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Cell Protein Cross-linking by Erbstatin and Related Compounds

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ABSTRACT. The protein-tyrosine kinase inhibitor and stable erbstatin analogue methyl 2,5-dihydroxycinnamate **(4)** cross-links cell proteins by a non-physiological chemical mechanism (Stanwell *et al.*, *Cancer Res* **55:** 4950–4956, 1995). To determine the structural requirements for this effect, erbstatin **(1)** and fifteen related compounds, including caffeic acid phenylethyl ester **(9)** were synthesized and examined for their ability to induce cross-linking of cellular protein at concentrations ranging from low micromolar up to 1000 μM. Tests were conducted in NIH3T3 fibroblasts as well as mouse keratinocytes. Potent cross-linking of cellular protein was observed for a number of analogues, including erbstatin, at concentrations as low as 10–50 μM. The inactivity of methoxy and fluoro as compared with their corresponding dihydroxylated counterparts indicated that free aromatic hydroxyls were essential for cross-linking. Additionally, compounds containing phenyl rings with 1,4-dihydroxy substituents were more potent than those having 1,2-dihydroxylated patterns. As with the prototype compound **4,** cross-linking was induced at both 37° and 4°, suggesting a chemical rather than physiological mechanism. Consistent with the data, a mechanism of action is proposed which involves initial oxidation to reactive quinone intermediates that subsequently cross-link protein nucleophiles via multiple 1,4-Michael-type additions. Similar alkylation of protein by protein-tyrosine kinase inhibitors, such as herbimycin A, has been invoked. While the latter benzoquinoid ansamycin antibiotics contain performed quinone moieties, results of the present study suggest that other hydroxylated kinase inhibitors can potentially participate in similar phenomena. A large number of potential therapeutics, including HIV integrase inhibitors, possess polyhydroxylated nuclei. The non-specific nature of the protein cross-linking reaction demonstrated for these erbstatin analogues, and the fact that cross-linking can occur at micromolar concentrations, may limit the therapeutic usefulness of such compounds to specific applications. BIOCHEM PHARMACOL 52;3:475–480, 1996.

KEY WORDS. erbstatin; lavendustin A; herbimycin A; protein-tyrosine kinase; cross-link; oxidation

In addition to their normal function as critical mediators of growth factor and cytokine-mediated signal transduction, PTKs§ can also participate in a variety of pathogenic processes, including several cancers. For this reason PTK inhibitors have been the object of intense development [1, 2]. One of the first PTK inhibitors was the fermentation product erbstatin **(1),** which was shown to inhibit the EGFR PTK at low micromolar concentrations both in membrane preparations [3] and *in situ* [4]. In addition to a styryl-like vinyl side chain, erbstatin contains the 1,4-dihydroquinone moiety found in other PTK inhibitors such as the lavendustin A pharmacophore **(2)** [5] and as the quinone in herbimycin A **(3)** [6] (Fig. 1). Structure-activity relationship studies based on erbstatin's lead have revealed that a large number of polyhydroxylated styryls can also function as effective PTK inhibitors, with great diversity being possible in functionalization of the vinyl side chain [1, 2]. With few exceptions, however, ortho or para dihydroxylation of the aryl ring has been a requirement for PTK inhibitory potency for this class of compound [7, 8]. The necessity of polyhydroxylation is somewhat paradoxical, since these inhibitors have been postulated to function as peptidomimetics of tyrosyl residues (which contain a single aromatic hydroxyl), competing for substrate at the catalytic site [8]. That a more complex mode of interaction may occur in cellular systems was first indicated by the demonstration that erbstatin effectively inhibits protein kinase C, which phosphorylates serine and threonine, rather than tyrosyl residues [9]. Erbstatin or other erbstatin-like PTK inhibitors, have been shown subsequently to inhibit a variety of kinase-independent enzyme systems [10], including HIV integrase [11]. As a further indication that such molecules exhibit multiple interactions with biological systems, we have reported recently that the stable erbstatin analogue, methyl 2,5-dihydroxycinnamate **(4)** (see Fig. 2) [12], cross-

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§ *Abbreviations:* PTK, protein-tyrosine kinase; EGFR, epidermal growth factor receptor; and CAPE, caffeic acid phenylethyl ester.

