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Targeting Tumor Neovascularization

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20 Feb. 2001

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13. ABSTRACT (Maximum 200 Words) Tumor progression and angiogenesis are dependent on the expression and activation of matrix metalloproteinases (MMPs). To target invading tumor cells based on their cell-surface expression of active MMPs, a novel bacterial toxin was developed in which the normal furin activation site of the proteolytically activated alpha-toxin of <i>Clostridium septicum</i> was replaced with a recognition site for the MMPs. Cleavage of either wild-type or mutant alpha-toxin leads its oligomerization into heptameric, pore-forming complexes which insert into the cell membrane, killing the cell. MMP-targeted alpha-toxin was fully effective against HT-1080 fibrosarcoma cells, but significantly less effective against normal fibroblasts that do not constitutively express active MMPs on their surfaces. Similarly, MCF7 mammary carcinoma cells, which were not significantly susceptible to toxins, became susceptible when transfected with the cell-surface metalloproteinase, MT1-MMP. MDA-MB-231 mammary carcinoma cells, representing an advanced stage tumor, were subject to toxin-mediated cell death without MMP transfection. These results suggest that an MMP-activated alpha-toxin may be useful as part of an anti-tumor strategy.				
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Introduction

Tumor progression and viability are associated with the expression of MMPs, a family of extracellular enzymes that play critical roles in extracellular matrix turnover and cell migration (19). Because of their ability to degrade basement membrane barriers and to facilitate cell migration through stromal matrix, MMPs are directly involved in tumor cell invasion and metastasis, as well as the development of tumor vasculature through angiogenesis. While the efficacy of inhibiting MMPs in a clinical setting remains unclear, there are alternative, MMP-targeted treatment possibilities. We have developed one such approach based on toxin-mediated cell lysis. Alpha-toxin, which is secreted by the gram-positive anaerobe, *Clostridium septicum*, is implicated as the cause of gas gangrene. Alpha-toxin is secreted as an inactive pro-toxin (46.5 kDa), and is converted at the target cell surface to the active form (41.3 kDa) by furin-like proteinases (1, 6, 16). It is extremely toxic to both cultured cells and live organisms, and kills susceptible cells at concentrations lower than 10 ng/ml. A region of alpha-toxin encoding the residues, RGKRS, is the furin-dependent proteolytic activation site of alpha toxin. Once this region is cleaved, alpha-toxin monomers oligomerize into a heptameric, pore-forming complex which inserts into the cell membrane, killing the cell. Mutations in the furin cleavage site in alpha-toxin reduce this toxicity 1000-fold (6). We have developed mutant alpha-toxin in which the native furin cleavage site is replaced with MMP cleavage sites. Our goal was to determine if alpha-toxin can be specifically targeted to cells expressing activated MMPs on their cell surfaces. The cell surface association of activated MMPs is now thought to be an important component of cell invasion and migration. Such binding and activation has been demonstrated in MCF-7 human breast cancer cells, where binding of gelatinase B enables them to migrate. A new sub-class of MMPs, the membrane-type MMPs, are also expressed by a wide variety of cell types, particularly aggressive tumor cells. Thus, while MMP expression associated with the tumor environment is known to be due to both the tumor cells and adjacent stromal cells, the activation of MMPs and their binding to the cell surface is typically associated with tumor cells actively migrating through extracellular matrix (7-11, 13, 18, 20). We have evidence that it is possible to target alpha-toxin to such cells by incorporating MMP-specific activation in place of furin-specific activation.

Body

The research accomplishments described below directly address the "Statement of Work" for each specific aim of the original proposal. These results are largely extracted from a manuscript that is currently being written for publication. We have not included the results of every construct that was generated since most of these were inactive or poorly specific for tumor cells. We have summarized this work and presented those results which have the most potential for future work on this method of attacking the vasculature of tumor cells. We are very enthusiastic about these results and are currently advancing the work past our original aims and plan to submit an NIH proposal based on these results.

Specific Aim 1: Substitute protein sequences recognized by all MMPs or, specifically, by gelatinase A and MT-MMP-1 for the normal activation site of alpha toxin (AT^{GL-A-X})

Task 1: Generation and cloning of PCR-generated alpha toxin gene derivatives containing coding regions for gelatinase A.

Task 2: Expression in *E. coli* and purification of alpha toxin derivatives containing gelatinase A activation sequences.

