VOL. 52 NO. 12, DEC. 1999 THE JOURNAL OF ANTIBIOTICS pp. 1086 — 1094

Resorcylic Acid Lactones: Naturally Occurring Potent and Selective Inhibitors of MEK

ANNIE ZHAO*, SEOK H. LEE[†], MARINA MOJENA[a], ROSALIND G. JENKINS, DENIS R. PATRICK[b], HANS E. HUBER[b], MICHAEL A. GOETZ, OTTO D. HENSENS, DEBORAH L. ZINK, DOLORES VILELLA[a], ANNE W. DOMBROWSKI, RUSSELL B. LINGHAM and LEEYUAN HUANG

Merck Research Laboratories,
P. O. Box 2000, Rahway, NJ 07065, USA
[a] Merck Sharp & Dohme de España,
S. A. Josefa Valcarcel 38, 28027 Madrid, Spain
[b] Merck Research Laboratories,
770 Sumneytown Pike, West Point, PA 19486, USA

(Received for publication April 26, 1999)

A resorcylic acid lactone, L-783,277, isolated from a *Phoma* sp. (ATCC 74403) which came from the fruitbody of *Helvella acetabulum*, is a potent and specific inhibitor of MEK (Map kinase kinase). L-783,277 inhibits MEK with an IC_{50} value of 4 nM. It weakly inhibits Lck and is inactive against Raf, PKA and PKC. L-783,277 is an irreversible inhibitor of MEK and is competitive with respect to ATP. L-783,290, the *trans*-isomer of L-783,277, was isolated from the same culture and evaluated together with several semi-synthetic resorcylic acid lactone analogs. A preliminary structure-activity relationship is presented. Several independent cell-based assays have been carried out to study the biological activities of these resorcylic acid lactone compounds and a brief result summary from these studies is presented.

MEK[††] is a threonine/tyrosine specific kinase involved in the MAP kinase cascade that begins with the stimulation of the proto-oncoprotein Ras in response to a wide variety of extracellular stimuli such as nerve growth factor, insulin, platelet derived-growth factor and expression of some oncogenes[1,2]. Located directly upstream of MEK, the raf kinases are activated by Ras[4] and MEK is then phosphorylated and activated by Raf[3]. MEK consists of two dual specificity protein kinase isoforms, MEK1 and MEK2[13,14]. The activation of MEK1 by Raf involves the phosphorylation of two serine residues within the catalytic domain[15]. Available data suggest that dominant negative mutants of Raf block the activation of the MAP extracellular signal regulated kinase (ERK2) induced by growth factors and oncogenic $p21^{ras}$[16]. Furthermore, the constitutively activated forms of Raf and MEK are both oncogenic[5,6]. Therefore, specific inhibitors of Raf or MEK may be of use as potential anti-cancer therapeutic agents.

Two MEK inhibitors, PD098059 and Ro 09-2210, have been reported recently[17,18]. Ro 09-2210 inhibits activated MEK *in vitro* with an IC_{50} of 60 nM[18]. PD098059 inhibits MEK activity by blocking the Raf-mediated activation of MEK *in vitro* with an IC_{50} 2~7 μM and was shown to be a selective inhibitor of activation of MEK *in vivo*[19].

L-783,277 was discovered from organic extracts of a *Phoma* sp. (ATCC 74403) as a potent and selective inhibitor of MEK. Both L-783,277 and Ro 09-2210 are closely related to hypothemycin, a known resorcylic acid

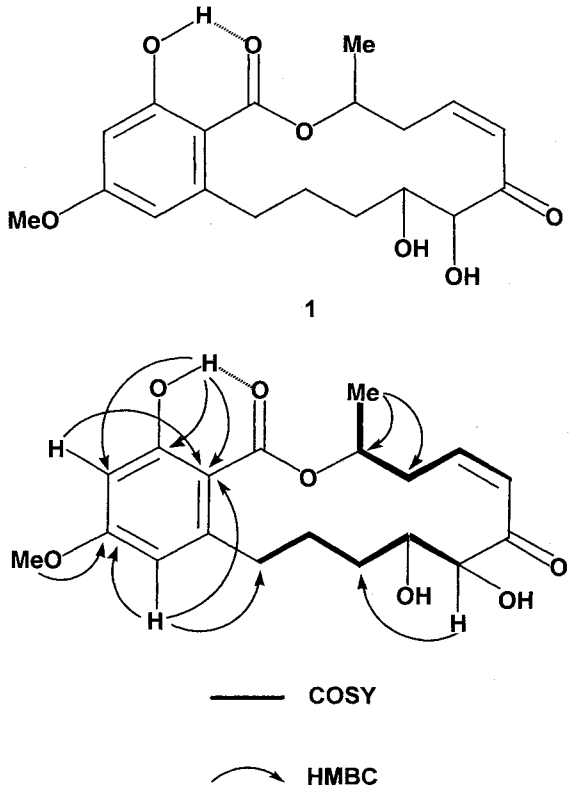
[†] Current address: Korea Food and Drug Administration, Division of Drug Evaluation, 5 Nokbun-Dong, Eunpyung-Gu, Seoul 122-704, Korea.