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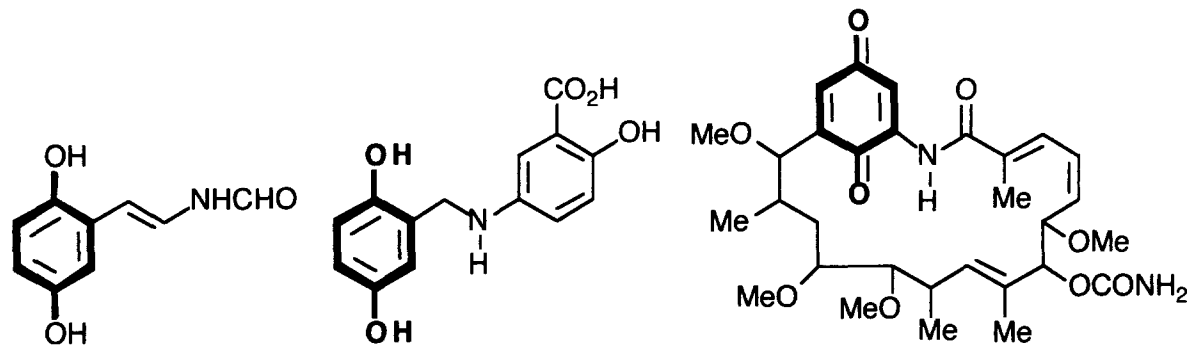

FIG. 1. Structures of erbstatin and structurally related PTK inhibitors.

links proteins and is cytotoxic to normal and neoplastic epithelial cells by a mechanism independent of PTK inhibition [13]. To further investigate this latter phenomenon, we prepared a variety of derivatives and examined their ability to induce cross-linking of cell proteins. We report herein the results of this study and the potential implications for other compounds of the erbstatin class.

MATERIALS AND METHODS

Inhibitors

Methyl 2,5-dihydroxycinnamate **(4)** was obtained commercially. The following inhibitors were synthesized according to the indicated procedures: erbstatin **(1)** [14]; compounds **5, 10, 11,** and **13–16** [11]; CAPE **(9)** [11, 15]; compound **17** [16]; and compound **18** [8]. Inhibitors **6–8** and **12** were prepared as outlined below:

β-PHENYLETHYL 2,5-DIMETHOXYCINNAMATE **(6).** A solution of *trans*-2,5-dimethoxycinnamic acid (520 mg, 2.5 mmol), β-phenylethyl alcohol (4.5 mL, 4.6 g, 38 mmol), and a catalytic amount of *p*-toluenesulfonic acid was stirred at reflux in a Dean Stark trap (overnight). Solvent was removed by rotary evaporation under reduced pressure, and crude product was purified by silica gel chromatography (5–25 μm silica; $CHCl_3$:hexane 2:3) to provide **6** as a yellow oil (220 mg, 28%): ^{1}H NMR ($CDCL_3$) δ 7.97 (d, 1H, J = 16.1 Hz), 7.35–7.22 (m, 5H), 7.03 (d, 1H, J = 2.9 Hz), 6.91 (dd, 1H, J = 3.0 Hz, 9.1 Hz), 6.85 (d, 1H, J = 9.0 Hz), 6.47 (d, 1H, J = 16.0 Hz), 4.42 (t, 2H, J = 7.2 Hz), 3.84 (s, 3H), 3.79 (s, 3H), 3.02 (t, 2H, J = 7.0 Hz).

β-PHENYLETHYL 2,5-DIFLUOROCINNAMATE **(7).** A mixture of 2,5-difluorobenzaldehyde (213 mg, 1.5 mmol), 760 mg (1.65 mmol) of (carboxymethyl)-triphenylphosphonium chloride, β-phenylethyl ester [m.p. 162–165° (dec); lit. [17] 148–151°], and powdered anhydrous K_2CO_3 (414 mg 3.0 mmol) in anhydrous dimethylformamide (5 mL) was stirred at ambient temperature (overnight). The crude reaction mixture was then partitioned between 0.5 N HCl in brine (100 mL)/ethyl acetate (2 × 50 mL), washed with brine (2 × 50 mL), and dried ($MgSO_4$); the solvent was removed by rotary evaporation under reduced pressure to yield a white solid. Purification by silica gel chromatography (5–25 μm silica; $CHCl_3$) gave an oil (559 mg) that was precipitated from petroleum ether (35–60°) at −78° to provide 7 as a white solid (295 mg, 68%): m.p. 35–36°; ^{1}H NMR ($CDCl_3$) δ 7.74 (d, 1H, J = 16.0 Hz), 7.36–7.19 (m, 5H), 7.09–7.03 (m, 3H), 6.48 (d, 1H, J = 16.3 Hz), 4.43 (t, 2H, 7.2 Hz), 3.02 (t, 2H, J = 7.0 Hz).

