BE-23372M, a Novel and Specific Inhibitor for Epidermal Growth Factor Receptor Kinase

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The fungal metabolite BE-23372M is a structurally novel protein kinase inhibitor. Its IC_{50} for epidermal growth factor (EGF) receptor kinase was 0.03 μM. IC_{50} values of BE-23372M for other protein tyrosine kinases, *erb*B-2, $p43^{v-abl}$, insulin receptor kinase, and $p60^{c-src}$ were 0.42, 1.0, 3.3, and 4.5 μM, respectively, and the IC_{50} for protein kinase C, a serine/threonine kinase, was 4.1 μM. Cdc2 kinase, casein kinases I and II and cAMP-dependent protein kinase were not inhibited by 20 μM BE-23372M. A kinetic study showed that BE-23372M was competitive with respect to the substrate peptide and to ATP. Autophosphorylation of solubilized EGF receptor kinase was clearly inhibited by 0.1 μM BE-23372M. Autophosphorylation of EGF receptor in A431 cells was also inhibited. These results show that BE-23372M is a potent and specific EGF receptor kinase inhibitor. It should be a valuable tool for EGF receptor kinase research.

Key words: EGF receptor — Protein tyrosine kinase — Inhibitor

Protein tyrosine kinases are known to play an important role in signal transduction of growth factors.[1,2] Their aberrant activation seems to be implicated in human clinical carcinogenesis.[3,4] Translocation of the *abl* gene is found in chronic myelogenous leukemia.[5] Amplification of the epidermal growth factor (EGF) receptor gene is often found in squamous cell carcinoma,[6] and amplification of its homologue, *erb*B-2, has been detected in breast, ovary and stomach adenocarcinoma.[7,8] Expression of *erb*B-2 protein is an indicator of poor prognosis in these cancers.[8–11] Activations of other tyrosine kinases such as *met*[12] and k-*sam*[13] in human stomach cancers have also been reported. Thus, inhibitors of these tyrosine kinases would be useful tools for the study of the relationships between tyrosine kinase activation and the formation, growth, and malignancy of these cancers. In fact, growth-inhibitory effects of some EGF receptor kinase inhibitors on cultured cell lines have been reported.[14–16] Their effects in *in vivo* antitumor evaluation systems[16–18] were limited, but these findings suggest that screening for tyrosine kinase inhibitors could be a valuable approach for the discovery of new anticancer agents. From this point of view, we have screened for EGF receptor kinase inhibitors and discovered BE-23372M. BE-23372M is a metabolite of a fungus of *Rhizoctonia* species and consists of an enollactone ring and two catechol rings. Chemical synthesis of BE-23372M was also successful. In the present paper, we report the inhibitory specificity, mode, and potency of BE-23372M.

MATERIALS AND METHODS

Reagents   BE-23372M was synthesized by a method which will be reported elsewhere. Its structure is shown in Fig. 1. The compound was dissolved in dimethylsulfoxide and subjected to assays. Plasmid pCOB2N[19] containing the entire c-*erb*B-2 cDNA was a generous gift of Dr. M. Yamamoto, Institute of Medical Science, University of Tokyo. Erbstatin[14] was kindly provided by Dr. K. Umezawa, Department of Applied Chemistry, Keio University. Other tyrosine kinase inhibitors, methyl 2,5-dihydroxycinnamate,[20] tyrphostin,[21] lavendustin A,[22] and 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid[22] were purchased from GIBCO/BRL (Gaithersburg, MD, USA). Genistein[23] was obtained from Funakoshi Pharmaceutical Co. (Tokyo). Staurosporine[24] and K252a[25] were obtained from Kyowa Medex Co. (Tokyo).

Cell culture   Human epidermoid cell line A431 was purchased from Dainippon Pharmaceutical Co. (Osaka) and maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid-NaOH (HEPES-Na), pH 7.4. *Spodopetera frugiperda* cell line Sf9 was provided by Dr. Y. Matsuura of the National Institute of Health, Tokyo. The cells were maintained in TNM-FH medium containing 10% FBS.

