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MEK INHIBITORS: THE CHEMISTRY AND BIOLOGICAL ACTIVITY OF U0126, ITS ANALOGS, AND CYCLIZATION PRODUCTS

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Abstract: In search of antiinflammatory drugs with a new mechanism of action, U0126 was found to functionally antagonize AP-1 transcriptional activity via noncompetitive inhibition of the dual specificity kinase MEK with an IC_{50} of 0.07 μM for MEK 1 and 0.06 μM for MEK 2. U0126 can undergo isomerization and cyclization reactions to form a variety of products, both chemically and in vivo, all of which exhibit less affinity for MEK and lower inhibition of AP-1 activity than parent, U0126. © 1998 The DuPont Merck Pharmaceutical Company. Published by Elsevier Science Ltd. All rights reserved.

Steroids have been used to treat inflammation successfully for many years, but they are plagued with serious side effects.[1] Mechanistically, steroid drugs and glucocorticoid (GC) hormones bind to the glucocorticoid receptor (GR) to form a complex which dimerizes and migrates to the nucleus (Figure 1). This complex interacts with the glucocorticoid response elements (GREs) in gene promoters resulting in the enhancement of transcription.[2] The GC/GR complex can also bind to the transcription factors AP-1 and NF-kB (NF-kB is not shown in Figure 1) which are regulators of the immune response genes.[3] Inhibition of these transcription factors

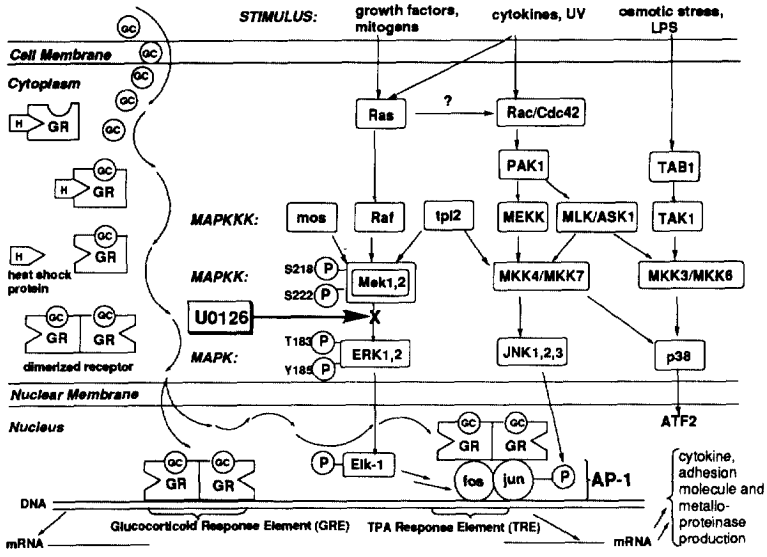

Figure 1. MAPK signalling cascades together with the mechanism of action of glucocorticoids.

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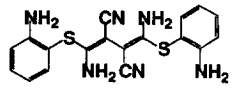
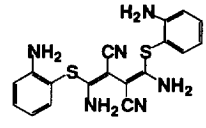
by the GC-GR complex is thought to be the mechanism by which steroids exert their anti-inflammatory and immunosuppressive effects, the results of which are inhibition of cytokine, adhesion molecule and metalloproteinase production.[3]

