Ro 09-2210 Exhibits Potent Anti-proliferative Effects on Activated T Cells by Selectively Blocking MKK Activity

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ABSTRACT: By using high throughput screening of microbial broths, we have identified a compound, designated Ro 09-2210, which is able to block anti-CD3 induced peripheral blood T cell activation with an $IC_{50} = 40$ nM. Ro 09-2210 was also able to block antigen-induced IL-2 secretion with an $IC_{50} = 30$ nM, but was considerably less potent at blocking Ca^{2+} flux stimulated by anti-CD3 treatment. To determine the mechanism of action of Ro 09-2210, we set up a transient expression system in Jurkat T cells using a variety of reporter gene constructs and showed effective inhibition of phorbol ester/ionomycin-induced NF-AT activation and anti-CD3 induced NF-AT with $IC_{50} = 7.7$ and 10 nM, respectively. Ro 09-2210 was also able to inhibit phorbol ester/ionomycin-induced activation of AP1 with $IC_{50} = <10$ nM. We further showed that Ro 09-2210 was unable to inhibit c-jun induced expression of AP1-dependent reporter constructs ($IC_{50} > 500$ nM), but was able to potently inhibit ras-induced AP1 activation ($IC_{50} = 20$ nM). This suggested that Ro 09-2210 was inhibiting an activator of AP-1 which was upstream of c-jun and downstream of ras signaling. To investigate further, we then purified a number of different kinases, including PKC, PhK, ZAP-70, ERK, and MEK 1 (a MKK), and showed that Ro 09-2210 was a selective inhibitor of MEK1 in vitro ($IC_{50} = 59$ nM).

Diseases such as rheumatoid arthritis and multiple sclerosis are pathological states resulting from an autoimmune response. One method to control the progression of the disease state is to attenuate the activation of T cells during relapses. However, the antigen(s) which trigger(s) the activation of T cells in these diseases is currently unknown. Nonetheless, the signal transduction cascade which is initiated upon T cell stimulation has been extensively studied and has highlighted opportunities to block T cell activation by pharmacological intervention.

The activation of T cells results in both tyrosine and serine phosphorylations of a number of substrates within minutes of TCR[1] ligation (*1*, *2*). The phosphorylation of the ITAM motifs on the ζ-chain within the TCR complex results in association of ZAP-70 (*3*). This relocation brings ZAP-70 proximal to the CD4-associated $p56^{lck}$ kinase which activates the kinase activity of ZAP-70 by phosphorylation at Y493 (*4*). The activated ZAP-70 is then able to transduce a signal via an as yet uncharacterized cascade which may involve SLP76 (*5*), vav (*6*), PLC-γ (*7*), and/or p120/130 (*8*).

TCR/CD3 stimulation also results in activation of ras which leads ultimately to activation of the broad specificity, proline-directed serine/threonine kinase ERK 1/2 (*9*). GTP-bound ras is known to be the activating ligand for raf kinase which plays a pivotal role in the activation of the MAP kinase cascade (*10*). Raf can be recruited to the membrane via a high affinity for activated GTP-bound form of ras (*11*). This relocation of raf permits phosphorylation and potential activation of raf by a diverse set of membrane-associated kinases. Indeed, many kinases are known to activate raf in vitro such as ceramide-activated kinase CAK (phosphorylates thr268 and thr269), src (phosphorylates tyr340 and tyr341), and/or PKC (phosphorylates ser497, ser499, and ser619) (*12*). An additional requirement for raf activation involves an oligomerization step which may occur independently of membrane localization and ras association (*13*, *14*).

MEK 1 is activated by raf which forms an inactive raf−MEK complex in the cytoplasm (*15*, *16*). Thus activated MEK1 is then able to activate ERK via phosphorylation on threonine and tyrosine residues.

Other kinase cascades may also be activated in stimulated T cells which lead to the activation of other MAP kinase family members such as JNK/SAPKs and p38. These kinase cascades ultimately result in the transcriptional upregulation of the IL-2 gene via the activation of AP-1 and other transcription factors including NF-kB and NF-AT (*17*−*22*).