Enzyme preparation and assay conditions   EGF receptor was partially purified from A431 cells using WGA agarose chromatography as described by Akiyama *et al.*[26] The enzyme assay was performed in 50 μl of assay

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Fig. 1.　Structure of BE-23372M.

tion at 37,000g for 30 min, the supernatant was used as the *erb*B-2 kinase. Expression of *erb*B-2 kinase was confirmed by tyrosine kinase assay, SDS-PAGE analysis, and Western blotting analysis using anti-erbB-2 antibody (c-neu Ab-3, Oncogene Science Inc., Uniondale, NY, USA). The enzyme assay was performed in 50 μl of the assay solution, containing 1 μU *erb*B-2 kinase, 20 mM HEPES-Na (pH 7.4), 5 mM MnCl$_2$, 150 mM NaCl, 20 μM [γ-^{32}P]ATP (9.25 kBq/assay), 1 mg/ml poly(EY), and 10% glycerol at 30°C for 10 min.

Insulin receptor kinase was partially purified from rat liver[31] and assayed[32] using 1 mg/ml poly(EY) as the substrate.

P60[c-*src*] and p43[v-*abl*] kinases, and their substrate peptide Raytide™, a modified gastrin analog, were obtained from Oncogene Science Inc., and assayed according to the manufacturer's protocols.

The cytosol fraction containing protein kinase C was prepared from mice cerebral tissues and assayed as described by Kikkawa *et al.*[33] using histone (lysine-rich, type III-S, Sigma) as the substrate. Casein kinases I and II, cdc2 kinase, and cAMP-dependent protein kinase were assayed as previously described.[27]

Intracellular autophosphorylation in A431 cells Semi-confluent A431 cells in a 24-well Multidish (Nunc Inc., Naperville, IL, USA) were washed three times with prewarmed D-MEM supplemented with 10 mM HEPES-Na (pH 7.4), and kept in 0.5 ml of medium for 1 h. BE-23372M was added to the culture at the concentrations indicated in the results section and the incubation was continued for a further 30 min. EGF was then added to 200 ng/ml and the incubation was continued for an additional 5 min. After the incubation, the cells were washed once with ice-cold phosphate-buffered saline and were lysed in 0.2 ml of SDS-PAGE sample buffer. The resulting lysate was briefly sonicated, and boiled for 5 min, then 10 μl aliquots were subjected to SDS-PAGE (7.5%) followed by semi-dry blotting as described by Kyhse-Andersen.[34] After blocking in Tris-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20, the blot was analyzed with monoclonal anti-phosphotyrosine antibody (Py-20, ICN, Costa Mesa, CA, USA) or anti-EGF receptor antibody (F4, Biomakor, Rehovot, Israel) and a Western blotting detection system (Amersham).

EGF binding to A431 cells A431 cells were cultured and treated as described in the above section except that 1.85 kBq/assay of 3-[^{125}I]iodotyrosyl EGF (Amersham) was also included at the final step of the incubation. After the incubation, the cells were washed 3 times with ice-cold medium and lysed in 0.5 ml of 0.1 N NaOH, 1% SDS, and 2% Na$_2$CO$_3$. Radioactivity bound to the cells was measured using a γ-counter. For the determination of nonspecific EGF binding, the cells were pretreated with

solution, containing 1 μU (1–2 μg protein) of 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), 20 μM [γ-^{32}P]ATP (9.25 kBq/assay, Amersham, Buckinghamshire, UK), 1 mg/ml poly(Glu, Tyr)4:1 (poly-(EY), Sigma, St. Louis, MO, USA), 0.1% Triton X-100, and 10% glycerol. The mixture was incubated at 30°C for 30 min. The reaction was terminated with 10 μl of 70% TCA and the resulting precipitate was collected using the MultiScreen Assay System (Millipore, Bedford, MA, USA). Radioactivity incorporated in the precipitate was counted by liquid scintillation methods. When the synthetic peptide substrate, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (RR-SRC, Peptide Institute Inc., Osaka) was used, the incorporated redioactivity was determined using P81 paper as described by Kitagawa *et al.*[27] The autophosphorylation reaction of solubilized EGF receptor was performed at 22°C for 2 min as described by Koland and Cerione.[28] The reaction was terminated by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. After boiling for 2 min, the samples were subjected to SDS-PAGE (7.5% polyacrylamide) followed by autoradiography.