3-(2,5-DIHYDROXYPHENYL)PROPANOIC ACID β-PHENYLETHYL ESTER **(8).** A solution of **5** (284 mg, 1.0 mmol) in MeOH (10 mL) was hydrogenated over 10% Pd·C (100 mg) under 40 psi H_2 in a Parr apparatus (5 hr). The reaction mixture was filtered through celite and solvent removed by rotary evaporation under reduced pressure to yield a brown syrup (278 mg). Purification by silica gel chromatography (5–25 μm silica; $CHCl_3$) provided **8** as a light brown syrup (263 mg, 92%): ^{1}H NMR ($CDCl_3$) δ 7.25–7.07 (m, 5H), 6.67 (d, 1H, J = 8.5 Hz), 6.51 (dd, 1H, J = 3.0 Hz, 8.5 Hz), 6.46 (d, 1H, J = 3.0 Hz), 4.22 (t, 2H, J = 7.0 Hz), 2.83 (t, 2H, J = 7.1 Hz), 2.77–2.71 (m, 2H), 2.63–2.57 (m, 2H).

β-PHENYLETHYL 3,5-DIMETHOXYCINNAMATE **(12).** Reaction of 3,5-dihydroxybenzaldehyde and (carboxymethyl)-triphenylphosphonium chloride, β-phenylethyl ester as reported above for the synthesis of **7** gave **12** as a white crystalline solid (57%): m.p. 150–153°; ^{1}H NMR ($CDCl_3$ with DMSO-d_6) δ 9.48 (s, 2H), 7.43 (d, 1H, J = 16.0 Hz), 7.36–7.24 (m, 5H), 6.50 (d, 2H, J = 1.8 Hz), 6.35 (d, 1H, J = 16.1 Hz), 6.32 (d [apparent], 1H, J = 1.8 Hz), 4.34 (t, 2H, J = 6.9 Hz), 2.97 (t, 2H, 6.8 Hz).

Biological

Experimental procedures are as previously reported [13]. A brief summary is provided.

CELL CULTURE. NIH3T3 fibroblasts were obtained from ATCC, Rockville, MD, and were routinely passaged in