[††] Abbreviations used: MAP kinase (MapK), mitogen-activated protein kinase; ERK, extrcellular regulated kinase; MEK, MAP kinase kinase; PKA, cyclic-AMP-dependent protein kinase A; PKC, protein kinase C; BSA, bovine serum albumin; DTT, dithiothreitol; GST, glutathione-*S*-transferase; DMSO, dimethyl sulfoxide; ATP, adenosine 5′-triphosphate. EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)*N*,*N*′-tetraacetic acid; MBP, Myelin Basic Protein; SPA, scintillation proximity assay.

Table 1. The ^{1}H and ^{13}C NMR assignments for L-783,277 in CD_2Cl_2 (400 MHz).

Position	δ ^{13}C ppm	δ ^{1}H ppm
1	172.2	
3	74.0	5.40 m
4	37.6	2.54 (ddt, 2.8, 17.2, 2.8)
		3.32 (dt, 17.6~11.2)
5	146.7	6.24 (dt, 2.4, 11.6)
6	126.7	6.37 (dd, 3.2, 11.6)
7	200.3	
8	81.5	4.64 (d, 2.4)
9	73.6	3.78 m
10	33.5	~1.15 m
		~1.48 m
11	29.4	~1.48 m
		~1.69 m
12	37.0	~2.47 m
		2.94 (ddd, 3.2, 11.2, 14.8)
12a	148.0	
13	109.7	6.28 (d, 2.8)
14	164.8	
15	99.4	6.31 (d, 2.8)
16	166.6	
16a	105.0	
17	21.2	1.40 (d, 6.4)
18	55.9	3.78 s
C16-OH		12.40 s

Fig. 1. COSY ^{1}H-^{1}H and HMBC long-range ^{1}H-^{13}C correlations of L-783,277.



lactone identified as an antifungal agent[7,8]. A recent patent[27] describes the uses for the compounds of this class as inhibitors of protein kinases, especially tyrosine kinases. This paper will present the biological activities of L-783,277 and several other resorcylic acid lactones as inhibitors of MEK.

Materials and Methods

Methods

Fermentation

The identification and fermentation of the culture that produced L-783,277 will be reported separately by DOMBROWSKI *et al.*[25]

Isolation and Structural Determination of L-783,277

The novel L-783,277 and the known *trans* isomer L-783,290 were both purified from fermentations of *Phoma* sp. (ATCC 74403) which came from the fruitbody of *Helvella acetabulum*. Methylethylketone extracts were defatted with hexanes then subjected to a sequence of column chromatographies on silica gel and preparative HPLC to afford the two compounds. Yields of L-783,277, crystallized from methanol, reached 600 mg/liter in early fermentations[25].

The structure of L-783,277 was established on the basis of NMR and MS data. The molecular formula was determined as $C_{19}H_{24}O_7$ (calculated *m/z* 364.1538, found *m/z* 364.1530) by scanning high-resolution electron-impact mass spectrometry (HR-EIMS) and the molecular weight of 364 confirmed by negative and positive electrospray ionization (ESI). The ^{13}C NMR and ^{1}H-^{13}C HMQC data (see Table 1) confirmed the carbon count of 19 and the number of carbon-bound protons as 21 thereby suggesting three exchangeable OH protons. Analysis of the ^{1}H-^{1}H COSY (see bold lines, Figure 1), HMQC and long-range ^{1}H-^{13}C HMBC (see arrows, Figure 1) data indicated the structure **1**, isomeric with the zearalenone-like metabolite

LL-Z1640-1 but with the *cis* ($^3J_{5,6}$=11.6 Hz) double bond in the $\Delta^{5(6)}$ position instead of being conjugated with the aromatic ring.[26]

Raf, MEK Protein Kinase Assays

Both Raf and MEK protein kinases were assayed in the same assay buffer containing 25 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.2 mM Na_3VO_4, 0.1 mg/ml bovine serum albumin, 2 mM β-mercaptoethanol, and 10 μM [γ-^{33}P]ATP (20 μCi/ml). The substrates for Raf and MEK kinase assays were 25 μg/ml of kinase-inactive MEK1, 30 μg/ml of kinase-inactive GST-MapK, respectively. Fermentation extracts or purified compounds were diluted in 50% aqueous DMSO. The final DMSO concentrations were 2.5% for both assays. Reactions were run at room temperature and terminated with an equal volume of 100 mM EDTA, 100 mM sodium pyrophosphate. Phosphorylated product was captured in 96-well Immobilon-P filter plates (Millipore). Filters were washed 5 times with H_2O and counted on a TopCount microplate scintillation counter in the presence of Microscint (Packard).