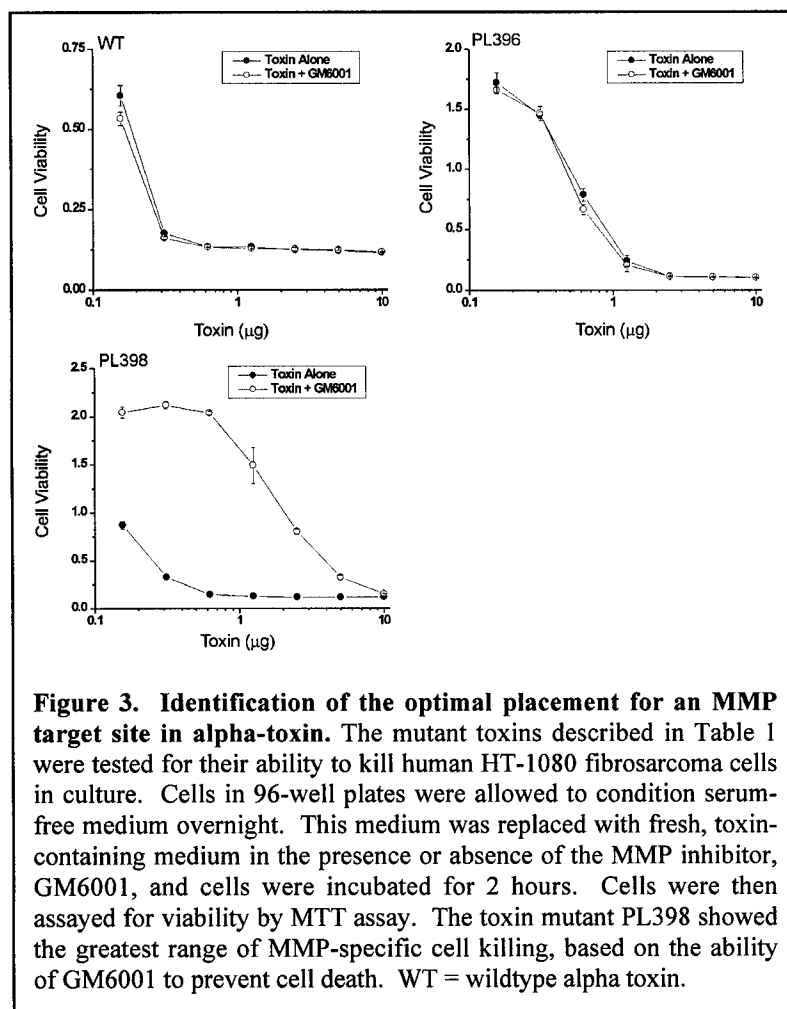
Research Accomplishments.

The tasks outlined in Specific Aim 1 have been met and are summarized below. These mutants contained either the PQGIAG or PLGIAG sequences which are the best published targets for gelatinases A and

Specific Aim 3: Evaluate whether the AT^{GL-A-X} constructs can be specifically activated only by cells producing active gelatinase A and MT-MMP-1

Task 4: Evaluation of AT^{GL-A-X} derivatives for activation and toxicity by cultured endothelial cells

Task 5: Evaluation of AT^{GL-A-X} derivatives for activation and toxicity by cultured NOS cells for activation by MT-MMPs



mutants, AT^{PL398} and AT^{PQ398} were identified as the best candidates for further study, based on the ability of GM6001 to fully rescue cells at toxin concentrations up to almost 1 $\mu\text{g}/\text{ml}$, which is completely toxic to these cells in the absence of inhibitor (Fig. 3). Nearly identical results were observed for AT^{PQ398} and so are not repeated here. However, for some experiments AT^{PQ398} was used instead of AT^{PL398} .

At toxin concentrations that were fully toxic to HT1080 cells, cell death was virtually complete within 2 hours, with little increase over a 24-hour period (Fig. 4). For this reason, a two-hour toxin incubation was used for the remainder of the experiments presented.

a. Evaluation of alpha toxin constructs on MMP producing cells

We evaluated the toxicity of alpha-toxin mutants when incubated with HT1080 fibrosarcoma cells in culture. These cells constitutively activate gelatinase A, and are extensively used as an *in vitro* model of an invasive cell phenotype (5). To differentiate between MMP- and non-MMP-dependent cell toxicity, parallel culture wells were pre-incubated with the potent hydroxamic acid-based MMP inhibitor, GM6001 (17). Toxins previously shown to be resistant to MMP-mediated activation, including AT^{PL394} , AT^{PL396} , AT^{PL402} , and wild-type, showed no MMP-dependent cell toxicity, and retained much of the wild-type toxin's ability to kill cells. An example of the lack of MMP-dependent toxicity is shown in Fig. 3 for AT^{PL396} . As can be seen there is little difference between the killing activity of the wildtype toxin as compared to the PL396 mutant in the presence or absence of the MMP inhibitor GM6001.