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[1] Abbreviations: GST, glutathione-S-transferase; PKA, protein kinase A; BSA, bovine serum albumin; DTT, dithiothreitol; MBP, myelin basic protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*,*N*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MKK, MAP/ERK kinase; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SV40, simian virus 40; SDS−PAGE, sodium dodecyl sulfate−polyacryamide electrophoresis; NF-AT, nuclear factor-activated T cells; TK, thymidine kinase; TRE, TPA-responsive element; PDBu, phorbol 12,13-dibutyrate; PHA, phytohaemoglobin; ERK, extracellular regulated kinase; aa, amino acid; JAK, Janus kinase; IL-2, interleukin 2; PTK, protein tyrosine kinase; IC_{50}, inhibitory concentration at 50% activity; PBT, peripheral blood T cells; iono, ionomycin; EBV, Epstein Barr virus; AP1, activator protein 1; NF-kB, nuclear factor κ B; I-kB, inhibitor κ B; PhK, phosphorylase kinase; PKC, protein kinase C; TCR, T cell receptor complex; ITAM, immune receptor tyrosine-based activation motif; ZAP-70, ζ chain associated protein 70 kD.

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We have been interested in identifying small molecular inhibitors of protein kinases which may be used to modulate this kinase cascade and therefore attenuate T cell proliferation by inhibiting the production of IL-2. Ro 09-2210 was originally isolated from a fungal broth FC2506 and subsequent assays indicated that Ro 09-2210 displayed inhibitory activity toward MEK (IC_{50} = 59 nM). We show evidence that Ro 09-2210 is a very effective blocker of T cell activation and/or proliferation with an IC_{50} = 50 nM for inhibition of IL-2 secretion. We show data that the mechanism of action involves the inhibition of ras signaling but not jun or MAPK inhibition. Finally, transfection experiments also suggest that raf and MEK are the likely targets for Ro 09-2210.

MATERIALS AND METHODS

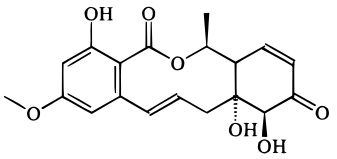
Baculovirus containing human recombinant $p56^{lck}$ was kindly supplied by A. Hayes (Roche, Basel). Phosphocellulose paper (P81) was obtained from Whatman, and AG-1X-8 resin from BioRad. $p56^{lck}$ antibodies and GST-MAP kinase (agarose conjugated) were obtained from UBI. Rabbit skeletal muscle MAPK was the generous gift of L. M. Graves (University of Washington). Ro 09-2210 was isolated from the mycelial cake of *Curvularia* sp. by Ms. K. Ilida (Nippon Roche Research Centre).

Enzyme Assays. T cell PKC was partially purified from peripheral blood T cells by ion-exchange chromatography by the method outlined in ref *23*. Bovine heart PKA (*23*), rabbit skeletal muscle phosphorylase kinase (*24*), and human recombinant $p56^{lck}$ (*25*) and ZAP-70 (*5*) assays were performed as previously described.

MAP kinase activity was assayed by measuring the incorporation of radio-labeled phosphate into myelin basic protein. Assay mixtures contained 100 μM ATP, 1 mg/mL BSA, 1mM DTT, [γ-^{33}P] ATP, 0.3 mg/mL MBP in 25 mM β-glycerophosphate pH 7.3, 1.25 mM sodium EGTA, and 10 mM $MgCl_2$. The reaction was initiated by the addition of 2.5 μg of GST-MAPK to a total reaction volume of 35 μL and allowed to proceed for 10 min at 37 °C before stopping by the addition of 20 μL of stop mix (100 mM EDTA, 6 mM adenosine). Phosphorylated products were then measured as described (*23*).

MEK activity was assayed in a coupled reaction, entailing the activation of GST-MAPK by preincubation with MEK, in the presence or absence of inhibitor. The activity of MAPK was then measured as above. Preincubation assay mixtures contained 200 μM ATP, 12.5 μg of GST-MAPK and 250 ng of MEK in 25 mM β-glycerophosphate pH 7.3, 1.25 mM sodium EGTA, and 10 mM $MgCl_2$. The reaction was initiated by the addition of MEK to a total reaction volume of 15 μL and allowed to proceed for 5 min at 30 °C before the addition of 15 μL of MAPK reaction mixture (described above). The assay was continued for a further 15 min before addition of stop mix.

Recombinant GST-MEK was incubated with 50 μCi [γ-^{33}P] ATP in 25 mM β-glycerophosphate pH 7.3, 1.25 mM sodium EGTA, and 10 mM $MgCl_2$ buffer for 20 min at 30 °C to determine autophosphorylation. The assay was terminated by the addition of protein disruption buffer, the products were separated by SDS−PAGE, and the results were visualized by autoradiography and analyzed on a Phosphor-imager.



Ro 09-2210

FIGURE 1: Structure of Ro 09-2210.

For inhibitor studies, compounds were dissolved in dimethylsulfoxide such that the final concentration of solvent in each assay was 10%. Solvent-only controls were included in each experiment.