The cytoplasmic domain (from Met-692 to Pro-1260) of human *erb*B-2 kinase was obtained using a baculovirus expression system with a strategy similar to that reported for the expression of rat *neu* protein.[29] Briefly, 1.78 kbp *Mro*I-*Aat*I fragment of *erb*B-2 cDNA was cloned into *Xma*I-*Eco*RI sites of plasmid pVL13-93.[30] Recombinant baculovirus was then prepared using the BaculoGold™ transfection system (Pharmingen, San Diego, CA, USA). Recombinant virus-infected Sf9 cells were lysed in hypotonic buffer consisting of 20 mM HEPES-Na (pH 7.4), 10 mM NaCl, 0.2 mM EDTA, 2 μg/ml each aprotinin, leupeptin and antipain, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. After centrifuga-

100-fold excess of radioinert EGF prior to the labeled EGF addition. Specific EGF binding was obtained by subtracting the nonspecific binding from total binding.

RESULTS

Effect on various protein kinases Table I shows the IC$_{50}$ values of BE-23372M on various protein kinases. The most potent effect of BE-23372M was seen on EGF receptor kinase, with an IC$_{50}$ value of 0.03 μM when poly-(EY) or RR-SRC was used as the substrate. Baculovirus-expressed erbB-2 kinase was inhibited with an IC$_{50}$ value of 0.42 μM, approximately one-fourteenth of that for EGF receptor kinase. An effect of BE-23372M on the p43^{v-abl} kinase was seen at 1.0 μM. The effects of BE-23372M on insulin receptor kinase and p60^{c-src} kinase were less potent, with IC$_{50}$ values of 3.3 μM and 4.5μM, respectively. Among serine and threonine kinases, BE-23372M up to 20 μM could not inhibit the activities of cdc2 kinase, casein kinases I and II, or cAMP-dependent

protein kinase by 50%. Only protein kinase C was moderately inhibited by BE-23372M with an IC$_{50}$ of 4.1 μM.

Table II compares the effect of BE-23372M with those of other known kinase inhibitors in our assay systems. BE-23372M was the most potent EGF receptor kinase inhibitor among the compounds tested. Typical-dose response kinetics of BE-23372M, erbstatin, and staurosporine on both kinases are shown in Fig. 2.

Kinetic analysis For the kinetic studies on the inhibitory effect of BE-23372M on EGF receptor kinase, RR-SRC was used as the phosphate acceptor. Fig. 3 shows the results. BE-23372M was competitive with respect to not

Table I. Inhibitiry Effect of BE-23372M on Various Protein Kinases

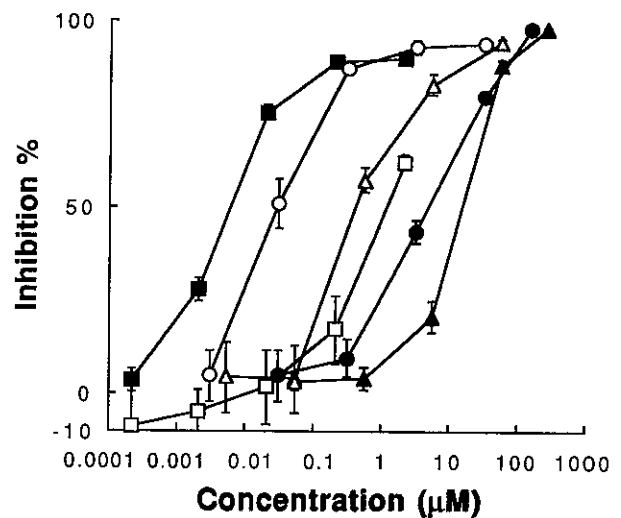| Kinase	Substrate	IC$_{50}$ (μM)
EGF receptor kinase	poly(EY)	0.03
	RR-SRC	0.03
erbB-2 kinase	poly(EY)	0.42
Insulin receptor kinase	poly(EY)	3.3
p43^{v-abl} kinase	Raytide	1.0
p60^{c-src} kinase	Raytide	4.5
Protein kinase C	Histone (lysine rich)	4.1
Cdc2 kinase	Histone H1	>20
Casein kinase I	Casein	>20
Casein kinase II	Casein	>20
cAMP dependent kinase	Histone H2B	>20

Fig. 2. Typical dose response kinetics of BE-23372M, erbstatin, and staurosporine on EGF receptor kinase and protein kinase C. ○ and ●, BE-23372M; △ and ▲, erbstatin; □ and ■, staurosporine on EGF receptor kinase and protein kinase C, respectively. Assay was done in triplicate. Bars represent SD.

