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A New Class of Mechanism-Based Inhibitors of Transglutaminase Enzymes Inhibits the Formation of Cross-Linked Envelopes By Human Malignant Keratinocytes

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SUMMARY

A series of tyrosinamidomethyl dihydrohaloisoxazole compounds, designed as mechanism-based inhibitors of bovine epidermal transglutaminase enzyme, was examined for effects on the formation of cross-linked envelopes by human SCC-9 malignant keratinocytes. Compounds inhibited ionophore-induced envelope formation in a manner that reflected their capacity to inhibit transglutaminase activity. Preincubation and inhibitor wash-out studies indicated that the inhibitor must be present at the time of cell activation by ionophore in order to inhibit envelope formation. The stereospecific nature of the inhibitory activity of these compounds on both transglutaminase activity and cross-linked envelope formation makes this class of compounds an important tool in the study of transglutaminase-mediated events at the cellular level.

One feature of keratinocyte terminal differentiation is the formation of an internal, cell membrane-associated, cross-linked envelope (1). In the process of cross-linked envelope formation, cytosolic proteins, including involucrin, become covalently attached to particulate proteins in a detergent (SDS)-resistant manner through ϵ-(γ-glutamyl) lysine isopeptide bonding (2). This "cross-linking" is catalyzed by cellular transglutaminase activity. Normal keratinocytes in culture express two types of transglutaminase, particulate types and cytosolic types (3, 4). The capacity of cells to form cross-linked envelopes correlates with the activity of the particulate enzyme (5).

In order to probe the biological consequences of the modulation of transglutaminase activity, a series of mechanism-based inhibitors of the enzymes has been developed (6). These compounds are derivatives of tyrosinamidomethyl halodihydroisoxazole and collectively will be called halodihydroisoxazoles.[2] In this report, we present the results of studies of the effects of these compounds on the process of ionophore-induced cross-linked envelope formation by a human malignant keratinocyte cell line, SCC-9 (squamous cell carcinoma) (7). This cell line has a predominance of the particulate transglutaminase activity (8).

Experimental Procedures

Materials. Cells were obtained from the American Type Culture Collection (Rockville, MD). All chemicals, with the exception of the enzyme inhibitors discussed below, were obtained from Sigma Chemical Company (St. Louis, MO). Cell culture materials were obtained from GIBCO Laboratories, (Burlington, Ontario) or Flow Laboratories (Mississauga, Ontario) except for the fetal bovine serum, which was obtained from Hyclone Laboratories (Logan, UT).

Compounds containing the CBZ *N*-protecting group were prepared as previously described (9). Compounds containing the MNP *N*-protecting group were prepared in the same manner as the CBZ analogs but starting with the requisite *N*-protected tyrosine. This was prepared in two steps, by condensing L-tyrosine methyl ester with 2-(*S*)-(6-methoxy-2-naphthyl)propionic acid using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/dimethylaminopyridine/ethyl acetate followed by hydroxide hydrolysis of the intermediate ester in methanol.

Cell culture. SCC-9 cells were cultured as described by Rheinwald and Beckett (7) but with the following modifications. Cells were cultured without 3T3 fibroblast feeder layer support by seeding the cells at higher densities at subculture than originally described (i.e., 1/4 final confluent density instead of 1/16). Cells cultured under these

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[2] IUPAC designation is 2-(1-benzyloxymethamido)-*N*-[(3-halo-4,5-dihydroisoxazol-5-yl)methyl]-3-(4-hydroxyphenyl)propanamide in the case of the CBZ *N*-substitution and 2-[2-(6-methoxynaphthyl)propanamido]-*N*-[(3-halo-4,5-dihydroisoxazol-5-yl)methyl]-3-(4-hydroxyphenyl)propanamide in the case of the MNP *N*-substituted compounds.

ABBREVIATIONS: SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; CBZ, carbobenzyloxy; MNP, 2-(*S*)-(6-methoxy-2-naphthyl)-propanyl; MDC, monodansylcadaverine.

conditions retained the capacity to form cross-linked envelopes upon treatment with ionophore.

Cross-linked envelope assay. The assay for cross-linked envelope formation competence was based on the procedure of Rice and Green (10). The assay was divided into two stages; a preincubation stage, in which cells were preincubated with inhibitors, and a subsequent induction stage, in which ionophore was added and cross-linked envelope formation was induced. For the induction stage, a time-course study demonstrated that the maximum cross-linked envelope formation occurred between 20 and 30 min after the addition of ionophore and that 75–80% of the cells formed envelopes. The time of the induction stage for testing inhibitors was set at 10 min (51–77% of the cells formed envelopes, on 11 separate test days). For the preincubation stage, cells in culture medium were incubated with compounds for various times at 37°, in a 5% CO_2 in air atmosphere, before addition of ionophore. Each reaction tube contained a final volume of 500 μl of Dulbecco's modified Eagle medium 2 × 10^5 cells, and compounds at various concentrations with a final DMSO vehicle concentration of 0.1%. Ionophore was added at a final concentration of 50 μg/ml in 5 μl of ethanol. Envelope formation was quenched by the addition of 55 μl of 20% SDS. After quenching, 1 mg of dithiothreitol was added to each tube and the tubes were allowed to stand for at least 20 min before the extent of envelope formation was determined. Envelopes were counted using a hemocytometer and a phase-contrast inverted microscope (10). In dose-response studies, each compound was tested at five concentrations, each in triplicate, and the effects were compared with controls in which DMSO was present.

