et al.*[19]. cAMP-dependent protein kinase was assayed as described previously[20].

Cell Culture and Proliferation Assay

A431 human epidermoid carcinoma cells were cultured in DULBECCO's modified EAGLE's medium (DMEM) supplemented with 10% fetal calf serum (FCS), and MKN-7 human stomach cancer cells were grown in RPMI 1640 supplemented with 10% FCS. Both cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cells were seeded into 96-well microtiter plates (2×10^3 cells/well) and incubated for 24 hours. The test sample, dissolved in dimethyl sulfoxide, was added in serial dilutions. After addition, the plates were incubated for 72 hours. Cells were fixed with 50% trichloroacetic acid and stained with 0.4% sulforodamine B. The dye was extracted from the stained cells with 10 mM tris(hydroxymethyl)aminomethane solution. The absorbance of the extract was measured at 540 nm. IC_{50} value was calculated from the absorbance in the presence and absence of the inhibitor.

Results

Description of Producing Organism

Strain F23372 was characterized by the fast-growing mycelium covering 90 mm petri plates of 5 media at 28°C in 3 days (Table 1).

Neither sexual spores nor asexual spores were observed on the media used, while the scleotia were seen on potato-dextrose agar medium. The scleotia were black or brown and irregular in shape and size. Hyphae were light to dark brown and stout, 7.2~8.5 μm in diameter. Side branches originated in the

Table 1. The cultural characteristics of strain F23372.

Agar medium	Growth	Aerial mycelia	Soluble pigment
Czapek-Dox agar	Good	Abundant, reddish brown	++
Czapek-yeast extract agar	Good	Abundant, dark brown	++
Potato-dextrose agar	Good	Abundant, brown	++
Malt extract agar	Good	Moderate, light brown	−
Malt extract-peptone agar	Good	Abundant, light brown	+
Cornmeal agar	Poor	Poor, white	−

Observation after incubation at 28°C for a week.

Fig. 1. Photomicrograph of hyphae of strain F23372.

The inserted scale is 10 μm.

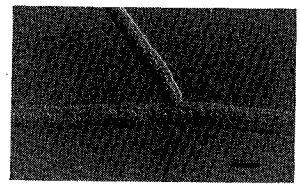

Table 2. The inhibitory effect of BE-23372M on protein kinases.

Protein kinase	Substrate	IC_{50} (μM)
EGF receptor kinase	Poly(Glu:Tyr)	0.02
EGF receptor kinase	RR-SRC	0.03
Protein kinase C	Histone (Lys rich)	4.5
cAMP-dependent protein kinase	Histone H2B	>20

distal part of the cell, were constricted at the point of origin and had septa formed close to the hypha. Clamp-connections were not seen (Fig. 1). The nuclear staining revealed that the strain is multinucleate.

Based on these cultural and morphological characteristics, strain F23372 was identified as *Rhizoctonia solani* (Agonomycetes)[21].

Isolation

After heating at 90°C for 10 minutes, the culture broth (*ca.* 14 liters), was premixed with Celite and filtered. The mycelial cake obtained was dispersed and soaked in 12 liters of acetone, and the mixture was stirred at room temperature for 1 hour. After filtering, the acetone extract was concentrated to about 1.8 liters, which was adjusted to pH 3 with 1 N HCl and extracted twice with ethyl acetate (each 1.8 liters). The ethyl acetate extract was combined and concentrated to dryness.

The residue obtained was dissolved in chloroform and applied to silica gel column (15 g, Kieselgel 60, Merck), which was washed with chloroform and then eluted with a mixture of chloroform-methanol-formic acid (100:10:1). The active fractions were collected and evaporated to dryness. The residue was dissolved in a mixture of chroloform-methanol-ethanol-water (5:2:2:1) and applied to a column of Sephadex LH-20 (2.5 i.d. × 50 cm, Pharmacia), and then developed with the same solvent mixture mentioned above. The active fractions were concentrated and applied to Sephadex LH-20 column chromatography again. The active fractions thus obtained were concentrated to give 5.7 mg of BE-23372M in the form of a reddish orange solid substance.

Biological Activities

To study the selectivity of BE-23372M, the inhibitory activity against protein kinase C and cAMP-dependent protein kinase together with EGF receptor kinase was examined. BE-23372M exhibited the strongest inhibitory activity against EGF receptor kinase with IC_{50} values 0.02 μM for poly(Glu:Tyr) 4:1 substrate, and 0.03 μM for RR-SRC substrate. On the other hand, IC_{50} values against protein kinase C and cAMP-dependent protein kinase were 4.5 μM and >20 μM, respectively (Table 2). These results indicate that BE-23372M is a potent and selective inhibitor of EGF receptor kinase.

The effect of BE-23372M on the cell proliferation was examined, using two human cancer cell lines A431 and MKN-7. A431 cells are known to have overexpressed EGF receptor[28], and MKN-7 cells are reported to be expressing *erb*-B2[29]. BE-23372M showed growth inhibitory activities against both cell lines. IC_{50} values against A431 and MKN-7 were 8 μM and 24 μM, respectively.

Discussion

Intracellular signalling pathways mediating the effect of growth factors and oncogenes on cell proliferation and transformation present a challenging new class of target sites for anticancer drug development. These pathways include GTP-binding proteins, protein tyrosine kinases, protein serine/threonine kinases, nuclear transcription factors and so on. Among them, protein tyrosine kinases are the most extensively studied, and much effort is being given to the search for protein tyrosine kinase inhibitors.

Genistein, isolated from *Pseudomonas* sp., was competitive inhibitor of the binding of ATP to protein tyrosine kinases[22,23]. Erbstatin was isolated from *Streptomyces* sp. as a potent EGF receptor kinase inhibitor, and blocked the peptide site of EGF receptor[12,24]. Lavendustin A, isolated from *Streptomyces griseolavendus*, was one of the most potent EGF receptor kinase inhibitors[25]. Beside naturally occurring inhibitors, some synthetic compounds are developed as EGF receptor protein kinase inhibitors[13,14,26,27].

BE-23372M, isolated from *Rhizoctonia solani* F23372, is a new member of these protein tyrosine kinase inhibitors.

BE-23372M was a potent inhibitor of EGF receptor kinase (IC_{50} 0.02~0.03 μM). The compound did not block the binding of EGF to its receptor (data not shown). The selectivity of this compound for the inhibition of EGF receptor kinase compared with protein kinase C and cAMP-dependent protein kinase was more than 150-fold (Table 2). Thus, the compound has been proved as a potent and selective inhibitor of EGF receptor kinase. The effects of BE-23372M on the other protein tyrosine kinases and on EGF receptor autophosphorylation in addition to the kinetic studies will be described elsewhere[30].

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