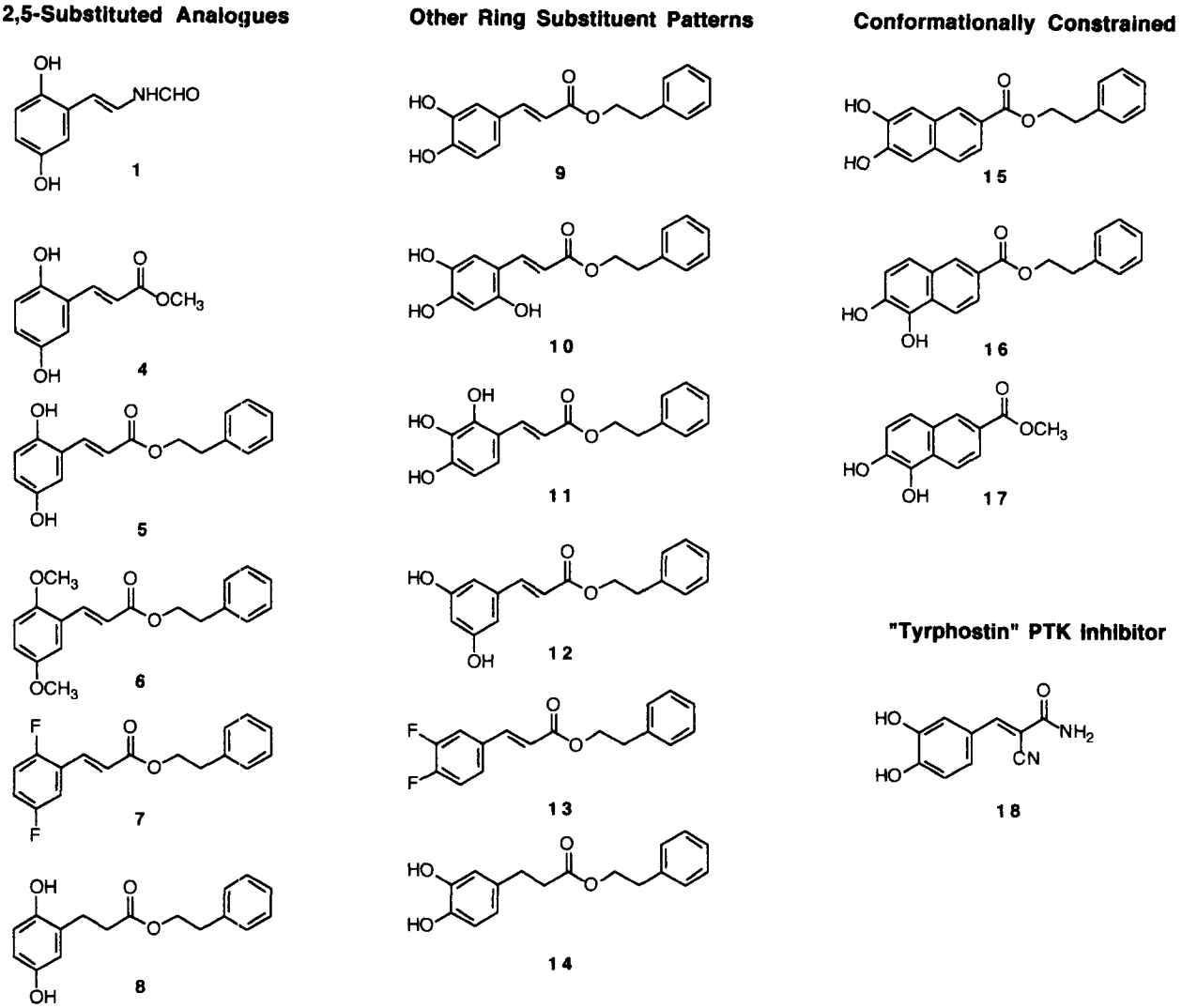

FIG. 2. Structures of compounds tested.

Dulbecco's minimum essential medium with penicillin-streptomycin, 2 mM glutamine and 1 mM pyruvate, with 10% newborn calf serum. Mouse keratinocytes were isolated from BALB/c newborn mouse skin and grown in Eagle's minimal essential medium with 8% fetal bovine serum (chelexed) and 0.2% penicillin/streptomycin solution with 0.05 mM Ca^{2+}.

CROSS-LINKED ENVELOPE ASSAY. The assay measures insoluble cross-linked protein envelopes as previously described [18] with modifications [13]. Cross-linked envelopes were prepared by scraping monolayers into 2% SDS, 20 mM dithiothreitol in phosphate-buffered saline. Unattached cells were pelleted from medium and were pooled with attached cells. Samples were boiled (10 min) and were applied to RC60 membranes over a dot blot apparatus. Soluble material was washed through the membrane [13]; then remaining cross-linked protein spots were fixed and stained as described [13]. Spots were excised and eluted with 1% NH_3OH in 66% methanol (overnight). Absorbance of the eluate was measured on a Titertek plate reader at 600 nm.

RESULTS AND DISCUSSION

During a preliminary study designed to examine the ability of PTK inhibitors to promote terminal differentiation of epidermal keratinocytes, it was found that the stable erbstatin analogue methyl 2,5-dihydroxycinnamate (**4**) induces cross-linking of cellular protein [13]. Since erbstatin (**1**) shares structural aspects common to other natural product PTK inhibitors, and has additionally served as the prototype for a wide variety of synthetic PTK inhibitors [1, 2], it was of interest to examine what features were important for this cross-linking effect, as similar cross-linking could potentially be exhibited by these other agents. In initial experiments it was found that erbstatin itself and the β-phenylethyl ester **5** were able to induce cross-linking of approximately the same order as the methyl ester **4**. This seemed to indicate that the ester functionality was not a critical determinant of cross-linking, and the β-phenylethyl ester group was utilized in many of the subsequent derivatives (**6–16**). This ester group was chosen because of its chemical stability, and because it is found in the related cinnamate, CAPE (**9**), which is a widely studied antipro-