Cross-linked envelope data analysis. Data are expressed as the per cent inhibition of envelope formation based on the number of envelopes in each sample relative to that in vehicle only treatments. Log dose-response curves were plotted and the concentration of compound reducing the control envelope formation by 50% (IC_{50}) was estimated from the graph. In cases in which the IC_{50} value was within the concentration range in which the compound was tested, a separate, nonparametric, one-way analysis of variance (Kruskel-Wallace) (11) was performed, followed by multiple comparisons of controls versus each dose level. In order to determine statistical significance the Dunn-Šidák method (12) was used to reflect experiment-wise rather than comparison-wise α levels.

Enzyme inhibition assays. Enzyme inhibition data are cited for comparison purposes only and will be published in greater detail in a separate communication. Bovine epidermal transglutaminase was prepared according to the method of Folk and Chung (13) but with the following modifications. The enzyme was isolated from lyophilized bovine snout by homogenization in a Waring blender and chromatography of the 8000 × *g*, 30 min, supernatant on DEAE-Sepharose Cl-6B in 5 mM Tris buffer, pH 7.5, containing 1 mM EDTA, with elution at 0.2 M NaCl of a 0 to 0.7 M gradient in this buffer.

Transglutaminase activity was measured by monitoring the rate of enzyme-catalyzed incorporation of MDC into CBZ-L-glutaminylglycine. Assay mixtures (150 μl) consisted of enzyme in Tris buffer, pH 8.1, containing 10 mM calcium chloride, 0.74 mM dithiothreitol, 1.5 mM MDC, 5% DMSO, and inhibitor at different concentrations in DMSO. At various times after inhibitor addition, 40 mM CBZ-L-glutaminylglycine was added for 30 min at 37° and then the reaction was quenched by the addition of 80 μl of EDTA (100 mM) that contained 40 μM internal fluorescent standard (dansyl-glycyl-tryptophan). Products were separated by high performance liquid chromatography on a 25-cm Ultrasphere ODS 5-μm column using a SpectraPhysics SP 8700 solvent delivery system.

The first-order rate constant (k) for the time-dependent loss of enzyme activity was obtained by nonlinear regression using the equation $y = Ae^{(-kt)}$ where y is the percentage of remaining enzyme activity observed at the incubation time t (min) compared with inhibitor-free control and A is the constant describing the initial value of y at time 0 min. The second-order rate constant $k/[I]$ was obtained by dividing the first-order rate constant by the inhibitor concentration.

Results

Time course of cross-linked envelope formation. The time course of cross-linked envelope formation was followed under control conditions, in the presence of 0.1% DMSO, for at least 2 hr. DMSO was necessary to increase compound solubility, although this concentration was associated with detectable inhibition (less than 1%) of cross-linked envelope formation. Cross-linked envelope formation occurred at a relatively rapid rate for the first 10 min after the addition of ionophore X537-A (50 μg/ml) and at a slower rate thereafter (Fig. 1). The maximum number of cells forming envelopes usually ranged from 60 to 70% of the total cells per tube.

The effects of the halodihydoisoxazole compound RS10823 on cross-linked envelope formation were examined, because this compound inhibited bovine epidermal transglutaminase in a time-dependent fashion (Fig. 2) and, furthermore, the inhibition was calcium-dependent (Fig. 3). Bovine epidermal trans-