Cellular Studies. T cell studies were performed by the methods previously detailed (*26*). In summary, fresh human blood from volunteers was obtained and PBT were isolated using Ficoll-Hypaque. The isolated purified PBT were washed in RPMI-1640, resuspended in RF10 media, and dispensed in 96-well plates at 10^5 cells/well. PDBu and ionomycin were added to a final concentration of 20 and 200 nM, respectively, with 1 nM to 1 μM Ro 09-2210 (or appropriate DMSO control) and incubated for 2 days at 37 °C. The cells were then pulsed with 1 μCi of tritiated thymidine and incubated a further 6 h at 37 °C. PBT proliferation experiments using α-CD3 (UCHT1) were set up in a similar fashion in 96-well plates with 5 replicate wells with 10^5 resuspended cells/well and 100 ng/mL α-CD3 and a 3 day incubation. The IC_{50} values for n = 13 experiments are included with standard deviations and sem values as calculated in Excel Version 5.0.

Transient Expression Experiments. The expression constructs pTKluc, p(NF-AT)$_3$luc, pTREluc, and pNFluc have been described in ref *27*. pEF-empty and pEFras are described in ref *28*. Jurkat E17 T cells were resuspended in RPMI1640 at 3×10^7 cells/mL and electroporated with 20 μg of reporter gene construct. Aliquots (0.5 mL) were divided into 6 well dishes. After overnight recovery, each dish was pretreated with various amounts of Ro 09-2210 (as indicated) for 1 h prior to stimulation with 50 ng/mL PDBu + 2 μg/mL ionomycin or 2 μg/mL anti-CD3, and cells were then incubated for a further 24 h at 37 °C. Results represent the cell extract luciferase activity (mean ± standard error) of triplicate cultures.

RESULTS

Ro 09-2210 Inhibits Peripheral Blood T Cell Activation. T cells were isolated from peripheral blood by density gradient centrifugation and cultured for 3 days with a variety of agonists in the presence of Ro 09-2210 (see Figure 1 for chemical structure) at differing concentrations. Figure 2 shows representative experiments which reveal the antiproliferative properties of Ro 09-2210 upon T cell stimulated with either PDBu + ionomycin (Figure 2A) or anti-CD3 (Figure 2B). The IC_{50} values for inhibition of proliferation are shown in Table 1. It is clear that stimulation by either phorbol/ionomycin, PHA, or anti-CD3 is potently inhibited by the compound (IC_{50} = 58−65 nM), whereas bypassing membrane receptors via treatment with phorbol ester/ionomycin is slightly more resistant to inhibition (IC_{50} = 139 nM). An alternative end-point to assay for inhibition of T cell activation is to measure the amount of IL-2 secretion. In addition, we were interested in whether Ro

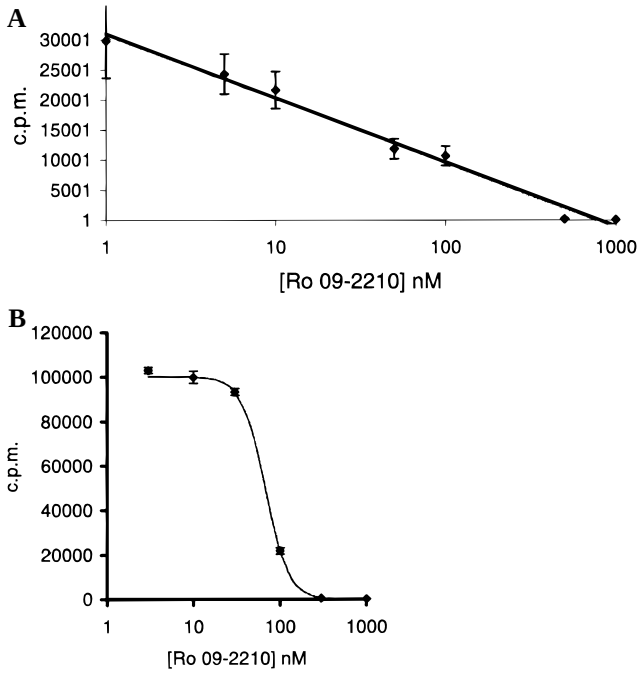


FIGURE 2: PBT from fresh blood were isolated by Ficoll/Hypaque, washed in RPMI-1640, resuspended in RF10 media, and dispensed in 96-well plates at 10^5 cells/well. Cells were then treated with (A) 20 nM PDBu and 200 nM ionomycin for 2 days, or with (B) 100 ng/mL α-CD3 (UCHT1) for 3 days. Ro 09-2210 was added to each experiment in the range from 1 nM to 1 μM The cells were then pulsed with 1 μCi of tritiated thymidine and incubated a further 6 h. Results represent mean cpm (and sem) incorporated for 5 replicate wells for each data point.