Inhibition of the transcription factors AP-1 and NF-kB without interaction with GREs would make for an ideal antiinflammatory agent, since it is believed that interaction with GREs produces the unwanted side effects of steroids.[4] Screening of the DuPont library of compounds yielded U0126 (**1**), which does not interact with the GR and therefore the GREs, but does inhibit AP-1 transcription activity (IC_{50} = 1.0 μM ± 0.2, n = 21).[5] Investigation of the cellular signaling cascades causing AP-1 activation led to the discovery that U0126 selectively inhibits MEK (a MAP kinase kinase or MAPKK), a dual specificity kinase in the mitogen activated protein kinase (MAPK) cascade (Figure 1).[5] MEK phosphorylates the threonine and tyrosine (T183 and Y185) residues on ERKs 1 and 2 resulting in their activation.[6] Activated ERK, in turn, phosphorylates Elk-1 leading to transcriptional activation of the cFos and cJUN genes, resulting in AP-1 activation.[7] For activation, MEK requires phosphorylation of two of its residues, S218 and S222.[6] Introduction of acidic residues to replace the serines coupled with truncation of an alpha-helical region in the N-terminal domain results in a constitutively active form of MEK which was used in investigating the SAR around U0126.[8]

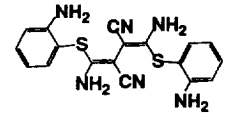
Seven MAPKKs have been identified: MEK 1 & 2, MKK3, MKK4, MEK 5, MKK6, and MKK7.[5] U0126 binds selectively to only MEK 1 and MEK 2 with IC_{50}s = 0.07 μM ± 0.02 (n = 6) and 0.06 μM ± 0.02 (n = 2), respectively. U0126 inhibition of MEK is noncompetitive with respect to ERK and ATP, suggesting that it binds in a unique site in MEK.[5] This may explain its selectivity against a wide variety of other kinases.[5] As a result of MEK inhibition, U0126 has been shown to block the production of a variety of cytokines and metalloproteinases involved in the inflammatory response.[9]



1: U0126, *Z,Z*-isomer



2: *Z,E*-isomer



3: *E,E*-isomer

U0126, synthesized in the late 1950's by W. J. Middleton[10], is quite stable in its crystalline state *even after four decades of storage!* There are three possible isomers of U0126: *Z,Z* (**1**); *Z,E* (**2**); and *E,E* (**3**). In solution, U0126 exists at equilibrium as a mixture of *Z,Z*- and *Z,E*-isomers, the rate of isomerization being dependent on the solvent. Using the Middleton synthesis procedure, a mixture of the *Z,Z*- and *Z,E*-products were formed, the *Z,Z*-isomer predominating. Both of these isomers were separated by HPLC and tested for MEK inhibition. The *Z,Z*-isomer was also crystallized from ethanol. X-ray crystal structure determination confirmed its structure and isomeric configuration.[11] The *E,E*-isomer was isolated after precipitating unexpectedly from refluxing methanol during an attempted large scale recrystallization.

The *Z,Z*-isomer appears to uniquely contain the MEK inhibitory activity. The *Z,E*-isomer had an IC_{50} of 0.5 μM (n = 1). However, HPLC analysis of the biological screening assay immediately after the IC_{50} determination revealed that 12% of the *Z,E*-isomer had already isomerized to the *Z,Z*-isomer. The presence of 12% *Z,Z*-isomer could account for the observed IC_{50}. The *E,E*-isomer also isomerized under assay conditions, and it would only take the presence of 3% of the *Z,Z*-isomer to yield the observed IC_{50} of 2.6 μM (n = 1). Thus the *Z,Z*-isomer is most likely the major contributor to the observed IC_{50} value.

Prolonged storage of U0126 in DMSO resulted in the diminution of U0126 peaks and formation of new

peaks by HPLC. DMSO stock solutions of U0126 tested for AP-1 suppression activity yielded diminishing activity with time: freshly prepared U0126, IC_{50} = 0.2 μM; 1 week old, 0.3 μM; 1 month old, 0.3 μM; 2 months old, 0.5 μM; 6 months old, 24.8 μM (all IC_{50}s, n = 1). Middleton previously showed that U0126-type compounds cyclized to pyrroles in dilute HCl.[10] Although our prolonged storage byproducts were never assigned structures, their ready formation coupled with the ability of U0126 to cyclize in dilute acid led us to speculate that U0126 cyclization products could also form in the binding assay, in the cellular AP-1 assay or in vivo. These cyclization products might also be major contributors to the observed activity of U0126.