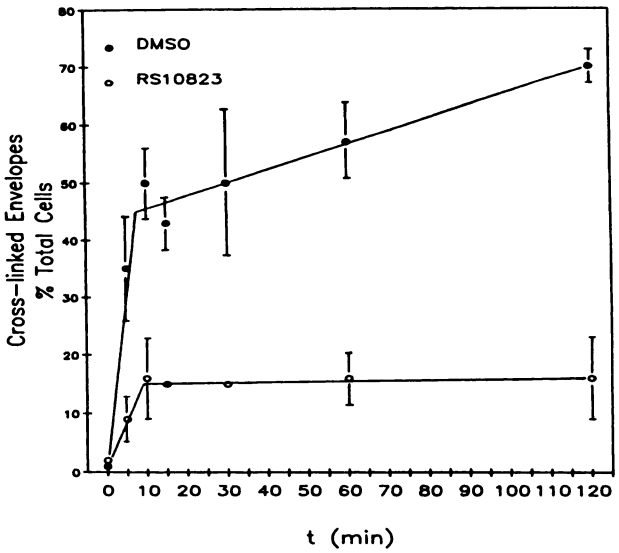


Fig. 1. Time course of cross-linked envelope formation in the presence and absence of RS10823. Cells were preincubated with compound (100 μM) or vehicle (DMSO, 0.1%) for 15 min before the addition of ionophore (X537-A), and envelope formation was terminated after various times. Envelope formation is expressed as the number of cells forming envelopes as a percentage of the total cell number. Each value is the mean and standard deviation of three determinations.

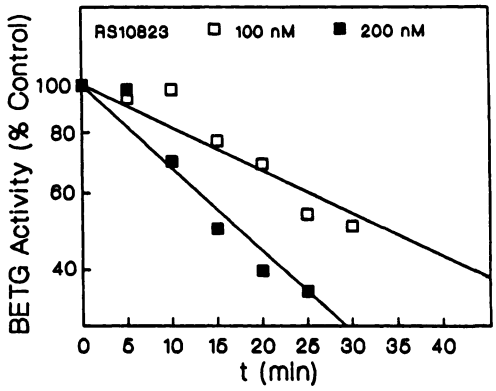


Fig. 2. Time-dependent inhibition of bovine epidermal transglutaminase (*BETG*) by RS10823. The enzyme activities in the presence of inhibitor are plotted on a logarithmic *y*-axis as a percentage of the corresponding inhibitor-free control activities.

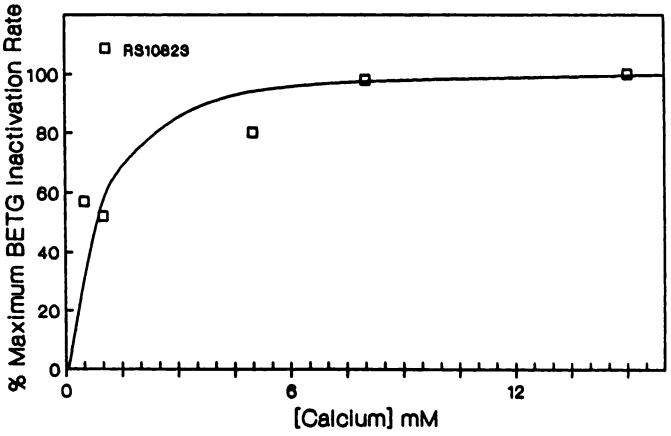


Fig. 3. Calcium-dependent inhibition of bovine epidermal transglutaminase (*BETG*) by RS10823. Enzyme activities were determined using the assay buffer described in Experimental Procedures but with variable calcium concentrations. The second-order rate constants for inhibition by RS10823 were calculated at the different calcium concentrations and are plotted as a percentage of the highest measured rate constant.

glutaminase had a calcium requirement for half-maximal activation in the range of 0.1 to 0.5 mM calcium (data not shown), as reported for other intracellular transglutaminases (14). Enzyme activation with calcium is, therefore, a necessary requirement before mechanism-based inactivation by RS10823 can occur. Cross-linked envelope formation progressed for 10 min in the presence of 100 μM RS10823, although the rate was significantly reduced compared with controls (Fig. 1). No further increase in envelope formation occurred after this time. The percentage of cells forming cross-linked envelopes after 2 hr with ionophore was reduced by RS10823 to 17%, from 70% in controls.

Effects of preincubation with RS10823. Compounds related to RS10823 have been characterized as irreversible inactivators of bovine epidermal transglutaminase (9). Tests were run, therefore, to determine whether irreversible inhibition could be demonstrated in cell studies. In one study, cells were preincubated with compound (100 μM) for various times (2–30 min) before the addition of ionophore to induce cross-linked envelope formation. The inhibitory effects of the compound were maximal after as little as 2 min of preincubation and inhibition did not increase with increasing preincubation time (Fig. 4). In other studies, cells were preincubated with compound (100 μM) for 30 min at 37° and then were washed free of inhibitor before ionophore addition and the induction of cross-linked envelope formation. The time-courses of envelope formation, with and without preincubation with inhibitor, were essentially the same (Fig. 5). These studies suggest that, to be effective, inhibitor must be present at the time of the induction of envelope formation and that preincubation of cells with inhibitor has no apparent effect on any transglutaminase involved in cross-linked envelop formation.