Table 1: The Effects of Ro 09-2210 on a Range of T Cell Responses

assay system	agonist	end point	IC_{50} (nM)
PBT + 2 μg/mL PHA (4 days)	mitogen driven	proliferation	65 ± 10 ($n = 2$)
PBT + 100 ng/mL anti CD3	anti-CD3	proliferation	58 ± 12 ($n = 13$)
PBT	PDBu/iono	proliferation	139 ± 48 ($n = 5$)
PBT	anti-CD3	Ca^{2+} flux	7500
13.13.18 autologous T cell	EBV antigen	IL-2 release	30 ($n = 3$)
13.13.18 autologous T cell	anti-CD3	IL-2 release	46
Jurkat T cell line	anti-CD3	IL-2 release	16

09-2210 would block a more physiological stimulus for T cell activation such as a specific antigen-induced activation event. An IL-2 response from the $CD4^+$ mouse T cell hybridoma line 13.13.18 was induced by overnight culture with the HLA-DR^+ Daudi B cell line, (or with solid-phase anti-CD3) in the presence of Ro 09-2210. Table 1 shows effective inhibition of IL-2 secretion, with either stimuli, with IC_{50} values in the range 30−50 nM. These results were not due to toxicity since concentrations >10 μM were required to cause 50% reduction in MTT cytotoxicity assays (data not shown).

It is clear that TCR proximal signaling events initiated by anti-CD3 treatment are not inhibited by low concentrations of Ro 09-2210. For example, Table 1 shows that Ca^{2+} flux is only inhibited at relatively high concentrations (IC_{50} = 7500 nM). This would suggest that Ro 09-2210 is acting downstream of the TCR. In support of this, bypassing the TCR with phorbol ester + ionomycin still results in the inhibition of proliferation by Ro 09-2210 (139 nM).

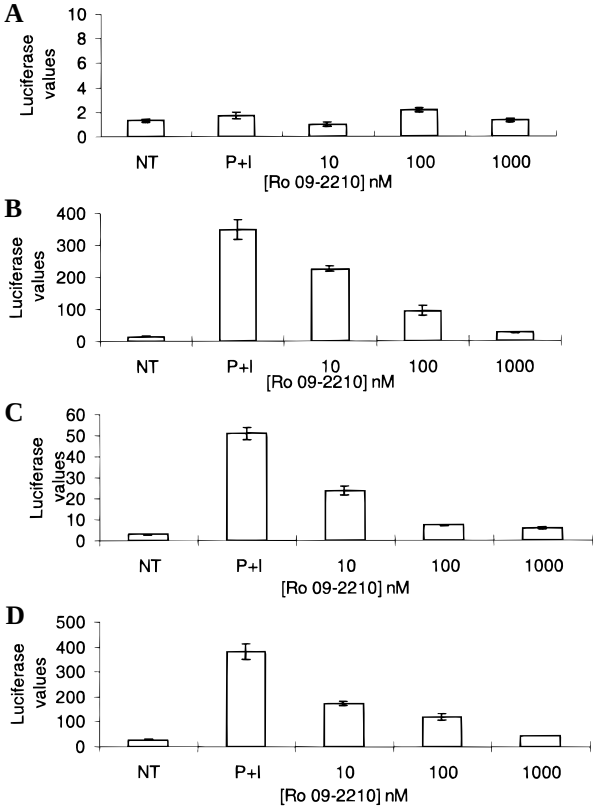


FIGURE 3: Jurkat E17 T cells were resuspended in RPMI1640 at 3 × 10^7 cells/ml and electroporated with (A) 20 μg of pTKluc, (B) 20 μg of pNFluc, (C) 20 μg of $p(NFAT)_3$luc, or (D) 20 μg of pTREluc. Aliquots (0.5 mL) were used to seed 6 well dishes. After overnight recovery, each transfection aliquot was pretreated with various amounts of Ro 09-2210 (as indicated) for 1 h prior to stimulation with 50 ng/mL PDBu + 2 μg/mL ionomycin, and cells were then incubated a further 24 h. Results represent the cell extract luciferase activity (mean ± standard error) of triplicate cultures.