U0126 was subjected to a variety of reaction conditions and a number of cyclization products were isolated and characterized (Figure 2). All of these cyclization products exhibited weaker inhibition of MEK compared to parent U0126 (inactive is defined as < 50% binding at 10 μM). Bisthiazoline analog **7** showed a 7-fold decrease in MEK inhibition and over a 40-fold decrease in AP-1 suppression activity compared to U0126. The bisthiazoline structure of **7** may not allow for effective cell penetration in the cell-based AP-1 assay.[5] Methylation of the nitrogens of **7** yielded inactive **8**. This result could reflect either the lack of hydrogen bonding by the now methylated nitrogens or steric hindrance by the methyl groups. It is unclear why monothiazoline product **6** is a weaker inhibitor of MEK compared to its bisthiazoline analog **7**. Compounds **9**, **10**, and **11** were poor inhibitors, suggesting that the pharmacophore which gives rise to good inhibition is the vinylogous cyanamide substructure found in compounds **1**, **6**, and **7**. To test whether biological transformation

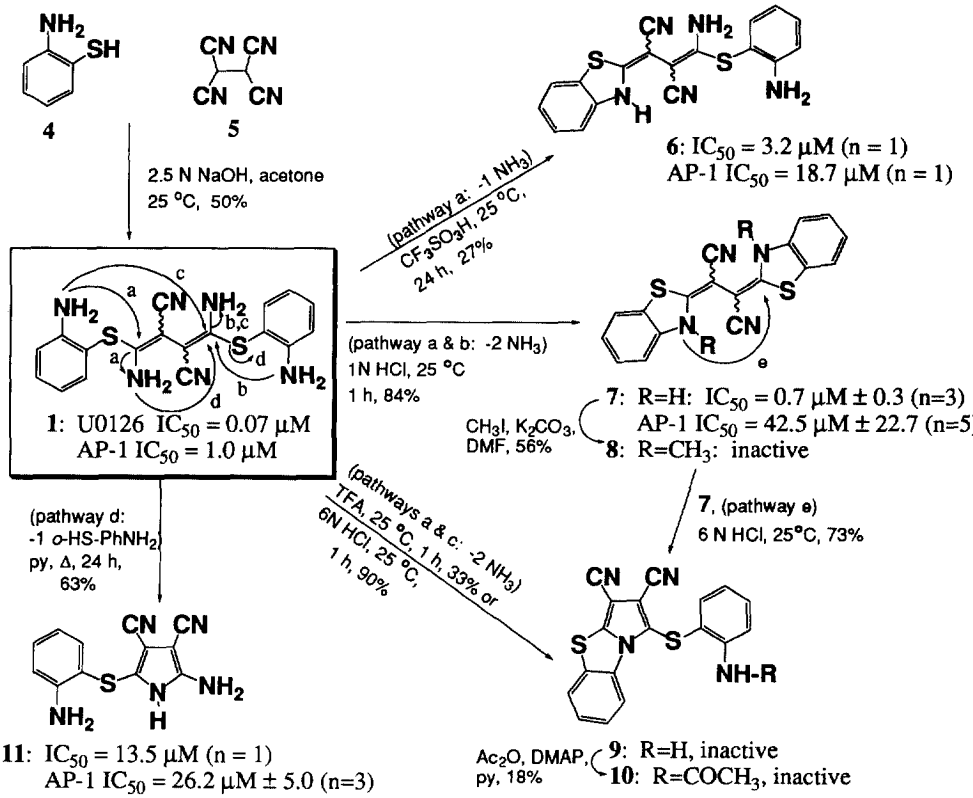
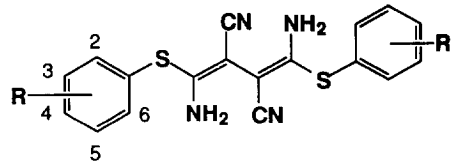


Figure 2. Cyclization products of U0126 together with IC_{50}s for MEK and AP-1 activity.