With toxin mutants AT^{PL397} , AT^{PL398} , and AT^{PL399} , a significant amount of cell death was blocked by the inclusion of GM6001 (not shown), suggesting that much of the toxicity was MMP-mediated. However, of these three

Previous studies have demonstrated that cleavage of alpha-toxin prior to cell association typically leads

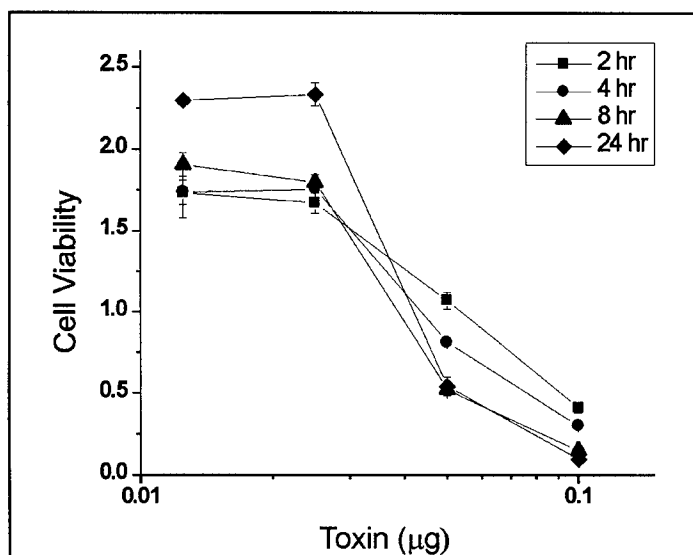


Figure 4. Time dependence of mutant alpha-toxin cytotoxicity. Cultured HT-1080 cells were incubated with PL398 for up to 24 hours in fresh medium, then analyzed for viability using the MTT assay. As can be seen little difference in cell killing is observed whether the toxin was incubated with the cells for 2 hours or 24 hours.

to premature oligomerization and poor toxicity (16). Because HT1080 cells constitutively express activated gelatinase A on their cell surfaces and in their conditioned medium, we predicted that the presence of activated gelatinase A in medium would not induce productive toxin activation. When cells were incubated with toxin in conditioned medium versus fresh medium, little difference was seen in PL398-mediated cell toxicity (fig. 5). This suggests that the presence of an active MMP at the cell surface, not in the medium, is critical for toxin susceptibility.

c. Activation of AT^{PQ398} by MMP producing and non-producing cells

An important milestone in the development of a clinically useful, MMP-targeted toxin will be to eliminate, as much as possible, the non-MMP-mediated cell toxicity. We compared the effectiveness of the toxin mutant, AT^{PQ398}, which has similar properties to AT^{PL398}, on cultured human lung fibroblasts versus HT1080 cells. Normal human lung fibroblasts (5387

cells) do not constitutively express activated gelatinase A on their cell surfaces, and, while these cells were more susceptible to wild-type alpha-toxin, they were somewhat less susceptible than HT1080s to AT^{PQ398} (Fig. 6). *Importantly, while HT1080 cells could be rescued by pre-treatment with GM6001, little MMP-mediated cell death was observed in cultures of 5387 fibroblasts.* Therefore, the noninvasive cell phenotype of the 5387 fibroblasts did not significantly activate AT^{PQ398} and AT^{PQ398} in an MMP-dependent manner. Only on the model

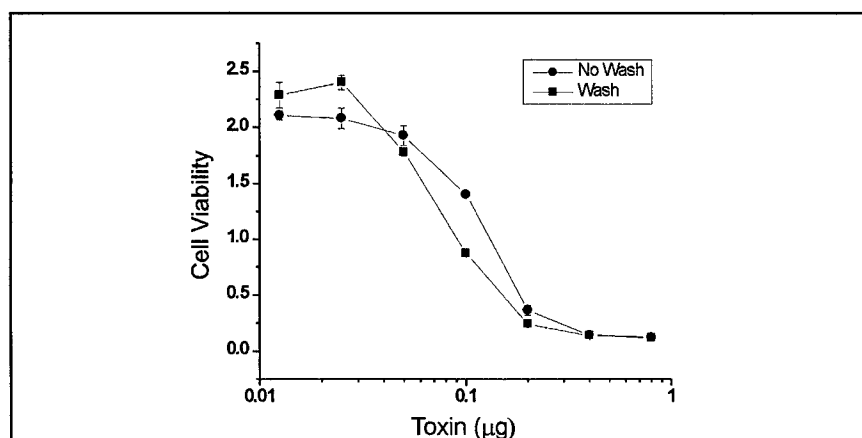


Figure 5. Cell toxicity of alpha-toxin mutants is dependent on cell-surface MMPs. HT-1080 fibrosarcoma cells in culture were allowed to condition their medium for 24 hours. This leads to the accumulation of both active and latent gelatinase A (MMP-2) in the medium, as well as the presence of active gelatinase A at the cell surface. Mutant toxin PL398 was added either directly to cultures without a change of medium (no wash), or was added in fresh medium (wash). No significant difference in cytotoxicity was noted suggesting that cell-surface proteolysis is critical for toxin

invasive cells, the HT1080s, did a significant extent of MMP-based cell killing occur. Hence, the AT^{PQ398} mutant (as well as the AT^{PL398} mutant) exhibited selective activation by MMPs on the MMP-expressing HT1080 cells.

d. Can the specificity of the AT^{PQ398} mutant be maximized by altering other potential non-MMP activation sites with the sequence of alpha toxin?