The same protocol was followed as described above when different concentrations of ATP were used.

Lck Protein Kinase Assay

The Lck protein kinase assay using the SPA technique was performed as described[20,21,24].

Protein Kinase C (PKC) Assay

The procedures for the PKC assay are modified from those developed by GOPALAKRISHNA *et al.*[22] Fermentation extracts or purified compounds (5 μl) in 50% aqueous DMSO were mixed with 0.06 nM PKC, 4 μM MBP as substrate, 10 μM ATP and 0.7 μCi of [γ-^{33}P]ATP in final volume of 100 μl containing 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.25 mM EGTA, 0.4 mM $CaCl_2$, 100 μg/ml BSA, 0.32 mg/ml phosphatidylserine, 0.032 mg/ml diacylglycerol and 1 mM β-mercaptoethanol. The mixtures were incubated for 20 minutes at room temperature and filter plates were processed as described above.

Protein Kinase A (PKA) Assay

The procedures for the PKA assay are modified from those developed by PITT and LEE[23]. Fermentation extracts or purified compounds (5 μl) in 50% aqueous DMSO were mixed with 7 nM PKA, 10 μM Kemptide as substrate, 10 μM ATP and 0.7 μCi of [γ-^{33}P]ATP in final volume of 100 μl containing 40 mM Tris pH 7.5, 20 mM $MgCl_2$, 0.1 mM EDTA, 100 μg/ml BSA and 1 mM β-mercaptoethanol. The mixtures were incubated for 30 minutes at room temperature and filter plates were processed as described above.

Enzyme-inhibitor Preincubation Studies to Examine whether L-783,277 is a Time-dependent Inhibitor of MEK

Serial dilutions of compounds were mixed with MEK in enzyme dilution buffer A (25 mM Hepes, 0.2 mM sodium orthovanadate, 0.5 mM EDTA and 100 μg/ml BSA) for either 0 or 60 minutes at room temperature. Substrate and ATP mix containing 30 μg/ml catalytically inactive GST-MapK, 10 μM ATP, 1.4 μCi of [γ-^{33}P]ATP, 5 mM $MgCl_2$, and 2.4 mM β-mercaptoethanol in final volume of 100 μl was then added to initiate the MEK kinase assay. The reactions were stopped after 90 minutes at room temperature and processed as described above.

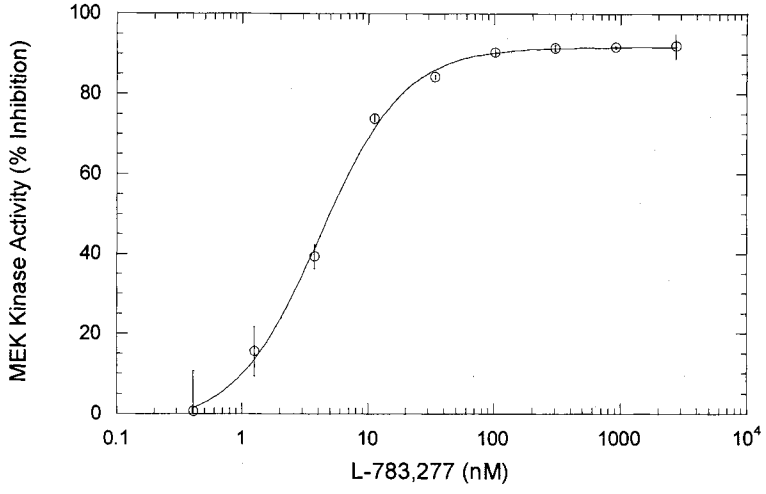
Assay to Examine whether L-783,277 is a Reversible Inhibitor of MEK