Effect of RS10823 on trypan blue exclusion by cells. One reason for a lack of effect of RS10823 on cells after preincubation and wash-out may be the inability of the compound to penetrate the cell membrane in the absence of ionophore stimulation. The capacity of cells to exclude trypan blue (0.4%) was examined, therefore, in the presence of various concentrations of RS10823, in the presence or absence of ionophore (50 μg/ml). RS10823 had no significant effect on trypan blue exclusion in the control or ionophore-stimulated

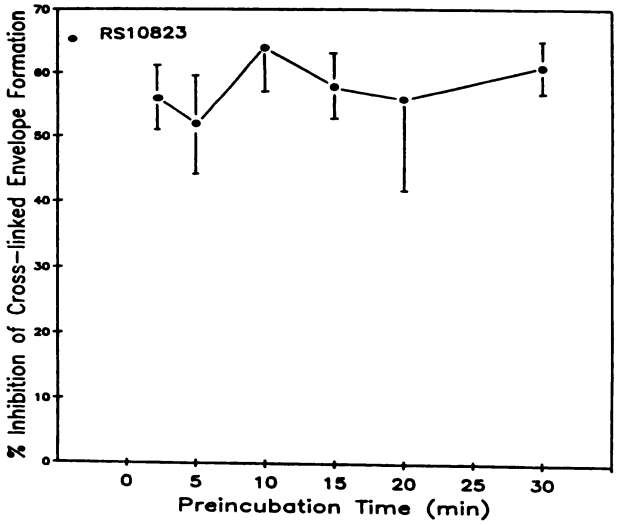


Fig. 4. Effects of inhibitor preincubation time on the inhibition of cross-linked envelope formation. Cells were preincubated with RS10823 for various times (2–30 min) before the induction of cross-linked envelope formation. Percentage of inhibition was calculated from the number of cells forming envelopes after treatment with inhibitor relative to inhibitor-free controls. Each value represents the mean and standard deviation of three determinations.

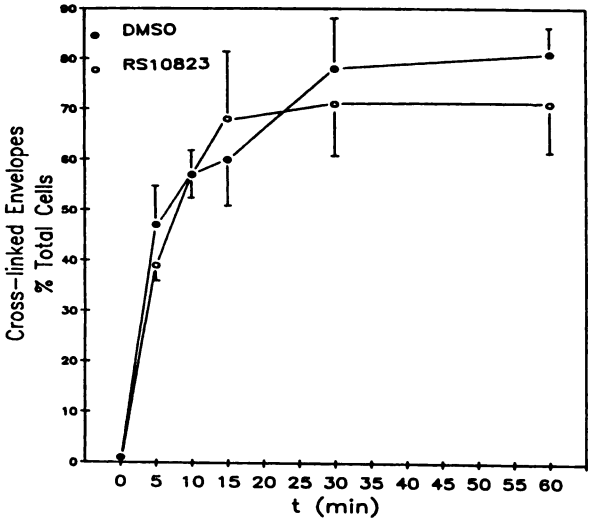


Fig. 5. Effects of pretreatment with RS10823. Cells were preincubated with RS10823 (100 μM) or vehicle (DMSO, 0.1%) for 15 min before washing and subsequent induction of cross-linked envelope formation with ionophore. The number of envelopes in each sample was measured at the indicated times. Envelope formation is expressed as the number of cells forming envelopes as a percentage of the total cell number. Each value represents the mean and standard deviation of three determinations.

cells (Fig. 6). The ionophore itself caused an increase in the number of cells accumulating dye, from less than 10% in the controls to as high as 80% in the presence of ionophore. There was, however, no difference in the release of lactate dehydrogenase between control and ionophore-treated cells (data not shown). It appears, therefore, that the ionophore causes a selective increase in the permeability of the cell membrane and this may facilitate the passage of inhibitor molecules into the cell without allowing the loss of larger intracellular proteins.

Comparison of 5*S*- and 5*R*-stereoisomers. This class of compounds may inhibit transglutaminases by attack of an enzyme active site cysteine thiol on the inhibitor ring. The