Table II. Effect of Various Protein Tyrosine Kinase Inhibitors on EGF Receptor Kinase and Protein Kinase C

Compound	IC$_{50}$ (μM)[a]	
	EGF receptor kinase	Protein kinase C
BE-23372M	0.03±0.003	4.1±1.0
Erbstatin	0.63±0.19	20±4.1
Methyl 2,5-dihydroxycinnamate	1.2±0.33	52±15.3
Tyrphostin	21±8.0	114±12
Lavendustin A	0.06±0.028	14±6.7
2-Hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid	0.25±0.13	114±59
Genistein	5.1±0.86	>370
Staurosporine	0.96±0.16	0.003±0.0016
K252a	9.5±1.52	0.05±0.016

a) Mean±SE of three independent experiments.

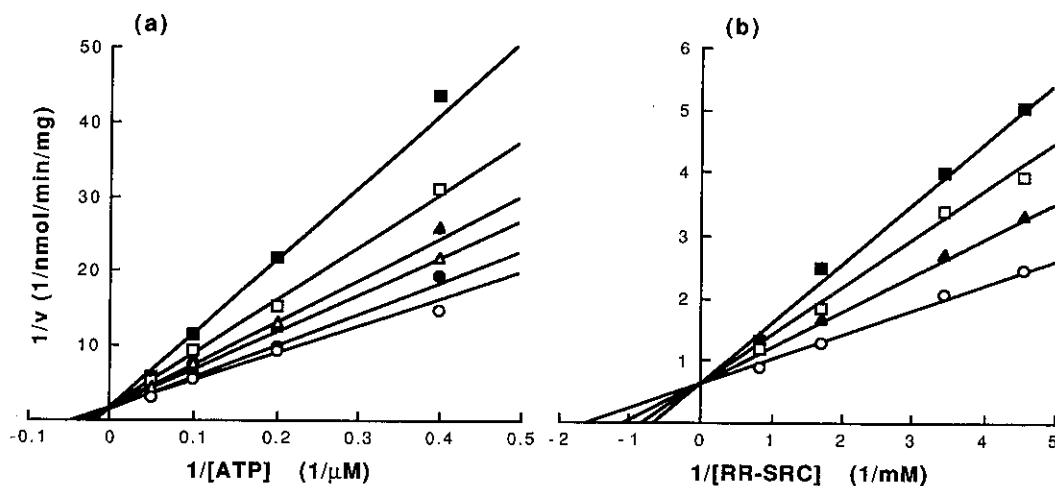Fig. 3. Effect of BE-23372M on the kinetics of the EGF receptor kinase activity. O, 0 n*M*; ●, 32 n*M*; △, 64 n*M*; ▲, 96 n*M*; □, 192 n*M*; ■, 320 n*M* BE-23372M. (a), versus ATP; (b), versus RR-SRC peptide.
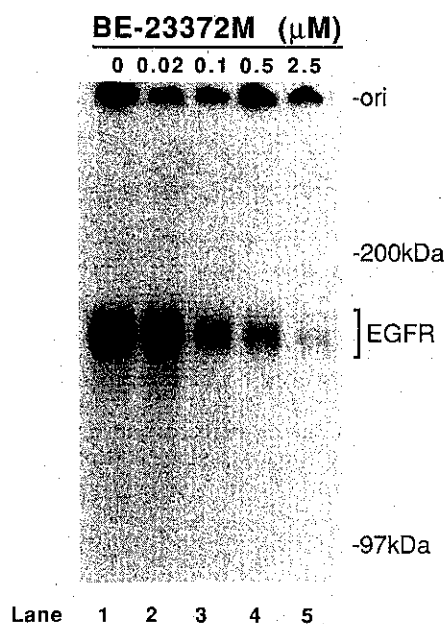

Fig. 4. Effect of BE-23372M on the autophosphorylation of solubilized EGF receptor *in vitro*. Molecular weight markers are indicated on the right. EGFR represents ~170 kDa EGF receptor.

only ATP (Fig. 3a), but also RR-SRC (Fig. 3b). The K_i values of BE-23372M for the substrates obtained from Dixon plot analyses were 0.16 μM each, which were 1/4000 and 1/170 of the K_m values in our assay system (RR-SRC; 640 μM, ATP; 27 μM), respectively.