As shown in Fig. 6 concentrations of AT^{PQ398} of 0.1 to 1.0 μg/ml demonstrated maximal cell toxicity that could be nearly completely blocked by GM6001. In an attempt to improve this MMP-dependent toxicity range, we engineered additional mutants (as described under specific aim #1 above) based on AT^{PQ398} in which selected basic amino acid residues upstream of this site were mutated to serine residues (see Fig. 1). Specifically, these mutants removed

additional basic residues within the native activation site that could possibly serve as targets for non-MMP

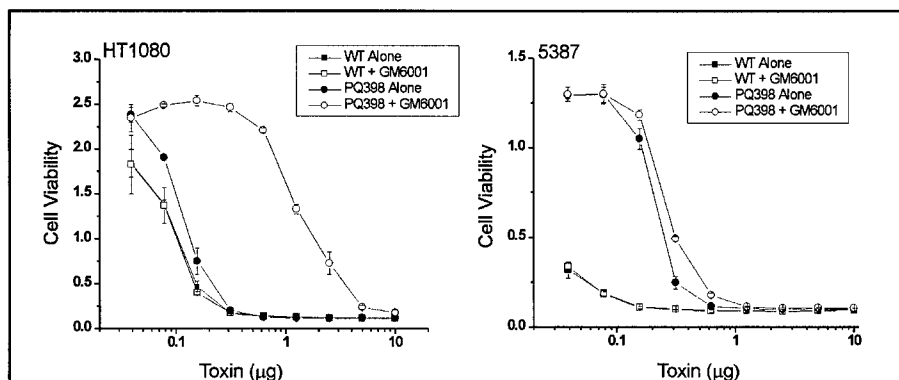


Figure 6. Mutant toxin AT^{PQ398} approaches wild-type toxin potency against cells expressing active MMPs on their cell surfaces. Cultured HT-1080 fibrosarcoma cells and human fetal lung fibroblasts (5387) were allowed to condition their medium for 24 hours, followed by incubation with fresh medium containing either wild-type or AT^{PQ398} toxins, and in the presence or absence of GM6001. Cell viability was then measured after 2 hours of toxin treatment. In HT-1080s, AT^{PQ398} toxicity approached that of wild-type toxin, and a significant amount of this toxicity was MMP-dependent, based on the ability of GM6001 to rescue cells. Normal fibroblasts, which *do not* constitutively express activated gelatinase A on their cell surfaces, were significantly more susceptible to wild-type toxin than AT^{PQ398} , and little of their susceptibility to AT^{PQ398} was MMP-dependent.

to believe that at least two other possibilities might be responsible for the non-MMP activation of the AT^{PQ398} and AT^{PL398} mutants; 1) that other non-MMP proteases might be recognizing residues other than lysines or arginines and thus activating the toxin or 2) that GM6001 may not be as potent an inhibitor of at least some

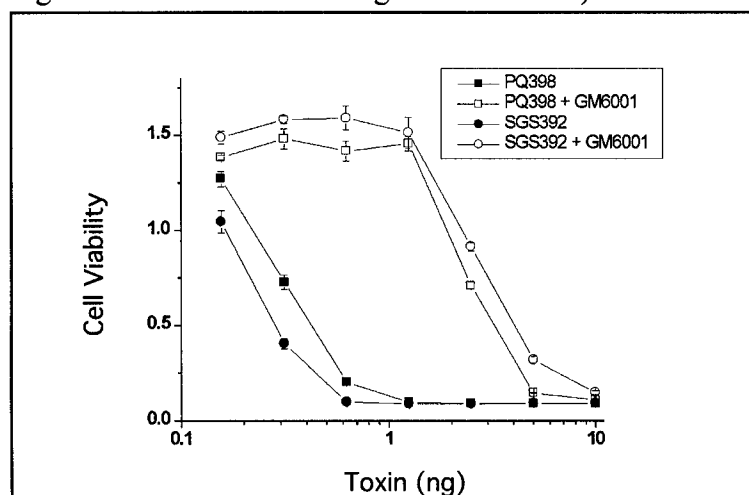


Fig. 7. Mutant toxin $AT^{PQ398/SGS392}$ exhibits MMP-dependent killing of HT1080 cells. $AT^{PQ398/SGS392}$ was tested and compared with the MMP-specific activity of AT^{PQ398} on HT1080 cells. As can be seen $AT^{PQ398/SGS392}$ exhibits a slightly better selectivity against MMP expressing cells than AT^{PQ398} .

proteases that could also activate the toxin. However, these mutants did not appreciably decrease the non-MMP activation of AT^{PQ398} (see as an example $AT^{PQ398/SGS392}$ in Fig. 7).