liferative agent [15]. The resulting series of β-phenylethyl cinnamates examined specific features necessary for cross-linking. Questions of particular interest centered on what role the nature and pattern of aromatic substituents played. Compounds **5–7** maintained the 2,5-substitution pattern of erbstatin, with the hydroxy groups being replaced by methoxy and fluoro substituents in **6** and **7**, respectively. Compounds **9–12** examined the efficacy of hydroxyl substitution patterns other than the 2,5-arrangement, while compounds **15** and **16** replaced the phenyl ring with a naphthalene system. As previously reported, the bicyclic naphthalene arrangement serves as a conformationally constrained mimic of CAPE (**9**) in which the phenyl ring is held in specific conformations relative to the vinylic side chain [11].

The series of sixteen compounds (Fig. 2) was screened for cross-linking in NIH3T3 cells at 37° using concentrations from 250 to 1000 μM (data not shown). Compounds **6, 7, 9, 12, 13,** and **15** did not exhibit appreciable activity within this concentration range and were not examined further. The remaining compounds were then examined more carefully. As shown in Fig. 3, the tyrphostin compound **18** was partially active at a 1 mM concentration in NIH3T3 cells, while compounds **11** and **14** induced extensive cross-linking, but only at the highest concentrations (500 and 1000 μM). Compounds **1, 4, 5, 8, 10, 16** and **17** showed significant cross-linking at concentrations as low as 250 μM, with compounds **1** and **17** showing appreciable cross-linking at 50–75 μM. The compounds were next examined at 37° in normal mouse keratinocytes, at a lower concentration range: from 10 to 100 μM (Fig. 4A). In these cells, little cross-linking was observed for compounds **11, 14** and **16** up to 100 μM, although extensive cross-linking was observed at 250–1000 μM (results not shown). Compounds **4** and **17** induced marked cross-linking down to 50 μM, with compounds **1, 5** and **10** doing so down to 25 μM. Compound **10** induced extensive cross-linking even at the lowest concentration of 10 μM. The most potent compounds (**1, 4, 5, 8, 10,** and **17**) were retested at 100 μM at a temperature of 4° (Fig. 4B). Significant cross-linking was observed with the entire set under these conditions, although compound **10** and, to a lesser degree, compound **17,** showed reduced potency. Finally, control assays were run at 37° and 4° using the nonspecific kinase inhibitor staurosporine (10 nM), which induces protein cross-linking by an enzymatic mechanism, with the induction of transglutaminases [19, 20]. As shown in Fig. 4C, the ability of staurosporine to induce cross-linking was almost eliminated at 4° relative to levels achieved at 37°, indicating that cross-linking occurs through a physiological process. In contrast, the inhibitors **1, 4, 5, 8, 10,** and **17** were able to induce cross-linking at both 37° and 4°, indicating that a chemical rather than a physiological process is probably involved.

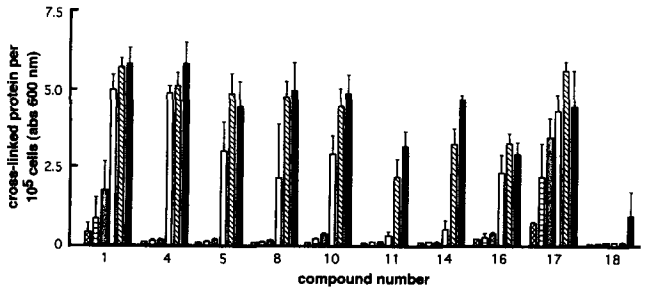

FIG. 3. Protein cross-linking after treatment with various analogues. NIH3T3 cells were incubated with analogues at 37° for 72 hr; then cross-linked protein was isolated and quantified as described in Materials and Methods. Key: checked bars, 50 μM; striped bars, 75 μM; grey bars, 100 μM; white bars, 250 μM; hatched bars, 500 μM; and black bars, 1000 μM. Results are the means ± SEM of three experiments, each conducted in duplicate.