Three separate enzyme-inhibitor binding reactions were set up simultaneously with reaction tube 1 containing MEK and 50% DMSO, reaction tube 2 containing MEK and staurosporine (300 nM), and reaction tube 3 containing MEK and L-783,277 (220 nM), in 400 μl of enzyme dilution buffer A. After 30 minutes at room temperature, aliquots (20 μl) were removed and assayed directly as preincubation controls. The remainder of the incubation mix was applied to separate Dextran plastic desalting columns (5 ml bed volume) equilibrated with enzyme dilution buffer A. The columns were eluted with enzyme dilution buffer A and a total of 12 fractions (400 μl each) from each column were collected. 20 μl of the eluates from fractions #3 to #10 were tested for MEK kinase activity together with the preincubation controls as described above. Protein elution profiles were monitored by fluorescence[12] using the fluoraldehyde reagent solution (OPA) following the manufacture's instructions. The conditions for studying the reversibility of L-783,277 against Lck were similar to the above procedure using Lck enzyme and Lck dilution buffer B (50 mM MOPS, 10 mM $MgCl_2$, 0.7 mg/ml BSA). Column fractions were tested for Lck activity as described[20,21,24].

Protein Kinases and Substrates

Human wild-type MEK1 and a kinase-inactive form of MEK1 (K97A, Lys97 to Ala substitution) with amino-terminal middle T-epitopes were produced from baculovirus-infected Sf9 cells. For that purpose MEK1 was cloned into the pVL1393 vector and expressed using the BaculoGold system (PharMingen). Activated MEK1 was obtained by co-infection with viruses expressing MEK1, c-

Fig. 2. The effect of L-783,277 on MEK kinase activity.



MEK kinase assays were performed in the presence of L-783,277 at the indicated concentrations. After 2 hours at room temperature, the assays were stopped and processed as described in the Methods section. The data shown are the means±SD (n=3) from a single experiment and are representative of three separate experiments.

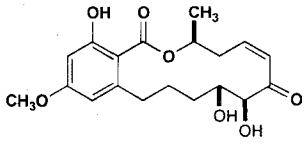
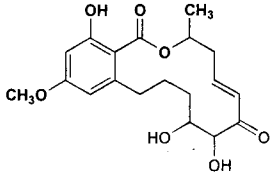
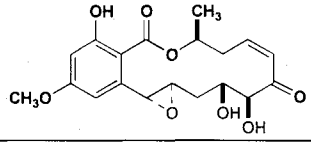
Raf, Lck, and activated H-Ras at multiplicity of infection of 10:2:2:2. The cells were lysed by sonication in 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM Na_3VO_4, 50 mM NaF, 1 mM EDTA, 1 mM $MgCl_2$, 1% Triton X-100, protease inhibitor cocktail (10 μg/ml benzamidine, 5 μg/ml each of leupeptin, aprotinin, pepstatin, 1 mM AEBSF), 1 mM dithiothreitol, 5 mM sodium pyrophosphate, and 25 mM sodium glycerolphosphate (buffer A). The soluble fraction was loaded onto a GammaBind Plus column (Pharmacia) preloaded with 17 mg of anti-middle T antibody (from Gernot Walter, UCSD) per ml of resin. The column was washed with three column volumes of each of the following: buffer A, then 25 mM Tris pH 8, 0.1 mM EGTA, 0.1 mM EDTA, protease inhibitor cocktail, 1 mM dithiothreitol, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate and 25 mM sodium glycerolphosphate (buffer B) containing 0.01% Triton X-100, and buffer B without Triton. Protein was eluted with 50 μg/ml of Glu-Tyr-Met-Pro-Met-Glu peptide in buffer B. Peak fractions were pooled and aliquots stored at −70°C in the presence of 0.1 mg/ml BSA and 20% glycerol. Recombinant full-length human c-Raf with a carboxyl-terminal middle T-epitope tag, Glu-Tyr-Met-Pro-Met-Glu were cloned, expressed, and purified as described for MEK1. A kinase-inactive form of MapK (K52R, Lys52 to Arg substitution) as substrate of MEK1 were expressed in *E. coli* as GST-fusion proteins in pGEX-4T vectors (Pharmacia) and purified over glutathione resin following the manufacturer's protocols.

The recombinant Lck kinase domain was purified and Lck substrate ([ε-aminohexanoyl]Biotin-EQEDEPEGIY-GVLE-NH2) was synthesized as described by PARK *et al.*[24]

Materials

ATP and [γ-^{33}P]ATP were obtained from Pharmacia and New England Nuclear, respectively. BSA, β-mercaptoethanol, calcium chloride, EDTA, EGTA, Hepes, Kemptide, sodium orthovanadate, sodium pyrophosphate were obtained from Sigma. Diacylglycerol and phosphatidylserine were from Avanti Polar Lipids, Inc. DMSO and methanol were from Fisher Scientific. Magnesium chloride was from Fluka. Phosphoric acid and Tris were from EM Science. PKA, PKC and MBP were obtained from Upstate Biotechnology (Lake Placid, NY). Dextran plastic desalting columns and fluoraldehyde reagent solution (OPA) were from Pierce. MultiScreen Immobilon-P™ filtration plates and MultiScreen-PH phosphocellulose plates were from Millipore. Adapter plates and Microscint-20™ scintillation fluid were from Packard.