Table 2: The Effects of Ro 09-2210 on Reporter Gene Activity

reporter gene	agonist	induction	IC_{50} (nM)
TK	phorbol ester + ionomycin	none	>1000 (basal)
AP1	phorbol ester + ionomycin	11	<10
NF-AT	phorbol ester + ionomycin	17	7.5
NF-AT	anti-CD3		10
NF-kB	phorbol ester + ionomycin	27	46
AP1	c-jun expression	4	>1000
AP1	v-ras	5	20

Ro 09-2210 Inhibits Activation of Transcription Factors. To further define the likely mechanism of action for Ro 09-2210, a series of reporter gene experiments were then set up using E17 Jurkat T cells electroporated with AP-1-, NF-kB-, and NF-AT-dependent luciferase expression constructs designated pTREluc, pNFluc, and pNF-ATluc, respectively. These constructs are based on the thymidine kinase promoter construct (pTKluc) which has been modified by insertion of relevant transcription factor binding sites. Figure 3A shows that Ro 09-2210 does not exhibit any nonspecific inhibitory effects on the TK promoter, but is able to potently inhibit PDBu/ionomycin-induced AP-1 and NF-AT transcription activity with an IC_{50} = <10 nM (Figure 3C,D and Table 2). The inhibition of PDBu/ionomycin-induced NF-kB activity is 4−5 times less potent with IC_{50} = 45 nM in transfected Jurkat E17 cells (Figure 3B, Table 2). As a further control

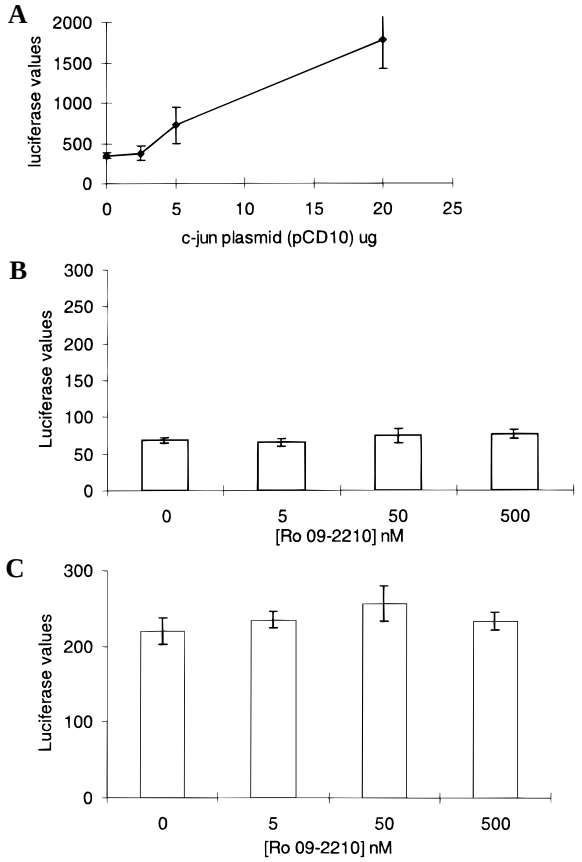


FIGURE 4: (A) Jurkat E17 T cells were resuspended in RPMI1640 at 3×10^7 cells/mL and 0.5 mL aliquots were electroporated with 6 μg of pTREluc and increasing amounts of pCD10. Each transfection was further incubated for 18 h. Results represent the cell extract luciferase activity (mean ± sem). (B,C) Jurkat E17 T cells were resuspended in RPMI1640 at 3×10^7 cells/mL and electroporated with either (B) 10 μg of pTREluc or (C) 10 μg of pTREluc + 10 μg of pCD10. Aliquots (0.5 mL) of each transfection were used to seed 6 well dishes. After overnight recovery, each transfection aliquot was pretreated with various amounts of Ro 09-2210 (as indicated) for 1 h prior to stimulation with 50 ng/mL PDBu + 2 μg/mL ionomycin, and cells were then incubated a further 24 h. Results represent the cell extract luciferase activity (mean ± standard error) of triplicate cultures.

for specificity, the SV40 early promoter was unaffected by Ro 09-2210 at up to 500 nM (data not shown).

The inhibitory effect on the AP-1 activity was further investigated using a c-jun expression plasmid (pCD10) to induce the cotransfected AP-1-dependent reporter gene construct, pTREluc (pCD10 uses the SV40 early promoter to transcribe the c-jun insert which was previously shown to be unaffected by Ro 09-2210, see above). Figure 4A shows the activation of pTREluc in Jurkat T cells by cotransfection with increasing amounts of pCD10. Figure 4B,C shows that pCD10 was able to induce 5-fold higher expression from pTREluc compared to basal level, and both induced and basal expression of pTREluc were unaffected by Ro 09-2210 at up to 500 nM. This suggested that the inhibition of induced AP-1 was occurring upstream from activation of c-jun.