The data indicate structural relationships that enhance cross-linking ability. First, erbstatin and its synthetic derivatives **4, 5,** and **10** exhibited similar cross-linking patterns, indicating that structural features necessary for cross-linking do not reside exclusively in either the formamido or the ester functionalities. Second, for phenyl rings, the highest cross-linking potency was generally found with compounds containing aromatic substituents arranged in a 1,4-orientation. Changing substituent position, for example to the 1,3-orientation (compound **12**), resulted in inactivation. Additionally, these substituents must be free hydroxyls, with neither methoxy nor fluorogroups being adequate. In the naphthalene series, high potency was found with a 1,2-dihydroxy pattern when this was in the 5,6- (compound **17**) but not the 6,7-orientation (compound **15**).

Only compounds with free hydroxyls in the 1,2- or 1,4-orientation are capable of readily forming quinones. The fact that such an arrangement was required for cross-linking potency is consistent with a mechanism involving initial oxidation to a reactive quinone intermediate which can then undergo multiple additions of protein nucleophiles via Michael-type addition reactions (Fig. 5). Such a quinone species was found preformed in the natural product PTK inhibitor herbimycin (A) (**3**), where a central mechanism of its antiproliferative activity is thought to involve such a 1,4-addition process [21]. However, unlike herbimycin A, which potently inhibits PTK and cell growth at nanomolar concentrations [21], compounds **1** [1], **4** [13], **10, 11, 14,** and **17** required micromolar concentrations for their effects (NCI screen, unpublished results), which overlaps to some extent with the concentrations required for protein cross-linking. Variations in cross-linking potency due to other structural modifications are not explained easily. Although data suggest that cross-linking is a "chemical" and not a physiological effect (i.e. not enzymatically mediated), it is difficult to rationalize differences in cross-linking potency, for example between **9** and **14** or between **16** and **17,** solely on the chemical ability to form quinone species. Once oxidation to the reactive intermediate has occurred, cross-linking may rely on other structure-dependent interactions of the cross-linker with cellular proteins, and therefore efficacy may vary depending on the nature of the cellular