Table 2. Structures and IC_{50} results (in nM) of the resorcylic acid lactones tested in MEK kinase and several other protein kinases (RAF, PKC, PKA and Lck).

Compound	Structure	MEK	RAF	PKC	PKA	Lck
L-783,277		4	>13000	>13000	>13000	750
L-783,290		300	>55000	>50000	>50000	45000
Hypothemycin		15	>88000	>52000	>88000	2800

Each compound was titrated in triplicate in the indicated kinase assays. The average percent inhibitions were calculated and the IC_{50} of each compound was determined by a semi-log plot of the percent inhibition of the compound versus the concentration of compound tested.

Results and Discussion

L-783,277 is a Potent and Selective MEK Inhibitor

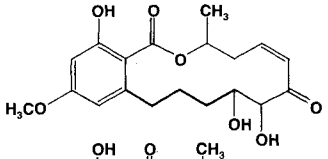
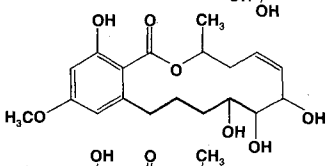
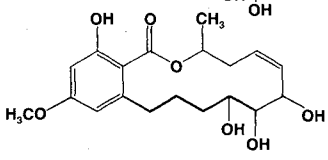
L-783,277, L-783,290 and hypothemycin were tested against several protein kinases to determine the specificity of these resorcylic acid lactones. L-783,277 is a potent inhibitor of MEK exhibiting an IC_{50} of 4 nM (Figure 2 and Table 2) and weakly inhibits Lck (IC_{50}=750 nM, a 188-fold difference, Table 2). L-783,277 does not inhibit Raf, PKC and PKA activities (Table 2), suggesting that L-783,277 is a potent and specific inhibitor of MEK. L-783,290 is a weaker inhibitor of MEK activity (IC_{50}=300 nM, Table 2) and does not inhibit other kinases. Hypothemycin is 2~3 fold less active than L-783,277 against MEK (IC_{50}=15 nM) and is a weaker inhibitor of Lck (IC_{50}=2800 nM, a 187-fold difference, Table 2).

L-783,277 is a Time-dependent and ATP Competitive Inhibitor of MEK

To determine if L-783,277 was a time-dependent inhibitor, L-783,277 was either pre-incubated with MEK for 60 minutes or mixed with the enzyme immediately before initiating the reaction with substrate. The results (Table 3) indicate that the inhibitory activity of L-783,277 increased with 60 minutes pre-incubation (IC_{50}=0.4 nM) when compared to no pre-incubation (IC_{50}=4 nM).

Preliminary experiments to determine the mode of inhibition of L-783,277 were performed by varying the ATP concentration in the assay at different L-783,277 concentrations. The IC_{50} values of L-783,277 at 2 μM, 10 μM and 100 μM ATP were 0.6 nM, 5.5 nM and 80 nM, respectively, suggesting that L-783,277 was a competitive inhibitor with respect to ATP. A detailed analysis of the effect of ATP on the association kinetics between MEK and L-783,277 will be reported by PATRICK *et al.* (manuscript in preparation). Similar results were obtained with L-783,290 and hypothemycin: the apparent inhibitory potential of these two compounds decreased as the ATP concentrations increased suggesting that they were ATP competitive inhibitors. All these data suggest that inhibition of L-783,277 is directed towards the MEK active site.

Table 3. MEK inhibition by L-783,277 is time-dependent as demonstrated in the pre-incubation study (assays were performed as described in Methods section).

Compounds	Structure	No pre-incubation	60 minutes pre-incubation
L-783,277		4	0.4
Dihydro-L-783,277, major isomer		1,600	200
Dihydro-L-783,277, minor isomer		20,000	4,200

IC_{50} values (in nM) were obtained as described in Table 1. Reduction of the α, β unsaturated ketone of L-783,277 dramatically decreased the potency of this compound (see text for details).