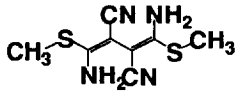
of U0126 leads to **6**, **7**, **9**, **11**, or other products, U0126 was dosed both ip and po in rats at 100 mg/kg. LC/ESI-MS of blood plasma samples taken at various time points up to 24 hr detected protonated molecules corresponding to the MW of U0126 (380), **6** (U0126 - 1 NH_3; 363), **7** and/or **9** (U0126- 2 NH_3; 346) and **11** (U0126 - C_6H_7NS; 255). No evidence for biological transformation of U0126 to more potent MEK inhibitors was found.

Table 1. Analogs of U0126 and their MEK and AP-1 Inhibitory Activities



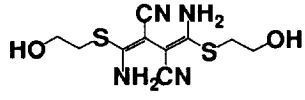
No.	R	MEK IC_{50} (μM) or % inh. at 10 μM*	(n)	Std. Dev.	AP-1 IC_{50} (μM)*	(n)	Std. Dev.	No.	R	MEK IC_{50} (μM) or % inh. at 10 μM*	(n)	Std. Dev.	AP-1 IC_{50} (μM)*	(n)	Std. Dev.
1	2-NH_2	0.07	6	0.02	1.0	21	0.21	**27**	2,6-di-Cl	2.8	1		6.5	5	4.1
12	all H	0.9	2	0.6	12.2	4	1.9	**28**	2-Ph	8.7%	1				
13	3-NH_2	4.8	3	0.6	18.1	3	7.0	**29**	3-Ph	54.6%	1				
14	2-CH_3NH-	19.1%	1					**30**	4-Ph	34.0%	1				
15	2-NH_2, 5-I	34.8%	1					**31**	2,3-benzo	26.0%	1				
16	2-NH_2-3,4-Benzo	2.1	1					**32**	3,4-benzo	54.0%	1				
17	4-NH_2	0.5	6	0.2	10.5	4	5.4	**33**	2-CH_3	2.0	1		13.3	2	0.3
18	4-CH_3CONH-	0.0%	1					**34**	2-CH_2CH_3	18.0%	1				
19	2-NO_2	41.0%	1					**35**	3-CF_3	32.0%	1				
20	2-OH	0.3	4	0.2		2	0.4	**36**	4-CF_3	33.0%	1				
21	4-OH	0.4	1					**37**	2-OCH3	4.5	1		16.5	2	9.0
22	2-F	0.9	2	0.3	7.6	2	4.7	**38**	2,5-di-OCH_3	18.0%	1				
23	2-Cl	0.9	2	0.1	6.6	3	1.6	**39**	3,4-di-OCH_3	0.0%	1				
24	2-Br	1.1	3	0.4	7.9	2	0.4	**40**	2-CH_2OH	51.0%	1				
25	3-Cl	1.9	1		18.4	2	3.2	**41**	2,3,4,5,6-F_5	17.0%	1				
26	4-Cl	2.1	1		21.7	2	16.5								

*MEK and AP-1 IC_{50} values were determined by the procedures described in reference 5.