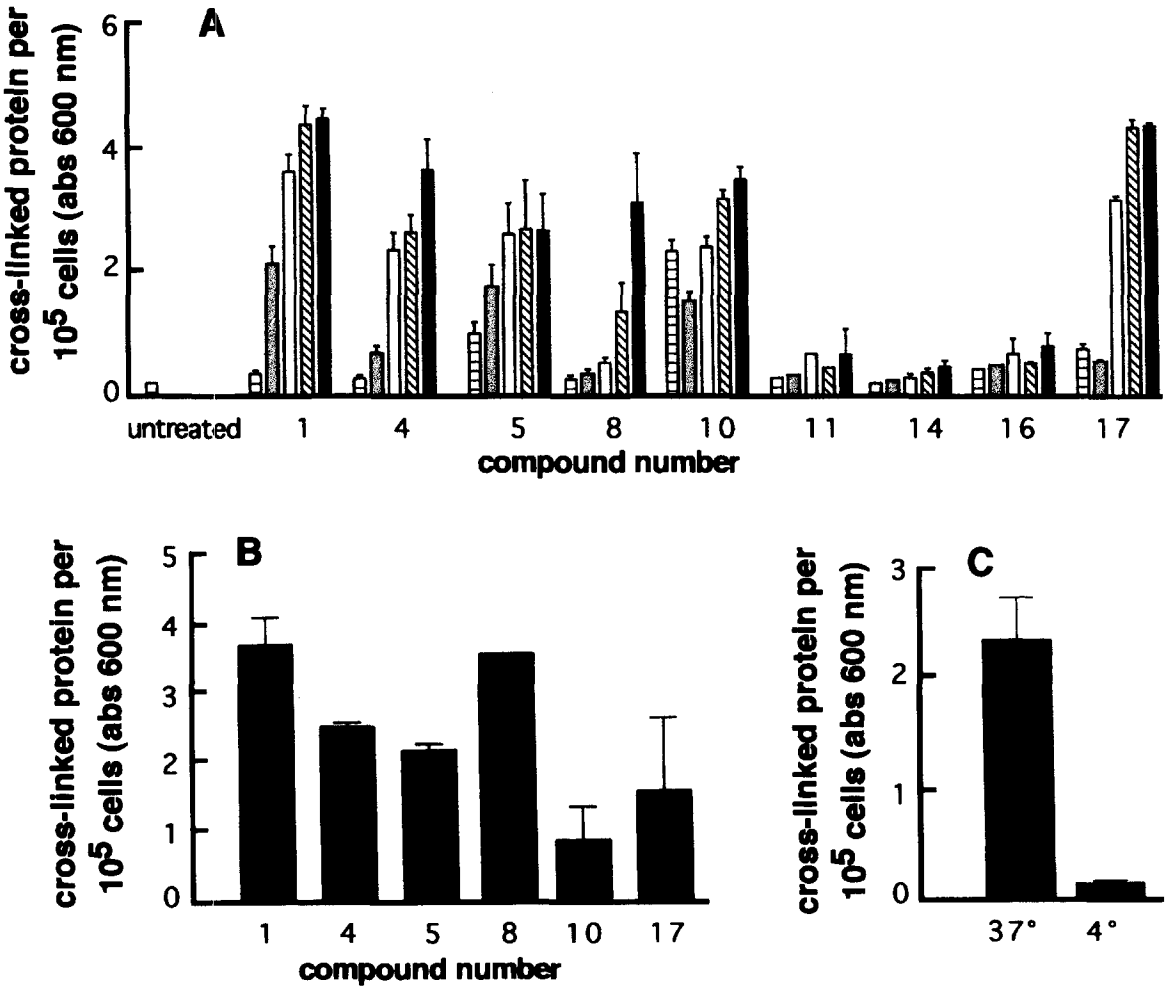

FIG. 4. Protein cross-linking under different conditions. Mouse keratinocytes were incubated with erbstatin analogues (panels A and B) or 10 nM staurosporine (panel C) for 72 hr prior to isolation and measurement of cross-linked protein. In panel A, cells were incubated with agents at 37°. In panel B, cells were incubated at 4° with a 100 μM concentration of each compound, and in panel C, the temperature of incubation with staurosporine was as indicated. (A) striped bars, 10 μM; grey bars, 25 μM; white bars, 50 μM; hatched bars, 75 μM; and black bars, 100 μM. In panels A and B, results are the means ± SEM of three experiments, each conducted in duplicate. In panel C, results are the means ± SD of one experiment ($N = 3$), which was repeated on two occasions with similar results.

constituents. For example, there is a noticeable cell line-dependence in cross-linking potency of some analogues in this study, as has also been observed previously [13]. Differences in cross-linking potency at this level may make a consistent structure-activity correlation within a series of analogues difficult to derive.

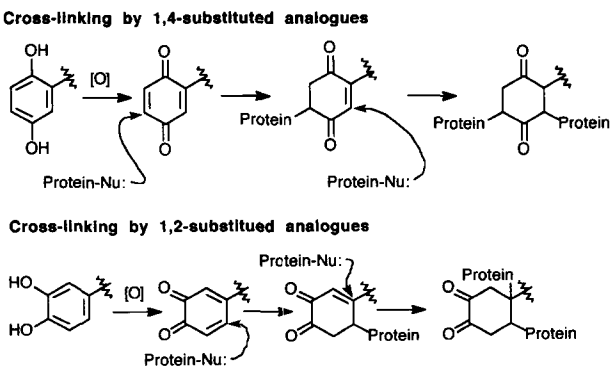

FIG. 5. Possible mechanism of cross-linking by erbstatin and related analogues.

Cellular material cross-linked by the erbstatin analogues could be visualized microscopically as portions of cell membrane at low concentrations and cell appeared to be "fixed" by the compounds at the highest concentrations (results not shown), as with the prototype compound methyl 2,5-dihydroxycinnamate (**4**) [13]. Cross-linking may contribute to cytotoxicity and limit the potential therapeutic applications of these types of compounds to treatment modalities in which protein cross-linking may be desirable, such as the topical treatment of warts or superficial neoplasms of the skin [13]. In conclusion, our results suggest that polyhydroxylated aromatic compounds, including erbstatin and related analogues, can mediate secondary effects, possibly via activation to quinones. A large number of potential therapeutics, including HIV integrase inhibitors [11], possess these kinds of polyhydroxylated aryl nuclei. Therefore, this phenomenon may be of general relevancy, since signifi-

cant cross-linking can occur at concentrations in the low micromolar range.

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