The α, β Unsaturated Ketone of L-783,277 is Critical for MEK Inhibitory Activity

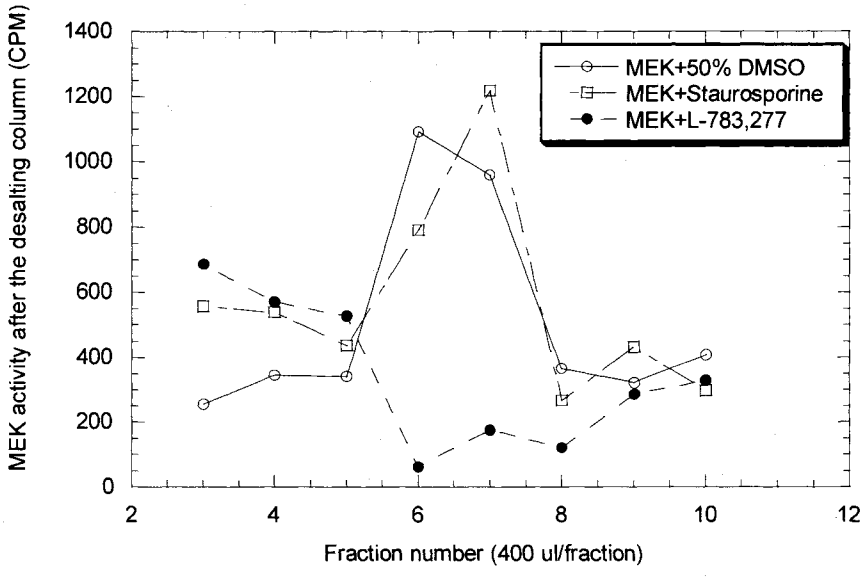
To examine whether the α, β unsaturated ketone of L-783,277 was essential for activity, this moiety was reduced with $NaBH_4$. The expected two isomers were weaker inhibitors of MEK with IC_{50} values of 1,600 and 20,000 nM (Table 3). These data suggest that inhibition of MEK by L-783,277 was most likely occurring through an interaction between the α, β unsaturated ketone of L-783,277 and the active site of MEK. This also potentially explains why preincubation of L-783,277 with MEK results in an apparent greater potency: exposure of MEK to L-783,277 promotes adduct formation between MEK and L-783,277 over time. Preincubation of the two hydroxyl isomers of L-783,277 with MEK increased their potency about 4 to 8 fold (Table 3). It is not clear whether this apparent greater potency is caused by a very low level L-783,277 present in these two compounds. This also may indicate that the isomers, as well as L-783,277, could be time-dependent inhibitors.

L-783,277 is an Irreversible Inhibitor for MEK but a Reversible Inhibitor for Lck

The data obtained from the preincubation study as well as from data with the reduced isomers of L-783,277 led us to examine whether L-783,277 was an irreversible inhibitor of MEK. A gel filtration technique was developed to examine the reversibility of L-783,277. Inhibitors were preincubated with enzyme and applied to the gel filtration column as described in the Methods section. Fractions were collected and tested as described.

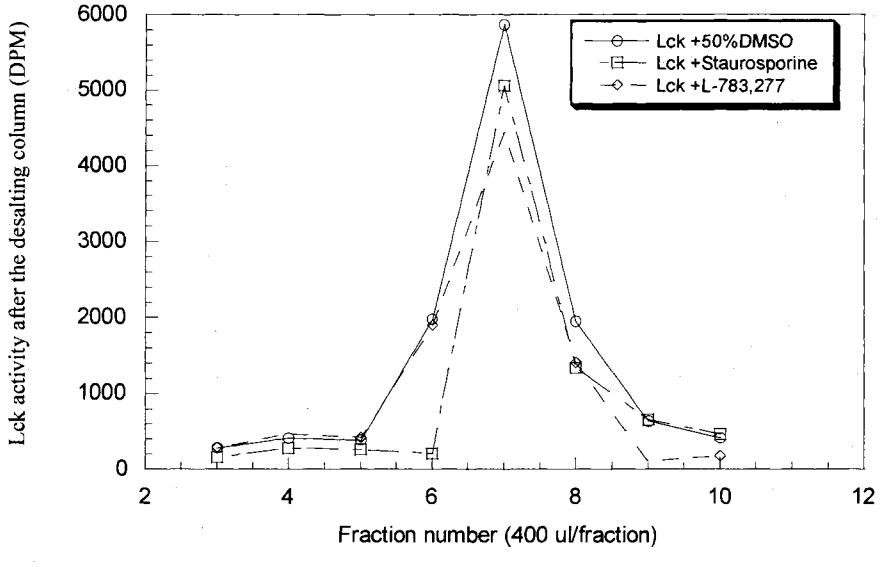
Staurosporine is a potent inhibitor of PKC, but a non-specific inhibitor of several other protein kinases[9~11], and inhibits MEK and Lck with IC_{50} values of 2 nM and 8 nM, respectively. Testing of aliquots removed before applying to the gel filtration column (preincubation controls) indicated that both Staurosporine and L-783,277 inhibited MEK activity. Following column separation, 10 fractions from each column were tested for MEK activity. Protein eluting profiles indicate that equivalent amounts of proteins (BSA and MEK) eluted from all columns, particularly in fractions #6 and #7 (data not shown). However, MEK activity was recovered only from samples preincubated with either DMSO or staurosporine. No detectable MEK activity from samples preincubated with L-783,277 was obtained in fractions #6 and #7 indicating that L-783,277 is an irreversible inhibitor of MEK (Figure 3). The apparent MEK activity present in fractions 3~5 and 8~10, that occurs for all three experimental conditions, is probably a result of a combination of factors that could include