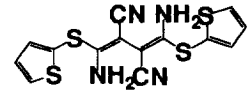
42

Inhibits 24% at 10 μM



43

Inhibits 0% at 10 μM



44

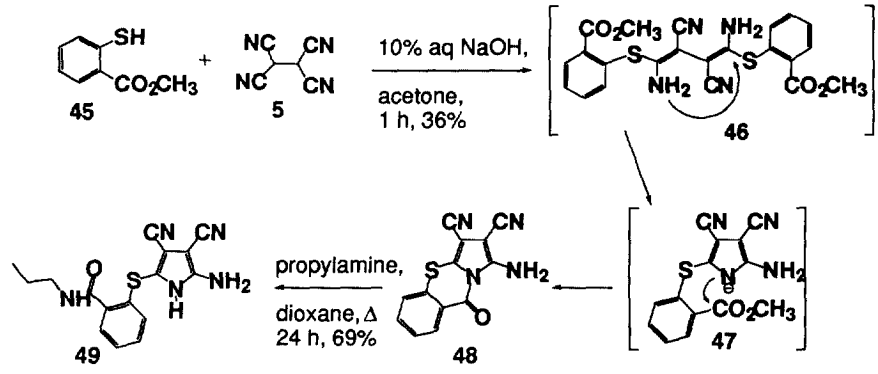
IC_{50} = 0.3 μM ± 0.1 (n = 2)
AP-1 IC_{50} = 1.2 μM ± 0.1 (n = 2)

The SAR around U0126 is summarized in Table 1. An electron-donating group is necessary in either the 2- or the 4-positions for good inhibition (compounds **1**, **17**, **20**, and **21**). The electron-rich bis-thiophene **44** is a stronger inhibitor (0.3 μM) of MEK than the corresponding unsubstituted benzene analog (**12**: 0.9 μM). Other heterocyclic thiols, such as 2-mercaptopyridine, failed to react with tetracyanoethane **5**. Substitution by electron-withdrawing groups resulted in weaker inhibition (**18**, **19**, **22-27**, **35**, **36**, and **41**). It appears that the

aromatic rings must be electron-rich for good inhibitory activity. Methylation of either the aryl amino or hydroxyl groups (**14**, **37-39**) lowered inhibitory activity, possibly due to steric hindrance and/or interruption of hydrogen bonding. Substitution by alkyl groups (**33**, **34**), which are slightly electron donating, resulted in weaker inhibition, most likely due to the lack of H-bonding capability. Introduction of a hydroxymethyl group (**40**) which is capable of H-bonding resulted in a poor inhibitor possibly due to steric effects compared to the smaller amino or hydroxyl (**1** or **20**). Additional aromatic substitution (**16**, **28-32**) resulted in decreased activity, again possibly due to steric hindrance. Removal of the two phenyl groups of U0126 and replacement with methyls (**42**) and 2-hydroxyethyls (**43**) lowered the inhibitory activity dramatically. Thus, no improvements over U0126 in either the IC_{50} or the AP-1 activity were obtained.

Reaction of 2-carbomethoxythiophenol **45** with **5** (Scheme 1) yielded not the corresponding U0126 analog **46**, but compound **48**. Subsequent ring opening with propylamine yielded amide **49** which helped in assigning the structure of **48**. Neither compound showed any significant inhibitory activity for MEK.