Ro 09-2210 Acts Downstream of Ras and Upstream of MAP Kinase. Ras proteins have been implicated in signaling from the TCR which results in activation of ERK2 (*9*). The effects of Ro 09-2210 upon ras-initiated signal transduction were investigated by cotransfecting an "activated ras"

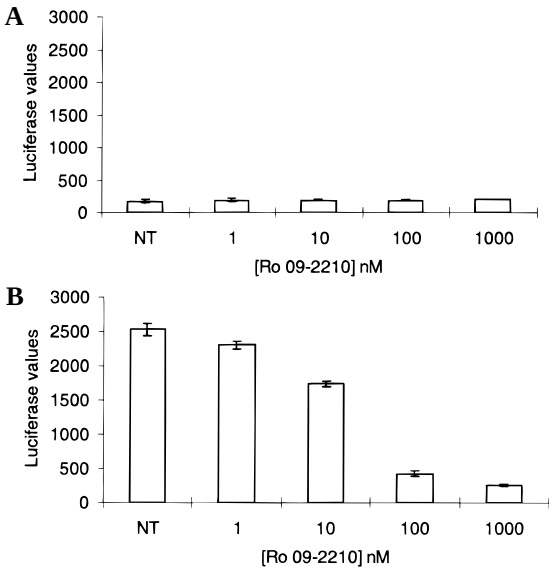


FIGURE 5: Jurkat E17 T cells were resuspended in RPMI1640 at 3×10^7 cells/mL and were electroporated with either (A) 20 μg of pTREluc + 40 μg of pEFempty or (B) 20 μg of pTREluc + 20 μg of pEFempty + 20 μg of pEFras. Immediately after electroporation each transfection aliquot was treated with various amounts of Ro 09-2210 (as indicated), and cells were then incubated a further 24 h. Results represent the cell extract luciferase activity (mean ± standard error) of triplicate cultures.

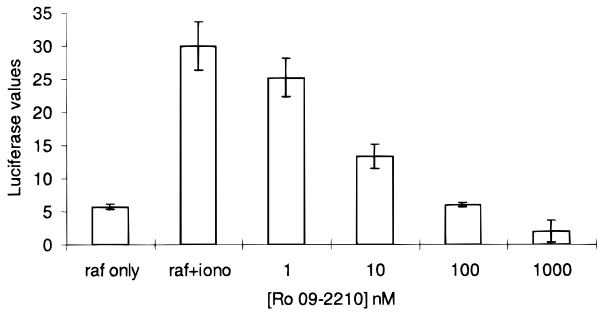


FIGURE 6: Jurkat E17 T cells were resuspended in RPMI1640 at 3×10^7 cells/mL and 0.5 mL aliquots were electroporated with 20 μg of pEF-raf + 20 μg of pNFATluc. After overnight recovery, some transfections were pretreated with various amounts of Ro 09-2210 (as indicated) for 1 h prior to stimulation with 2 μg/mL ionomycin, and cells were then incubated a further 24 h. Results represent the cell extract luciferase activity (mean ± standard error) of triplicate.

expression plasmid (designated pEFras which encodes the G12R, A59T, $p21^{Ha-ras}$ mutation) and the pTREluc reporter gene construct in E17 Jurkat T cells. Figure 5 shows that the activated $p21^{Ha-ras}$ was able to induce AP1 approximately 10-fold in T cells, which was inhibited by Ro 09-2210 at an IC_{50} = 20 nM. Western blotting controls on pEFras transfected T cell lysate using anti-ras monoclonals show no Ro 09-2210 mediated inhibition of ras expression plasmid (data not shown). Thus, the inhibition of ras-induced AP-1 activity was not due to diminished amounts of ras protein. One of the downstream effectors of ras is known to be raf kinase (*29*−*31*). Thus, pEF-raf was used to overexpress an active truncated form (aa 26−302) of c-raf and was shown to activate cotransfected pNF-ATluc by 5-fold in the presence of 2 μg/mL ionomycin (Figure 6). The presence of increasing amounts of Ro 09-2210 effectively blocked raf-mediated activation of the NF-AT reporter gene with an IC_{50} = 5 nM.