Fig. 3. Reversibility study of L-783,277 and staurosporine in MEK kinase assay following the assay procedures as described in Method section.



The data shown are representatives of three separate experiments. MEK activity was recovered only from the columns with (1) MEK+DMSO and (2) MEK+staurosporine, while there was no detectable MEK activity from the column with (3) MEK+L-783,277 in fractions #6 and #7.

Fig. 4. Reversibility study of L-783,277 in Lck kinase assay.



Lck activity was recovered from all three columns (Lck+DMSO, Lck+staurosporine and Lck+L-783,277).

variations in the elution of protein from the columns as well as in the assay.

We also evaluated the reversibility of L-783,277 against Lck using the same procedure with Lck replacing MEK. The results (Figure 4) indicate that Lck activity can be recovered from all three columns (Lck and DMSO, Lck and

staurosporine, and Lck and L-783,277), suggesting that L-783,277 is a reversible inhibitor of Lck.

These data suggest that there might be covalent binding between the α, β unsaturated ketone of L-783,277 and the active site of MEK. This result is consistent with the fact that the α, β unsaturated ketone of L-783,277 appears to be important for MEK inhibitory activity. In contrast, L-783,277 is a reversible inhibitor of Lck suggesting that the α, β unsaturated ketone does not form an irreversible adduct with Lck hinting that the active site of the two enzymes are dissimilar.

Several independent cell-based assays have been carried out (PATRICK *et al.*, manuscript in preparation) to study the biological activities of these resorcylic acid lactone compounds. L-783,277 was found to inhibit Ras-dependent MAP kinase phosphorylation in PSN-1 human tumor cells with submicromolar EC_{50}'s and inhibit the growth of several human epithelial tumor lines in soft agar with EC_{50} values in the 100 to 200 nM range; Furthermore, in nude mouse explant studies L-783,277 was able to significantly reduce tumor growth at 100 mpk. The discovery of L-783,277 and related resorcylic acid lactones will facilitate further study of the mechanism of activation/inhibition of the MAP kinase pathway and could lead to the identification of therapeutically useful anti-cancer agents.

Acknowledgments

We thank Ms. PATRICIA M. CAMERON, Drs. DENNIS M. ZALLER and YOUNG-WHAN PARK for providing the reagents for the Lck kinase assay. We also thank Ms. NANCY A. THORNBERRY for her advice and discussions on the ATP competition study.