Effect on the autophosphorylation of purified EGF receptor The effect of BE-23372M on the autophosphorylation of solubilized EGF receptor was determined by SDS-PAGE followed by autoradiography. As shown in Fig. 4, autophosphorylation of solubilized EGF receptor was clearly inhibited by 0.1 μM BE-23372M. Densitometric analysis of the autoradiogram indicated that the IC_{50} value of BE-23372M in this assay system was 0.085 μM.

Effect of BE-23372M on the autophosphorylation of EGF receptor in intact cells Next, the inhibitory effect of BE-23372M on the EGF receptor autophosphorylation induced by EGF in A431 cells was determined. Fig. 5a shows the results of Western blotting of A431 cellular proteins detected with anti-phosphotyrosine antibody. The assay was done in duplicate and the autophosphorylation of EGF receptor was clearly induced by the addition of EGF (lanes 1–4). When the cells were treated with BE-23372M prior to EGF addition, the autophosphorylation was inhibited, in accordance with the *in vitro* study. The inhibitory effect at 80 μM BE-23372M was equivocal (lanes 5 and 6), but clear inhibition was seen at 160 μM (lanes 7 and 8). The results of Western blotting analysis on the same samples with anti-EGF receptor antibody showed that the EGF receptor number was not apparently affected in this concentration range (Fig. 5b, lane 5-8). At 240 μM, EGF receptor autophosphorylation was strongly inhibited, and the EGF receptor level seemed to decrease slightly (Fig. 5a and b, lanes 9 and 10). Therefore, to rule out the possibilities that BE-23372M affected the EGF receptor number in the cell, and that it inhibited the EGF binding to the receptor, the effect of BE-23372M on [^{125}I]EGF binding

-EGF +EGF

BE-23372M (µM)

0 0 80 160 240

(a) -200kDa
EGFR
-97kDa

(b) -200kDa
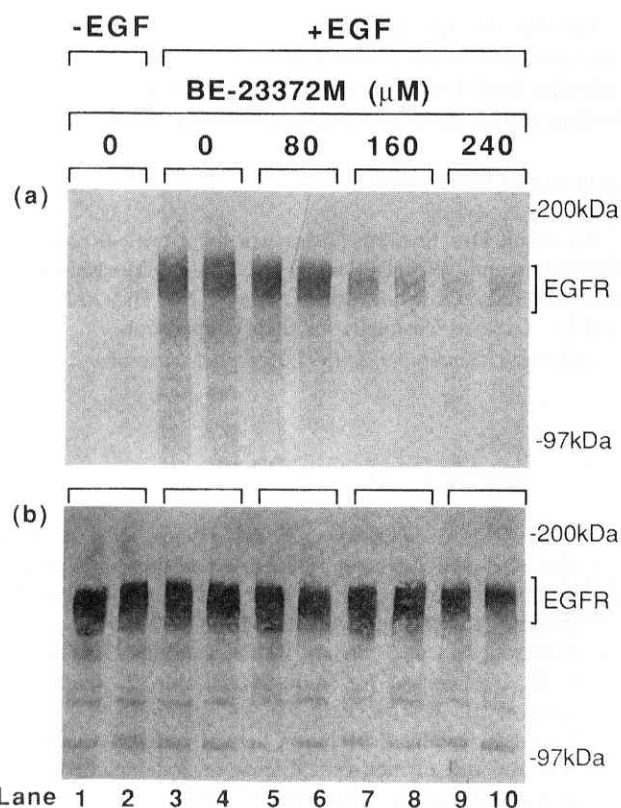EGFR
-97kDa

Lane 1 2 3 4 5 6 7 8 9 10

Fig. 5. Effect of BE-23372M on the autophosphorylation of EGF receptor in intact A431 cells. Molecular weight markers are indicated on the right. EGFR represents ∼170 kDa EGF receptor. (a), analyzed with anti-phosphotyrosine antibody Py-20; (b), analyzed with anti-EGF receptor antibody F4.