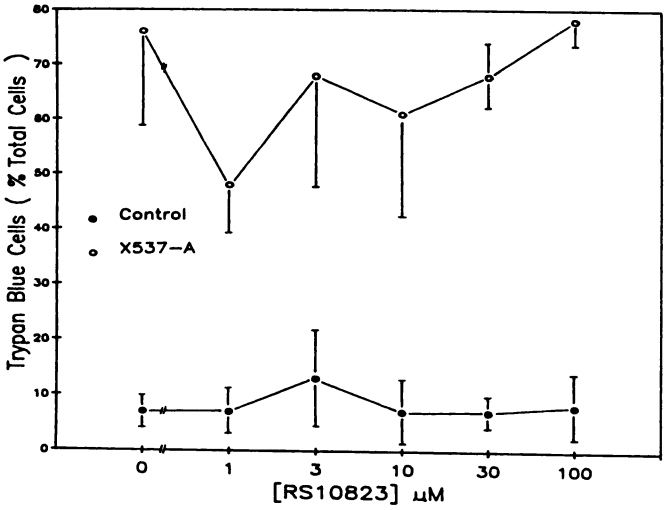


Fig. 6. Trypan blue staining of ionophore-treated and control cells in the presence of various concentrations of RS10823. Cells were preincubated for 15 min with compound or vehicle (DMSO, 0.1%) before the addition of ionophore. Ten minutes after the addition of ionophore, trypan blue (0.4%) was added for 2 min followed by cell washing. The stained cells were counted and the per cent trypan blue staining was determined based on the total cell count. Each value represents the mean and standard deviation of three deteriminations.

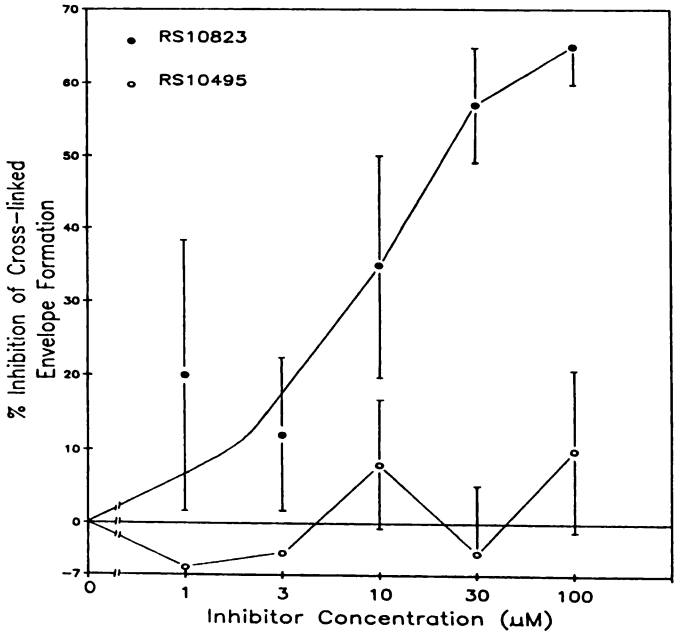
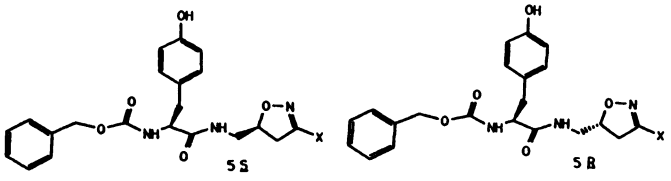


Fig. 7. Dose-response curves comparing 5*S*- and 5*R*-isomers, RS10823 and RS10495, respectively. Cells were preincubated with compounds for 15 min before the addition of ionophore (50 μg/ml) and envelope formation was terminated after 10 min. Per cent inhibition was calculated from the number of cells forming envelopes after treatment with compounds relative to that measured in control samples. Each value represents the mean and standard deviation of three determinations.

TABLE 1

Comparison of the inhibitions of transglutaminase activity and cross-linked envelope formation by CBZ-halodihydroisoxazoles

IC_{50} values for the inhibition of cross-linked envelope formation by SCC-9 cells and second-order rate constants for enzyme inhibition ($k/[I]$) were determined as described in Experimental Procedures.



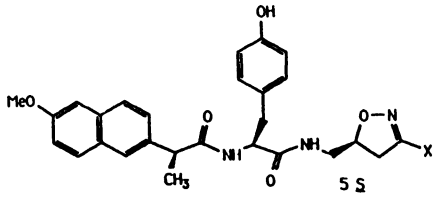
RS number	X	Chirality	BETG inactivation, $k/[I]$[a]	Envelope inhibition, IC_{50}[b]
			$M^{-1}\ min^{-1}$	μM
10598	Cl	5*R*	1,200	>100, >100
10025	Cl	5*S*	67,000	70, 100
10495	Br	5*R*	5,750	>100, >100
10823	Br	5*S*	219,000	70, 30
31819	H	5*S* + 5*R*	N[c]	N

[a] BETG, bovine epidermal transglutaminase. $k/[I]$ = second-order rate constant for inhibition.
[b] Independent determinations for inhibition of envelope formation are separated by commas.
[c] N, no inhibition.

TABLE 2

Comparison of the inhibitions of transglutaminase activity and cross-linked envelope formation by MNP-halodihydroisoxazoles

IC_{50} values for the inhibition of cross-linked envelope formation by SCC-9 cells and second-order rate constants for enzyme inhibition ($k/[I]$) were determined as described in Experimental Procedures.