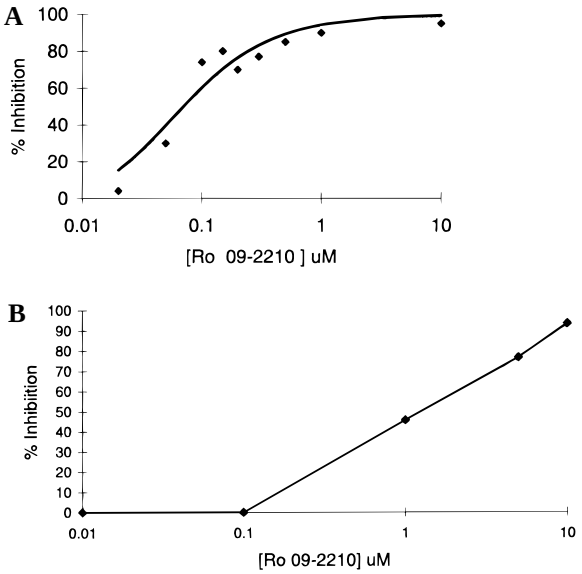


FIGURE 7: (A) The inhibition of rabbit skeletal MEK by Ro 09-2210. Purified MEK had a specific activity of 10 400 units/mg and was 98% pure by silver stain gel analysis (1 unit of activity being the amount of enzyme capable of activating recombinant ERK2 by 100 pmol/min after 15 min). The assay was performed as described in Materials and Methods. (B) The inhibition of rabbit skeletal MAPK by Ro 09-2210. The assay was performed as described in Materials and Methods.

Table 3: The Effects of Ro 09-2210 as an Inhibitor of a Number of Serine/Threonine- and Tyrosine-Specific Protein Kinases[a]

enzyme	IC_{50} nM
T cell PKC	>100 000 (2)
bovine heart PKA	>100 000 (2)
human recombinant ZAP-70	>100 000 (2)
human recombinant p56^{lck}	>100 000 (3)
rabbit muscle phosphorylase kinase	100 000 (2)
rabbit skeletal muscle MEK	58.7 ± 25.4 (n = 3)
human recombinant MAP kinase	990 ± 135.2 (n = 3)

[a] The specificity controls for the in vitro inhibitory activity of Ro 09-2210 were bovine heart PKA (1−2 units/μg protein), ZAP-70 (recombinant baculoviral source, 10% pure, only kinase present in preparation as determined by autophosphorylation), p56^{lck} (recombinant baculoviral source 30% pure, sole kinase activity in preparation as determined by autophosphorylation), rabbit muscle phosphorylase kinase (200−500 units/mg of protein), and rabbit skeletal muscle MEK (purified material had a specific activity of 10 400 units/mg and was 98% pure by silver stain gel analysis). The fold purification from rabbit skeletal muscle was 4500 with one unit defined as the amount of enzyme capable of activating recombinant ERK2 by 100 pmol/min after 15 min.

Ro 09-2210 Is a Potent Inhibitor of MEK in Vitro. The observation that the Ro 09-2210 sensitive target in the signal transduction pathway is upstream of AP-1 but downstream of ras prompted an investigation of the inhibitory activity of Ro 09-2210 on the MKK class of kinases. Indeed, Ro 09-2210 is a potent inhibitor of MEK purified from rabbit skeletal muscle (IC_{50} = 59 nM), but only a weak to moderate inhibitor of MAPK (Figure 7A,B, Table 3). Ro 09-2210 was also able to inhibit human recombinant MEK1 in an autophosphorylation assay (IC_{50} = 140 nM, data not shown).

PKC, ZAP-70, and p56^{lck} are all kinases which have been shown to play important roles in T cell signaling processes. However, no inhibitory activity was detected against these or a range of other serine/threonine- and tyrosine-specific protein kinases (IC_{50} > 100 μM, see Table 3).

DISCUSSION

We have described a compound that selectively inhibits dual specificity kinases, such as MEK, in vitro and exhibits potent antiproliferative effects in peripheral blood T cells activated by a number of different stimuli which initiate signaling via the TCR. We also observed that Ca^{2+} flux is not greatly affected by Ro 09-2210, which suggests that immediate proximal effectors of the TCR such as G-protein activated PLC-γ are not inhibited. Other potential targets include PTKs such as p56^{lck} and ZAP-70 which are activated during TCR signal transduction. Three observations suggest that this is not the case. First, we were unable to demonstrate inhibition of either of these enzymes in vitro. Second, bypassing the TCR with PDBu/ionomycin treatment still has Ro 09-2210 sensitive stages. Third, Ro 09-2210 is able to block ras-induced events in non-T cells such as NIH 3T3 cells, which do not contain p56^{lck} or ZAP-70 activity (data not shown).