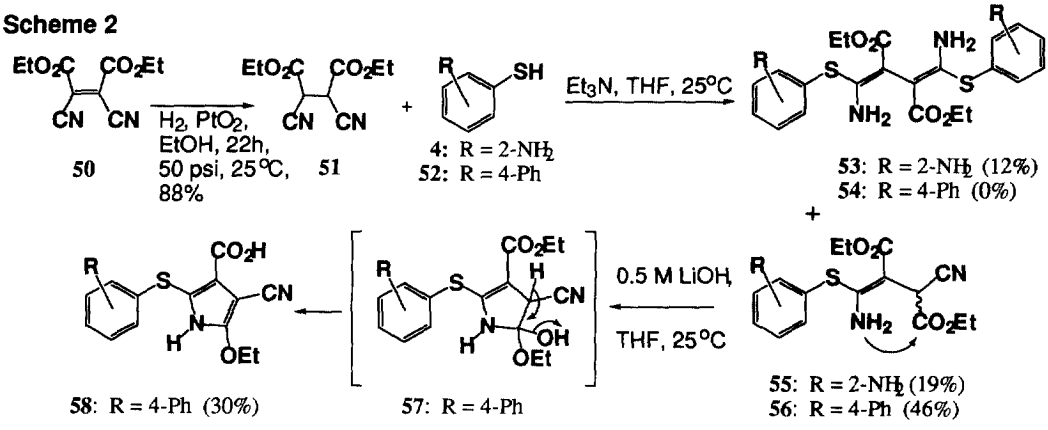
Scheme 1



45 5 46 47 48 49

Reacting 2-aminothiophenol **4** with 1,2-dicarboethoxy-1,2-dicyanoethane **51** (Scheme 2) afforded the corresponding biscarboethoxy analog **53** as one isomer together with some of the monoaddition adduct **55**. Although the reaction conditions were changed (Et_3N, THF instead of NaOH, acetone), the addition of thiols to tetracyanoethane **5** never yielded single isomers or monoadducts, regardless of the reaction conditions.

Scheme 2



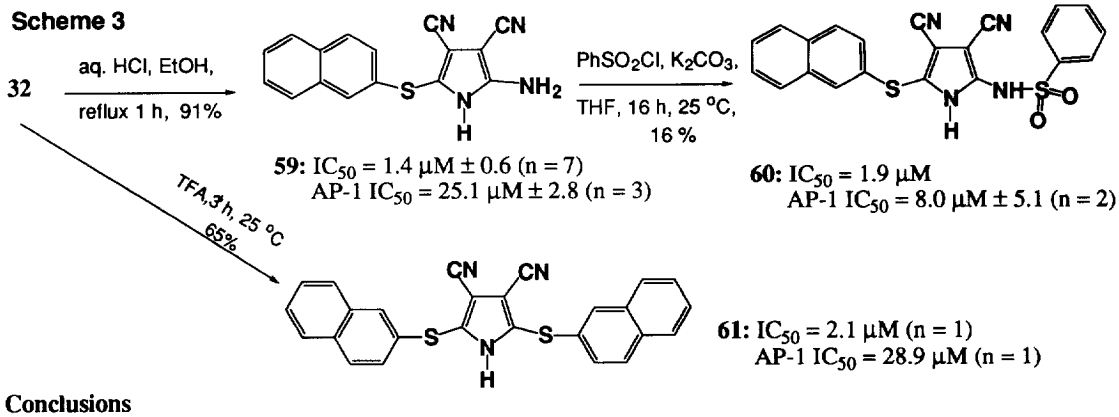
50 51 4: R = 2-NH2 52: R = 4-Ph 53: R = 2-NH2 (12%) 54: R = 4-Ph (0%) 55: R = 2-NH2 (19%) 56: R = 4-Ph (46%) 57: R = 4-Ph 58: R = 4-Ph (30%)

Somewhat unexpectedly, biphenylmercaptan **52** yielded only monoadduct **56**. Saponification of **56** surprisingly yielded tetrasubstituted pyrrole **58**. Compound **53** decomposed under these conditions and thus could not be

saponified. All of these compounds were devoid of inhibitory activity for MEK. Thus it appears that the cyano groups of U0126 are very important for MEK inhibition and cannot be replaced by carboethoxy groups.

All of the U0126 analogs in Table 1 were cyclized in refluxing aqueous HCl and ethanol[10] to yield 2-amino-3,4-dicyanopyrroles. The most potent 2-amino-3,4-dicyanopyrrole was **59** (Scheme 3). Pyrrole **59**, however, was poorly active in the cellular AP-1 suppression assay. Improvement could be made by sulfonylating the amino group to yield pyrrole **60** which retained the MEK inhibitory activity of **59**, while showing an improvement in the AP-1 suppression activity. In some instances, cyclization in TFA yielded symmetrical pyrroles. Thus, cyclization of **32** in TFA yielded **61** which exhibited good MEK but poor AP-1 inhibition.

Scheme 3



Conclusions

U0126 is a potent inhibitor of MEK and AP-1 transcriptional activity. Although it is readily susceptible to isomerization and cyclization, the *Z,Z*-isomer of U0126 appears to be the active moiety. Studies are presently underway to determine whether the novel mechanism of action displayed by U0126 will lead to anti-inflammatory activity in animal models.

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