RS number	X	Chirality	BETG inactivation, $k/[I]$[a]	Envelope inhibition, IC_{50}[b]
			$M^{-1}\ min^{-1}$	μM
48375	Cl	5*S*	23,600	5
48373	Br	5*S*	73,200	1, 3, 6

[a,b] See footnotes *a* and *b* of Table 1.

tetrahedral intermediate formed could then break down with the loss of the halide group and the subsequent formation of a stable thioimine adduct (9). The chirality at C-5 of the dihydroisoxazole ring was critical for potency in enzyme studies (Table 1). There was a marked reduction in inhibitory potency of the 5*R*-isomer compared with the 5*S*-isomer in this series of compounds. Isomers were, therefore, compared for effects on cross-linked envelope formation. The 5*S*-isomer, RS10823 (Table 1), caused a concentration-related decrease in envelope formation (Fig. 7) with an IC_{50} value of 30 to 70 μM (Table 1). At inhibitor concentrations of 30 and 100 μM, the inhibitory effects were statistically significant ($p < 0.01$). The 5*R*-isomer (RS10495) had no inhibitory effect on envelope formation over the same concentration range. In comparing another isomer set (RS10025 and RS10598; Table 1) the same preference for the 5*S*-stereoisomer was found.

Comparison of bromine- and chlorine-substituted compounds. Bromine-substituted compounds were more effective inhibitors of enzyme activity than the corresponding chlorine-substituted compounds (Tables 1 and 2). Results from the cross-linked envelope assay, however, did not reveal significant differences in potency between bromine- and chlorine-substituted compounds of similar stereochemistry. The importance of the halide leaving group on the dihydroisoxazole ring for envelope inhibition was supported by studies with RS31819,

in which hydrogen replaces the halide atom. RS31819 was inactive in both enzyme and cell assays (Table 1).

Comparison of the CBZ and MNP *N*-protecting groups and studies with other transglutaminase inhibitors. There was a correlation between bovine epidermal transglutaminase inhibitory activity and the inhibition of cross-linked envelope formation by the CBZ-substituted compounds (Table 1). RS48373, a compound in which the CBZ moiety was replaced by a MNP *N*-protecting group, was a more potent inhibitor of cross-linked envelope formation (Fig. 8; Table 2). The MNP function conferred enhanced inhibitory characteristics on the tyrosinamidomethyl halodihydoisoxazole core, with concentrations as low as 3 μM causing statistically significant inhibition ($p < 0.01$). This increased inhibitory activity was only evident at the cellular level, however, and not at the level of bovine epidermal transglutaminase activity (see data for RS48373, Table 2, versus RS10823, Table 1).

For comparison purposes, two other known enzyme modulators were examined for effects on cross-linked envelope formation. MDC, a synthetic amine that serves as an alternate substrate for transglutaminase, was found to block envelope formation in this system (Fig. 8). MDC was less potent than RS48373 and had a very steep dose-response profile. Cystamine, an inhibitor of transglutaminase, had no effect on cross-linked envelope formation at concentrations employed for the halodihydroisoxazoles (up to 100 μM; data not shown).

Discussion

Transglutaminases are calcium-dependent enzymes that catalyze the posttranslational modification of proteins through the exchange of primary amines for ammonia at the γ-carboxamide group of peptide-bound glutamine residues (14). An active site cysteine residue is crucial for enzyme activity. 3-Halo-4,5-dihydroisoxazoles render the enzyme inactive, most likely through the formation of stable thioimine inhibitor-enzyme adducts following the formation of tetrahedral intermediates (6, 9). The inhibitory activity of each subgroup of these compounds on cross-linked envelope formation by a human malignant keratinocyte cell line, SCC-9, reflects the inhibitory effects on transglutaminase isolated from bovine epidermis. The chiral nature of the compounds leads to eutomers (more potent isomers) and distomers (less potent isomers) (15) with substantial differences in potency. Lorand and Conrad (14) discussed strategies for probing transglutaminase-mediated events in biological systems and these include the use of synthetic amine substrates and the isolation and identification of ϵ-(glutamyl)lysine-cross-linked polymers. Pitfalls exist with both of these methodologies (14). Another approach, however, involves the use of noncompetitive enzyme inhibitors and the halodihydroisoxazoles represent one of the most active classes of inhibitors of transglutaminases and of cross-linked envelope formation. The correlation of effects on cross-linked envelope formation and transglutaminase activity with respect to stereochemistry (5*S* versus 5*R*) is most useful in that inactive isomers can be used as controls for most nonspecific effects.