We then demonstrated the ability of Ro 09-2210 to potently inhibit IL-2 secretion in activated T cells, and used various reporter gene constructs to show that this inhibition occurred at the transcriptional level. The specificity of this inhibition was illustrated by the inability to inhibit both the TK and SV40 early promoters. NF-kB transcription factor activity was inhibited with an IC_{50} = 46 nM, and both AP-1 and NF-AT activities were inhibited at IC_{50} < 10 nM. All three of these factors have been shown to be required to upregulate the IL-2 promoter (*17*−*21*). Thus we conclude that Ro 09-2210 is likely affecting signal transduction pathways that lead to IL-2 gene regulation. We note that the in vitro IC_{50} against MEK (59 nM) is lower than the cellular IC_{50} values against NF-AT and other reporter gene systems described above. This may be partly explained by the observation the Ro 09-2210 is able to inhibit other dual specificity kinases such as MKK 6, 4, and 7, albeit at 4−10-fold higher IC_{50} concentrations compared to MEK 1 (P. Cohen, personal communication). Thus, although Ro 09-2210 is unable to inhibit p90^{rsk}, JNK, p38, or MAPKAP kinase 2 (data not shown) directly at concentrations >10 μM, it is clear that the activation of these substrate kinases is likely to be partially blocked in the presence of Ro 09-2210, and this may explain the increased potency of the inhibitor in cellular systems. Other kinases which are not inhibited at submicromolar concentrations by Ro 09-2210 include MAP kinase, PKC, PhK, PKA, ZAP-70, and p56^{lck}. Thus, Ro 09-2210 appears to be specific to the dual specificity ser/tyr kinases.

Both ras and raf have been shown by previous investigators to be important for TCR signal transduction (*9*, *32*). Other workers have demonstrated that ras can activate the raf-MEK-ERK pathway (*33*). We have clearly shown that Ro 09-2210 can block signaling from a constituitively active ras construct. We were able to rule out ras as the target for inhibition by showing that Ro 09-2210 was also able to block constitutive raf signaling. Under these conditions, ras is no longer required for raf activation/signaling; thus Ro 09-2210 was inhibiting a target downstream from ras. In vitro experiments on purified MEK demonstrated that Ro 09-2210 was an effective inhibitor with an IC_{50} of 60 nM, which is similar to the IC_{50} values observed for the inhibition of PBT proliferation. We have been unable to purify sufficient active

protein to perform similar in vitro analyses on raf. Given the Ro 09-2210 selective inhibition of raf/MEK, it is interesting to note the Ro 09-2210 has a 4−5-fold reduced potency against NF-kB activation compared to NF-AT and AP-1 activation. It has been previously reported that raf was able to activate NF-kB via phosphorylation of I-kB (*34*, *35*). However, our data suggest that raf and NF-kB are on different pathways. This discrepancy can be explained either by cross-talk via a common mediator of both pathways or by assuming that raf is not the genuine I-kB kinase in vivo. Support for the latter assumption comes from experiments which have shown that the activation sites recognized by raf do not resemble the inducible I-kB phosphorylation sites (*36*). Nevertheless, our results would suggest that a Ro 09-2210 sensitive kinase is directly or indirectly involved in the activation of NF-kB. Work in other laboratories has identified a variety of other putative I-kB kinases including a MEKK/700 kD complex (*37*), a 42/44 kD kinase (*38*), casein kinase II (*39*, *40*), PKC-ξ (*41*, 42), CHUK/IKKα (*43*, *44*), and a PTK (*45*, *46*).

A MEK inhibitor designated PD098059 has been previously reported in the literature (*47*). Mechanisms of action studies have shown PD098059 is able to bind the inactive nonphosphorylated MEK1 and thus block raf-mediated activation of MEK with an $IC_{50} = 2-7\ \mu M$ (*48*). This is in contrast to Ro 09-2210 which is able to inhibit activated biochemically purified MEK in vitro with an $IC_{50} = 0.06\ \mu M$. Both compounds are similar in being unable to inhibit IL-1 signal transduction in murine fibroblasts.

Finally, we have previously shown that a selective PKC inhibitor Ro 32-8425 was also able to inhibit TCR signaling in stimulated Jurkat T cells (*27*). Comparison of the inhibitory profiles between the two compounds shows complementary effects. Thus, the PKC inhibitor appeared to be blocking signal transduction upstream of ras and was a more effective inhibitor of NF-kB rather than AP-1 or NF-AT. In contrast, Ro 09-2210 seems to be blocking signal transduction downstream of ras and was more effective against AP-1 and NF-AT compared to NF-kB. We are continuing to identify selective inhibitors of a variety of other kinases involved in TCR signaling and intend to generate a panel of such compounds which can be used to dissect kinase cascades (*49*, *50*) and ultimately be used in the clinic as the basis for chemotherapies to alleviate the symptoms of autoimmune diseases such as rheumatoid arthritis.

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