Table III. Effect of BE-23372M on EGF Binding to A431 Cells

BE-23372M (μM)	Specific EGF binding[a] (% of control, mean $\pm$ SD)
0	100 ± 1.5
80	104 ± 1.9
160	104 ± 3.2
240	112 ± 0.7

a) Specific [^{125}I]EGF binding to the control group was 7,329 ± 112 (mean cpm $\pm$ SD). Assay was done in triplicate.

to the receptor was investigated. The results are shown in Table III. BE-23372M did not show any inhibition of EGF receptor binding of [^{125}I]EGF in A431 cells under the same assay conditions. Overall, these results strongly suggested that BE-23372M directly inhibited intracellular EGF receptor autophosphorylation without affecting the receptor number or EGF-receptor binding.

Under the same assay conditions, the effects of other kinase inhibitors listed in Table II were weak, or not observed. Only genistein showed an inhibitory effect at 185 μM (data not shown).

DISCUSSION

Our present studies have proved that BE-23372M is the most potent EGF receptor kinase inhibitor among the tyrosine kinase inhibitors tested. BE-23372M inhibited not only exogenous substrate phosphorylation, but also EGF receptor autophosphorylation with IC$_{50}$ values of 30 nM and 85 nM, respectively. The inhibitory effect of BE-23372M was specific to EGF receptor kinase, with only weak inhibition of other protein kinases, including erbB-2, of which the kinase domain was reported to have 82% homology to that of EGF receptor kinase.[35] The EGF receptor kinase used in the study is a full-length glycoprotein, which was solubilized and purified from A431 cells, and the erbB-2 kinase is a soluble recombinant protein lacking extracellular and transmembrane domains. It is conceivable that these structural differences of the kinases might have affected the inhibition specificity of BE-23372M. However, kinetic studies strongly suggested that BE-23372M really acted on the catalytic site of EGF receptor kinase. RR-SRC, used in the studies, was also a good substrate for EGF receptor kinase, while it did not act as a substrate for our erbB-2 assay system (data not shown). Such substrate preferences might explain why BE-23372M was more specific to EGF receptor kinase.

The present studies also showed that BE-23372M had a unique mode of inhibition; it was competitive to both substrates. Traxler et al.[16] reported the design of a bisubstrate type inhibitor for EGF receptor kinase with tyrosine-mimicking and diphosphate-mimicking moieties in the compound. From the structural similarity to other peptide-competitive inhibitors such as tyrphostins,[21] the catechol moiety of BE-23372M might be a tyrosine mimic. However, it remains unclear which catechol moiety mimics tyrosine, and which part of the compound is a diphosphate mimic.

Recent studies have shown that the signals from the activated growth factor receptors were further transduced by a set of cytoplasmic proteins, which bind on the phosphorylated tyrosine residues of the receptor through their src-homology-2 domains.[36] Therefore, inhibition of EGF receptor autophosphorylation is considered important for the blockade of growth signal transduction. As in the case of solubilized EGF receptor, intracellular EGF receptor autophosphorylation was also inhibited by BE-23372M. The data suggest that BE-23372M will be a good tool for EGF receptor kinase research. A higher concentration of BE-23372M than in the case of the cell

free system was, however, needed for the inhibition of intracellular EGF receptor autophosphorylation. Similarly, approximately 25 μM BE-23372M was needed for the inhibition of the growth of A431 cells by 50% (data not shown). The reason why a high concentration of BE-23372M was needed for the inhibition of intracellular EGF receptor autophosphorylation might be poor membrane penetrability of BE-23372M. An inappropriate intracellular distribution of BE-23372M is another possibility. It is also possible that abundant intracellular ATP[37] might have weakened the effect of BE-23372M, since it was in part competitive to ATP.

Finally, derivatization of BE-23372M should lead to improvement of the activity against intact cells, affording valuable tools for EGF receptor kinase research, and for further elucidation of structure-activity relationships.

ACKNOWLEDGMENTS

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