Therefore, what is responsible for the non-MMP activation of AT^{PQ398} ? It clearly is not proteases that recognize basic residues since their substitution does not appreciably change the non-MMP activation. As can be seen in Fig. 3 we can see about a 10-20-fold difference in cell killing of the invasive HT1080 cells when AT^{PQ398} is incubated in the presence or absence of the MMP inhibitor GM6001. The successive replacement the other basic residues upstream and downstream of the PQ398 site resulted in only a slightly less non-MMP-mediated toxicity, particularly $AT^{PQ398/SGS392}$ (Fig. 7). This has lead us

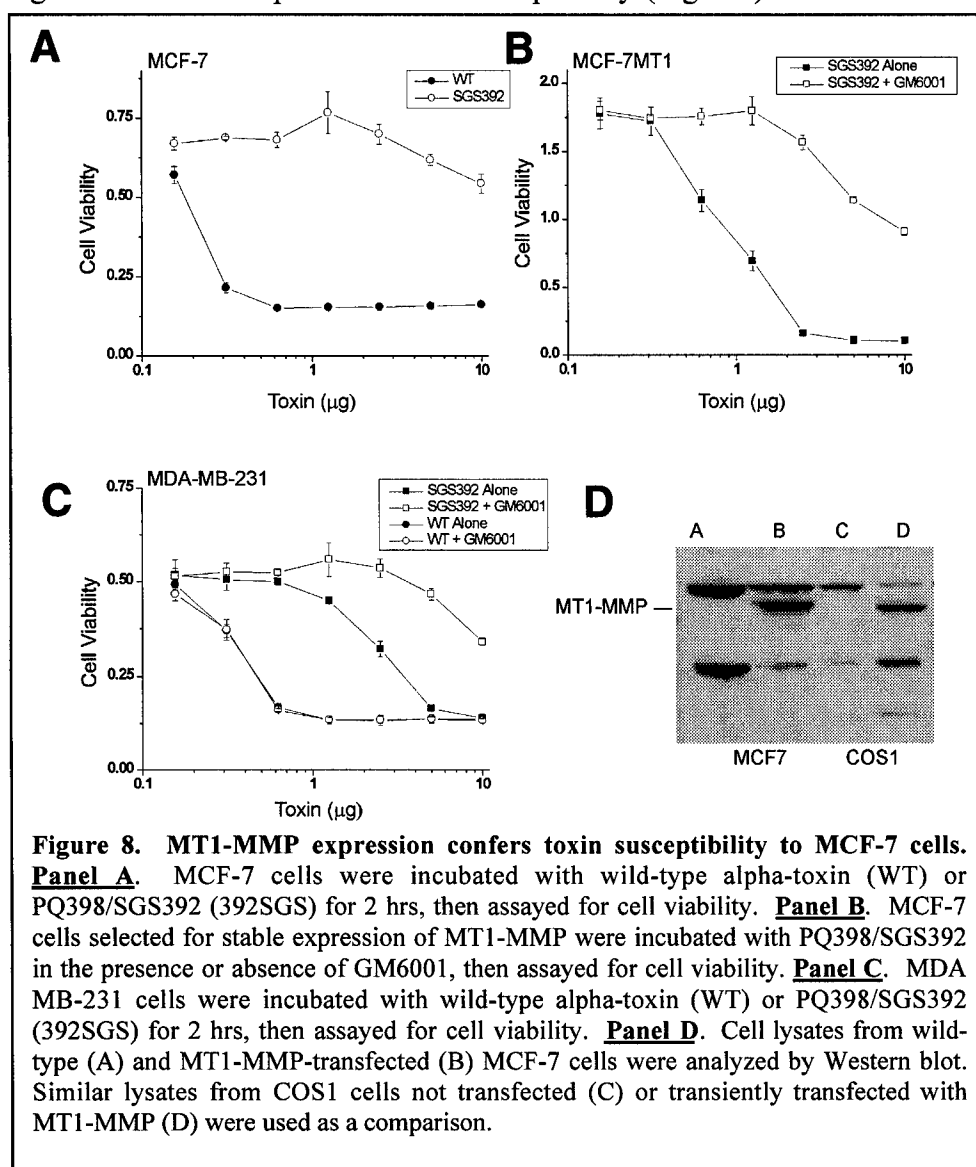
to believe that at least two other possibilities might be responsible for the non-MMP activation of the AT^{PQ398} and AT^{PL398} mutants; 1) that other non-MMP proteases might be recognizing residues other than lysines or arginines and thus activating the toxin or 2) that GM6001 may not be as potent an inhibitor of at least some MMPs and therefore these MMPs remain sufficiently active to cleave these mutants. As described below in section "f" we now have some evidence that perhaps the latter explanation may be at least partially true.

e. Activation of MMP site mutants of alpha toxin with cells specifically transfected and expressing the MT1-MMP