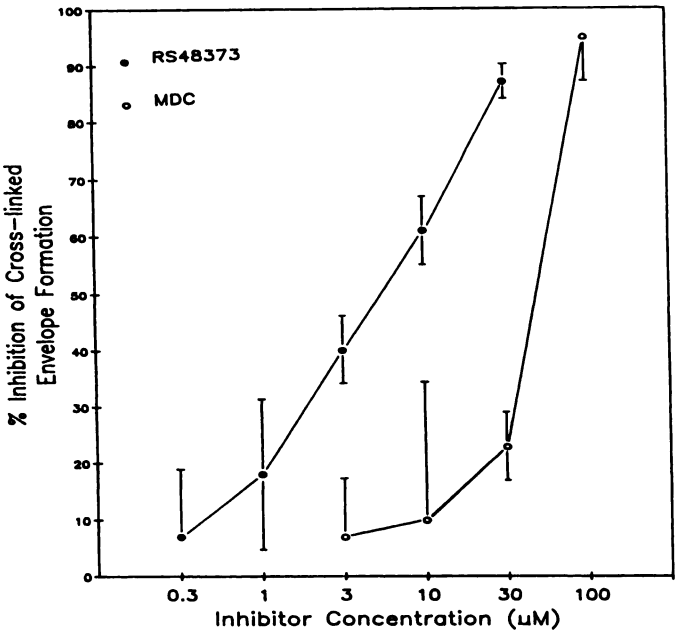


Fig. 8. Dose response of RS48373 and MDC. Cells were preincubated with RS48373 or MDC for 15 min before the addition of ionophore (50 μg/ml) and envelope formation was determined after 10 min. Per cent inhibition was determined from the number of cells forming envelopes after treatment with inhibitor relative to inhibitor-free controls. Each value represents the mean and standard deviation of three determinations.

The reason for the apparent biphasic time course of cross-linked envelope formation is not known. Time-course studies of envelope formation in the presence of the most potent enzyme inhibitor (RS10823) also demonstrated a 10-min initial rate of envelope formation and no further increase in envelope formation thereafter. Although this initial rate was significantly reduced compared with controls, the reason for its very presence is a matter of speculation. Preincubation studies and inhibitor-wash-out studies, which demonstrate that the inhibitor must be present at the time of ionophore-treatment to be effective, indicate two possible scenarios for inhibitor-cell interaction. It is possible that the enzyme is inactive and, therefore, uninhibitable by the halodihydroisoxazoles until ionophore treatment of cells, with the subsequent influx of calcium. Furthermore, the activated enzyme may interact with some of its cellular substrates before time-dependent inhibition can occur. In support of this concept is the requirement for calcium-dependent transglutaminase activation in order for these compounds to be inhibitory (Fig. 3). Alternatively, the cell membrane may block access of the inhibitor to the enzyme until ionophore treatment and the subsequent influx of compound, as appears to be the case with trypan blue (Fig. 6). This influx of inhibitor would probably be slower than the influx of cations and the resultant enzyme activation. The final effect observed, however, may also represent a combination of these proposals.

A number of other agents have been employed as transglutaminase inhibitors in biological studies. Cystamine is a compound that can serve as an alternate acyl acceptor for transglutaminase-mediated reactions but it is also a transglutaminase inhibitor containing disulfide bonds, and enzyme inhibition probably occurs through a disulfide-exchange reaction. It is used at concentrations of 20 mM to block cross-linked envelope formation (10) and was ineffective in our assay when used at concentrations of 100 μM or less. MDC is a synthetic amine that acts as an alternate substrate for transglutaminase. This compound did block envelope formation in our assay but had a rather irregular, steep dose-response profile (Fig. 8). MDC has nonspecific effects, including the inhibition of other cellular enzymes and the inhibition of cell attachment to the substra-

tum (16), and this reduces its utility as a specific probe of transglutaminase-mediated events. Lee and associates (17) reported the development of a series of α,ω-diaminoalkane derivatives of phenylthiourea, which were competitive inhibitors of guinea pig liver transglutaminase and blood coagulation factor XIIIa, and Lorand *et al.* (18) reported that 2-[3-(diallylamino)propionyl]benzothiophene inhibits calcium-enriched erythrocyte and activated platelet protein cross-linking but this occurred at high concentrations (300 to 600 μM). The CBZ-substituted halodihydroisoxazole compounds have the advantages of potency and the requirements for correct stereochemistry, and substitution with the MNP *N*-protecting group leads to a group of compounds with even greater efficacy on cross-linked envelope formation.

The human squamous cell carcinoma cell line SCC-9 was cultured without feeder layer support by plating at high cell densities. The use of such cell lines and methodology facilitated the rapid screening of compounds. Although such methods may impose selection pressure to favor fitter variants (7), the cells remain a useful model system for studying cell-mediated transglutaminase activity at the most basic level. This cell line, with its predominance of particulate versus cytosolic transglutaminases, is particularly suitable for studies of keratinocyte cross-linked envelope formation, because particulate enzymes play the major role in the process of cross-linked envelop formation (8).