References

1) SERGER, R. & E. G. KREBS: The MAPK signaling cascade. FASEB. J. 9: 726~735, 1995
2) VOJTEK, A. B. & C. J. DER: Increasing complexity of the Ras signaling pathway. J. Biol. Chem. 273: 19925~19928, 1998
3) KYRIAKIS, J. M.; H. APP, X. F. ZHANG, P. BANERIJEE, D. L. BRAUTIGAN, U. R. RAPP & J. AVRUCH: Raf-1 activates MAP kinase-kinase. Nature 358: 417~421, 1992
4) VOJTEK, A. B.; S. M. HOLLENBERG & J. A. COOPER: Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 74: 205~214,1993
5) COWLEY, S.; H. PATERSON, P. KEMP & C. J. MARSHALL: Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77: 841~852, 1994
6) KIZAKA-KONDOH, S.; K. SATO, K. TAMURA, H. NOJIMA & H. KAYAMA: Raf-1 protein kinase is an integral component of the oncogenic signal cascade shared by epidermal growth factor and platelet-derived growth factor. Mol. Cell. Bio. 12: 5078~5086, 1992
7) AGATSUMA, T., *et al.*: Revised structure and stero-chemistry of hypothemycin. Chem. Pharm. Bull. 41: 373~375, 1993
8) NAIR, M., *et al.*: Metabolites of Pyrenomycetes XIII[1]: structure of (+) Hypothemycin, an antibiotic macrolide from *Hypomyces trichothecoides*. Tetrahedron Letters 21: 2011~2012, 1980
9) TAMAOKI, T.; H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA: Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem. Biophys. Res. Commun. 135: 397~402, 1996
10) NAKANO, H.; E. KOBAYASHI, I. TAKAHASHI, T. TAMAOKI, Y. KUZUU & H. IBA: Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. J. Antibiotics 40: 706~708, 1987
11) RUEGG, U. T. & G. M. BURGESS: Staurosporine, K-252 and UCN-01, potent but nonspecific inhibitors of protein kinases. Trends Pharmacol. Sci. 10: 218~220, 1989
12) ROTH, M.: Fluorescence reaction for amino acids. Anal. Chem. 43: 880~882, 1971
13) CREWS, C. M.; A. ALESSANDRINI & R. L. ERIKSON: The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science 258: 478~480, 1992
14) ZHENG, C.-F. & K.-L. GUAN: Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. J. Biol. Chem. 268: 11435~11439, 1993
15) ALESSI, D. R.; Y. SAITO, D. G. CAMPBELL, P. COHEN, G. SITHANANDAM, U. RAPP, A. ASHWORTH, C. J. MARSHALL & S. COWLEY: Identification of the sites in MAP kinase kinase-1 phosphorylated by $p74^{raf-1}$. EMBO J. 13: 1610~1619, 1994.
16) SCHAAP, D.; J. VAN DER WAL, L. R. HOWE, C. J. MARSHALL & W. J. VAN BLITTERSWIJK: A dominant-negative mutant of raf blocks mitogen-activated protein kinase activation by growth factors and oncogenic $p21^{ras}$. J. Biol. Chem. 268: 20232~20236, 1993.
17) DUDLEY, D. T.; L. PANG, S. J. DECKER, A. J. BRIDGERS & A. R. SALTIEL: A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. USA 92: 7686~7689, 1995
18) WILLIAMS, D. H.; S. E. WILKINSON, T. PURTON, A. LAMONT, H. FLOTOW & E. J. MURRAY: Ro 09-2210 exhibits potent anti-proliferative effects on activated T cells by selectively blocking MKK activity. Biochemistry 37: 9579~9585, 1998
19) ALESSI, D. R.; A. CUENDA, P. COHEN, D. T. DUDLEY & A. R. SALTIEL: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J. Biol. Chem. 270: 27489~27494, 1995
20) PARK, Y.-W.; R. CUMMINGS & J. HERMES: Proximity high throughput kinase assays. 3rd Annual Meeting of the Society for Biomolecular Screening, 22~25 September, 1998, San Diego, CA, USA
21) KOLB, A. J.; P. V. KAPLITA, D. J. HAYES, Y.-W. PARK, C. PERNELL, J. S. MAJOR & G. MATHIS: Tyrosine kinase assays adapted to homogeneous time-resolved fluorescence. Drug Discovery Today 3: 333~342, 1998

22) GOPALAKRISHNA, R.; Z. H. CHEN, U. GUNDIMEDA, J. C. WILSON & W. B. ANDERSON: Rapid filtration assays for protein kinase C activity and phorbol ester binding using multiwell plates with fitted filtration discs. Anal. Biochem. 206: 24~35, 1992

23) PITT, A. M. & C. LEE: High throughput screening protein kinase assays optimized for reaction, binding, and detection totally within a 96-well plate. J. Biomolec. Screen. 1: 47~51, 1996

24) PARK, Y.-W.; R. T. CUMMINGS, L. WU, S. ZHENG, P. M. CAMERON, A. WOODS, D. M. ZALLER, A. I. MARCY & J. D. HEMES: Homogeneous proximity tyrosine kinase assays: Scintillation proximity assay versus homogeneous time-resolved fluorescence. Anal. Biochem. 269: 94~104, 1999

25) DOMBROWSKI, A.; R. JENKINS, S. RAGHOOBAR, G. BILLS, J. POLISHOOK, F. PELAEZ, B. BURGESS, A. ZHAO, L. HUANG, Y. ZHANG & M. GOETZ: Production of a family of kinase-inhibiting lactones from fungal fermentations. J. Antibiotics 52: 1077~1085, 1999

26) ELLESTAD, G. A.; F. M. LOVELL, N. A. PERKINSON, R. T. HARGREAVES & W. J. MCGAHREN: New zearalenone related macrolides and isocoumarins from an unidentified fungus. J. Org. Chem. 43: 2339~2343, 1978

27) GIESE, N. A. & N. LOKKER; Method and compositions for inhibiting protein kinases, US patent 5,728,726, March 17, 1998