MT1-MMP is expressed on the membrane surface of cells and is involved in the activation of gelatinase A and can function to cleave MMP target sequences in other proteins (15). The level of expression of MT1-MMP as well as gelatinase A has been correlated with the invasiveness of a variety of tumor types (2-4, 14). Therefore, we reasoned that MT1-MMP itself may be able to activate our MMP-dependent alpha toxin mutants at

the cell surface. To test this hypothesis that the membrane-restricted MT1-MMP could activate $AT^{PQ398/SGS392}$ we transfected MCF7 cells, which do not express gelatinase A, with the gene for MT1-MMP. The $AT^{PQ398/SGS392}$ toxin mutant was used to determine if cell-surface MT1-MMP expression could render human breast cancer cells susceptible to MMP-targeted alpha-toxin mutants. MCF7 mammary carcinoma cells

represent a model for *non-invasive, early stage tumors*, and do not express any appreciable amount of MT1-MMP or gelatinase A (12). Not surprisingly, they were not significantly susceptible to MMP-dependent $AT^{PQ398/SGS392}$ toxicity (Fig. 8A). Stable transfection of MT1-MMP into MCF7 cells, however, conferred significant MMP-dependent toxin susceptibility (Fig. 8B). The inclusion of medium containing gelatinase A



did not significantly increase this toxicity, suggesting that MT1-MMP alone was capable of activating alpha-toxin (not shown). Both wild-type and MT1-MMP-expressing MCF7 cells were equally susceptible to wild-type toxin. MT1-MMP transfection was confirmed by Western blot, and was compared with COS1 cells transiently transfected with the same plasmid construct (Fig. 8D). The more *invasive* mammary adenocarcinoma cell line, MDA-MB-231, was significantly more susceptible to $AT^{PQ398/SGS392}$ toxin than wild-type MCF7 cells, and this toxicity was MMP-dependent (Fig. 8C). Together, these data suggest that alpha-toxin mutants can be engineered to target active MMPs on the surface of tumor cells.

Overall these results demonstrate that the proteolytic activation site of alpha toxin can be engineered such that its activation can be redirected to activation by a different protease. Our studies show that we have

specifically altered this site such that it can be activated by tumor and angiogenic specific matrix metalloproteinases (MMPs) such as gelatinase A and B. In addition, we have shown that membrane-restricted MT1-MMP can also specifically activate these mutant toxins and suggests that activation probably takes place on the cell surface, as it does during the activation of native alpha toxin by the membrane-restricted proteinase furin (6).

f. What might be responsible for the non-MMP activation of the mutants AT^{PQ398} and AT^{PL398} ?

As is apparent from the data in Figs. 3, 6 and 7 we cannot completely eliminate the non-MMP activation of AT^{PQ398} , AT^{PL398} or $AT^{PQ398/SGS392}$. Why? A clue is revealed in Figs. 7A and 7B. When the noninvasive breast tumor cells, MCF-7, are treated with $AT^{PQ398/SGS392}$ we observe negligible cell death, even at the highest dose of the mutant toxin. Native toxin still kills these cells efficiently so they are not inherently resistant to the action of the toxin. However, once MCF-7 cells are transfected with the gene for MT1-MMP they become very

susceptible to AT^{PQ398/SGS392}, yet GM6001 cannot completely rescue the cells from killing. Therefore, it appears that, at least in this case, GM6001 does not completely inhibit MT1-MMP or MT1-MMP activates other MMPs that are not completely inhibited by GM6001. These data suggest that our toxin mutants may actually exhibit better specificity than is indicated. It is clear that AT^{PQ398/SGS392} has little effect on native MCF-7 cells, yet when these cells are transfected with and express only MT1-MMP, GM6001 cannot completely rescue them.

Key Research Accomplishments

- The furin proteolytic activation site alpha toxin could be altered to MMP-dependent activation sites
- In vitro cleavage of MMP-dependent alpha toxin mutants was accomplished with purified gelatinase B
- The position of MMP target sequences PQGIAG and PLGIAG within the native activation site of alpha toxin exhibited optimal cleavage by MMPs when substituted for residues 398-403
- The substitution of upstream basic amino acids at positions 392-394 improved the specificity of the activation by MMPs
- Model tumor cells (HT1080s) expressing active gelatinase A were efficiently killed by AT^{PQ398}, AT^{PL398} and AT^{PQ398/SGS392} mutant toxins, but cell death could be significantly reduced by the inclusion of the MMP inhibitor GM6001
- Surface bound MMPs and not extracellular MMPs appeared to be responsible for activation of the MMP-dependent alpha toxin
- Cells specifically expressing surface bound MT1-MMP could specifically activate AT^{PQ398/SGS392} whereas cells not expressing MT1-MMP did not activate AT^{PQ398/SGS392}

Reportable Outcomes

Manuscripts

Eric W. Howard, Lori M. Bentsen, Dawn L. Updike, Elizabeth C. Bullen, and Rodney K. Tweten.

Development of a Matrix Metalloproteinase-activated Toxin That Targets Tumor Cells. *in preparation*

Abstracts

Howard, E. and Tweten, R.K. (2000) Development of a novel, proteinase-activated toxin targeting tumor neovascularization. *Era of Hope Proceedings II*:561.