Further studies, using both SCC-9 cells and normal human keratinocytes, are now warranted. The reason for the potentiating effects of the MNP moiety on envelope formation but not enzyme inactivation are, as yet, unknown but could involve the interaction of this group with the cell membrane or, alternatively, the differences in the specificity of the keratinocyte particulate enzyme from the bovine epidermal transglutaminase used in the enzyme characterization. Studies correlating intracellular enzyme inhibition with the efficacy of the MNP versus the CBZ-substituted compounds should help uncover the discrepancy between the effects on envelope formation and enzyme activity reported here. This result will have implications for the understanding of the relation of the particulate transglutaminase with the cell membrane. Longer term studies, such as those comparing the suppression of transglutaminase activity by retinoic acid or these inhibitors, will address questions on the biological importance of the different forms of transglutaminase in normal and malignant cells (7, 8) and will provide insight into the treatment of acne and other diseases associated with alterations in transglutaminase activity (6, 19).

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References

1. Green, H. The keratinocyte as a differentiated cell type. *Harvey Lect.* **74**:101–139 (1979).
2. Simon, M., and H. Green. Enzymatic cross-linking of involucrin and other proteins by keratinocyte particulates *in vitro*. *Cell* **40**:677–683 (1985).
3. Thatcher, S. M., and R. H. Rice. Keratinocyte specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. *Cell* **40**:485–495 (1985).
4. Lichti, U., T. Ben, and S. H. Yuspa. Retinoic acid-induced transglutaminase in mouse epidermal cells is distinct from epidermal transglutaminase. *J. Biol. Chem.* **260**:1422–1426 (1985).
5. Thatcher, S. M., E. L. Coe, and R. H. Rice. Retinoid suppression of transglutaminase activity and envelope competence in cultured human epidermal carcinoma cells. *Differentiation* **29**:82–87 (1985).
6. Krantz, A., R. Billedeau, B. Bonaventura, A. L. Castelhano, L. Copp, D. Pliura, R. Smith, R. Spencer, T. Tam, and E. Thomas. Recent advances in the design of mechanism-based inhibitors of acyl transfer enzymes, in *The Molecular Basis of Action of Drugs and Toxic Substances* (N. Castagnoli, Jr., T. P. Singer, and C. C. Wang, eds.) Walter de Gruyter and Co., Berlin, 171–184 (1988).
7. Rheinwald, J. G., and M. A. Beckett. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. *Cancer Res.* **41**:1657–1663 (1981).
8. Rubin, A. L., and R. H. Rice. Differential regulation by retinoic acid and calcium of transglutaminases in cultured neoplastic and normal keratinocytes. *Cancer Res.* **46**:2356–2361 (1985).
9. Castelhano, A. L., R. Billedeau, D. H. Pliura, B. Bonaventura, and A. Krantz. Synthesis, chemistry and absolute configuration of novel transglutaminase inhibitors containing a 3-halo-4,5-dihydroisoxazole. *J. Bioorg. Chem.* **16**:335–340 (1988).
10. Rice, R. H., and H. Green. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* **18**:681–694 (1979).
11. Hollander, M., and D. A. Wolfe. *Nonparametric Statistical Methods.* John Wiley, New York (1973).
12. Sokal, R. R., and F. J. Rohlf. *Biometry,* 2nd Ed. W. H. Freeman and Company, New York (1980).
13. Folk, J. E., and S. L. Chung. Transglutaminases. *Methods Enzymol.* **113**:358–373 (1985).
14. Lorand, L., and S. M. Conrad. Transglutaminases. *Mol. Cell Biochem.* **58**:9–35 (1984).
15. Lehman, P. A. Stereoisomerism and drug action. *Trends Pharmacol. Sci.* **7**:281–285 (1986).
16. Cornwell, M. M., R. L. Juliano, and P. J. A. Davies. Inhibition of the adhesion of Chinese hamster ovary cells by the naphthylsulfonamides dansylcadaverine and *N*-(6-aminohexyl)-5-chloro-1-naphthlenesulfonamide (W-7). *Biochim. Biophys. Acta* **762**:414–419 (1983).
17. Lee, K. N., L. Fesus, S. T. Yancey, J. E. Girard, and S. I. Chung. Development of selective inhibitors of transglutaminase: phenylthiourea derivatives. *J. Biol. Chem.* **260**:14689–14694 (1985).
18. Lorand, L., N. Barnes, J. A. Brunar-Lorand, M. Hawkins, and M. Michalska. Inhibition of protein cross-linking in Ca^{2+}-enriched human erythrocytes and activated platelets. *Biochemistry* **26**:308–313 (1987).
19. DeYoung, L., S. Ballaron, and W. Epstein. Transglutaminase activity in human and rabbit ear comedogenesis: a histochemical study. *J. Invest. Dermatol.* **82**:275–279 (1984).

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