Patents

U.S. Provisional Patent: Modified Bacterial Toxin activated by metalloproteinases. Rodney Tweten and Eric Howard. Ref. No. 5834.012, May 11, 2000

Conclusions

The basic conclusion that can be reached for this work is that we can modify the proteolytic activation site of alpha toxin such that this toxin can be activated by alternative proteases. We have shown, for the first time that this can be used to specifically target the lethal action of alpha toxin to MMP producing cells.

Therefore, the processes of tumor neovascularization and the tumor itself can potentially be targeted with these alpha toxin mutants. Another advantage of this approach is that it is not tumor specific since all tumors must recruit blood vessels to grow once they exceed a minimum size.

“So what” does this mean for the treatment of breast cancer and other cancers? We are very excited about the possibilities of developing this toxin into a therapeutic for tumors. Why? First, we believe that we already have a potential therapeutic range for these the MMP-activated alpha toxin derivatives. Our best derivative exhibits at least a 10-fold difference between a toxin dose for normal cells versus cells that produce MMPs. However, the actual effectiveness of our MMP-dependent alpha toxin derivatives may actually be better than our data suggests. One should note that in Fig. 7 when we apply $AT^{PQ398/SGS392}$ to the noninvasive breast tumor MCF-7 cells we see less than 15% killing of the MCF-7 at the highest dose of $AT^{PQ398/SGS392}$. When these same cells are transfected with the gene for MT1-MMP, $AT^{PQ398/SGS392}$ is activated nicely and kills the cells. However, this killing effect can only be inhibited to 50% with the MMP inhibitor GM6001. Therefore, we believe that GM6001 may not be an effective inhibitor for all of the MMPs that are expressed by invasive tumor cell types. Hence, the fact may be that our MMP-dependent toxins may actually have a far larger therapeutic range than the cell culture suggests simply because the GM6001 inhibitor may not necessarily be a good inhibitor of all MMPs that are expressed in invasive tumor cells.

Second, we also believe that if we can optimize the therapy of invasive tumors with our MMP-dependent alpha toxin derivatives that the effective treatment regimen may be comparatively short. Since the MMP-dependent alpha toxin kills invasive tumor cells within just 2 hours it is possible that a therapy based on this approach may only require a short period of treatment (perhaps just hours or days) and not a lengthy treatment over a period of months, as is necessary for current chemotherapy. In addition, we hope that the side-effects observed with conventional chemotherapy may not be as severe with a MMP-activated toxin based regimen since its duration should be short and it should not affect normal cells which don't express active MMP.

Another important aspect of this approach that is potentially very beneficial is that this method of therapy should target most tumor types that require neovascularization for tumor growth and metastasis. Furthermore, since we are targeting a normal cellular process for tissue remodeling tumors cannot become resistant to this form of chemotherapy.

In summary we are very excited about the possibilities that these toxins may be useful for the treatment of breast and other cancers, particularly invasive tumors. We plan to carry on the research by submitting a proposal to the NIH within the next year.

References

1. Ballard, J., Y. Sokolov, W.-L. Yuan, B. L. Kagan, and R. K. Tweten. 1993. Activation and mechanism of *Clostridium septicum* alpha toxin. *Molec. Microbiol.* **10**(3):627-634.
2. Bjornland, K., J. O. Winberg, O. T. Odegaard, E. Hovig, T. Loennechen, A. O. Aasen, O. Fodstad, and G. M. Maelandsmo. 1999. S100A4 involvement in metastasis: deregulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in osteosarcoma cells transfected with an anti-S100A4 ribozyme. *Cancer Res.* **59**(18):4702-8.
3. Dalberg, K., E. Eriksson, U. Enberg, M. Kjellman, and M. Backdahl. 2000. Gelatinase A, membrane type 1 matrix metalloproteinase, and extracellular matrix metalloproteinase inducer mRNA expression: correlation with invasive growth of breast cancer. *World J Surg.* **24**(3):334-40.

4. **Ellenrieder, V., B. Alber, U. Lacher, S. F. Hendler, A. Menke, W. Boeck, M. Wagner, M. Wilda, H. Friess, M. Buchler, G. Adler, and T. M. Gress.** 2000. Role of MT-MMPs and MMP-2 in pancreatic cancer progression. *Int J Cancer*. **85**(1):14-20.
5. **Funahashi, Y., T. Wakabayashi, T. Semba, J. Sonoda, K. Kitoh, and K. Yoshimatsu.** 1999. Establishment of a quantitative mouse dorsal air sac model and its application to evaluate a new angiogenesis inhibitor. *Oncol Res*. **11**(7):319-29.
6. **Gordon, V. M., R. Benz, K. Fujii, S. H. Leppla, and R. K. Tweten.** 1997. *Clostridium septicum* alpha toxin is proteolytically activated by furin. *Infect. Immun*. **65**(10):4130-4134.
7. **Kossakowska, A. E., S. J. Urbanski, A. Watson, L. J. Hayden, and D. R. Edwards.** 1993. Patterns of expression of metalloproteinases and their inhibitors in human malignant lymphomas. *Oncology Research*. **5**(1):19-28.
8. **Majmudar, G., B. R. Nelson, T. C. Jensen, J. J. Voorhees, and T. M. Johnson.** 1994. Increased expression of stromelysin-3 in basal cell carcinomas. *Molecular Carcinogenesis*. **9**(1):17-23.
9. **McDonnell, S., M. Navre, R. J. Coffey, and L. M. Matrisian.** 1991. Expression and localization of the matrix metalloproteinase pump-1 (MMP-7) in human gastric and colon carcinomas. *Molecular Carcinogenesis*. **4**(6):527-33.
10. **Muller, D., C. Wolf, J. Abecassis, R. Millon, A. Engelmann, G. Bronner, N. Rouyer, M. C. Rio, M. Eber, G. Methlin, and a. I. et.** 1993. Increased stromelysin 3 gene expression is associated with increased local invasiveness in head and neck squamous cell carcinomas. *Cancer Research*. **53**(1):165-9.
11. **Naylor, M. S., G. W. Stamp, B. D. Davies, and F. R. Balkwill.** 1994. Expression and activity of MMPs and their regulators in ovarian cancer. *International Journal of Cancer*. **58**(1):50-6.
12. **Polette, M., C. Gilles, V. Marchand, M. Seiki, J. M. Tournier, and P. Birembaut.** 1997. Induction of membrane-type matrix metalloproteinase 1 (MT1-MMP) expression in human fibroblasts by breast adenocarcinoma cells. *Clin Exp Metastasis*. **15**(2):157-63.
13. **Powell, W. C., J. D. Knox, M. Navre, T. M. Grogan, J. Kittelson, R. B. Nagle, and G. T. Bowden.** 1993. Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. *Cancer Research*. **53**(2):417-22.
14. **Sardinha, T. C., J. J. Noguerras, H. Xiong, E. G. Weiss, S. D. Wexner, and S. Abramson.** 2000. Membrane-type 1 matrix metalloproteinase mRNA expression in colorectal cancer. *Dis Colon Rectum*. **43**(3):389-95.
15. **Sato, H., T. Takino, T. Kinoshita, K. Imai, Y. Okada, W. G. Stetler Stevenson, and M. Seiki.** 1996. Cell surface binding and activation of gelatinase A induced by expression of membrane-type-1-matrix metalloproteinase (MT1-MMP). *FEBS Lett*. **385**(3):238-40.
16. **Sellman, B. R., and R. K. Tweten.** 1997. The propeptide of *Clostridium septicum* alpha toxin functions as an intramolecular chaperone and is a potent inhibitor of alpha toxin-dependent cytolysis. *Molec. Microbiol*. **25**(3):429-440.
17. **Tournier, J. M., M. Polette, J. Hinnrasky, J. Beck, Z. Werb, and C. Basbaum.** 1994. Expression of gelatinase A, a mediator of extracellular matrix remodeling, by tracheal gland serous cells in culture and in vivo. *J Biol Chem*. **269**(41):25454-64.
18. **Wagner, S. N., C. Ruhri, K. Kunth, B. U. Holecek, M. Goos, H. Hofler, and M. J. Atkinson.** 1992. Expression of stromelysin 3 in the stromal elements of human basal cell carcinoma. *Diagnostic Molecular Pathology*. **1**(3):200-5.

19. **Westermarck, J., and V. M. Kahari.** 1999. Regulation of matrix metalloproteinase expression in tumor invasion. *Faseb J.* **13**(8):781-92.

20. **Wolf, C., M. P. Chenard, Durand, de, Grossouvre, P, J. P. Bellocq, P. Chambon, and P. Basset.** 1992. Breast-cancer-associated stromelysin-3 gene is expressed in basal cell carcinoma and during cutaneous wound healing. *Journal of Investigative Dermatology.* **99**(6):870-2.

Personnel

No other personnel, other than those originally stipulated on the proposal (PI, Co-PI, research technician), were paid from this grant.

Appendix

Appendix materials: Provisional Patent

Proprietary Data

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Applicant(s)

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DUNLAP, CODDING
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Title

Modified bacterial toxin activated by metalloproteinases

Preliminary Class

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Proprietary Data

PROVISIONAL APPLICATION

MODIFIED BACTERIAL TOXIN ACTIVATED BY METALLOPROTEINASES

The present invention contemplates toxins such as *Clostridium septicum* alpha toxin (Ballard et al., "Activation and Mechanism of *Clostridium septicum* Alpha Toxin," Molec. Microbiol., 10(3):627-634, 1993; Ballard et al., "The Primary Structure of *Clostridium septicum* Alpha Toxin Exhibits Similarity With *Aeromonas hydrophila* Aerolysin", Infect. Immun., 63(1):340-344, 1995, which are hereby explicitly incorporated herein by reference; and Figure 1) wherein the proteolytic activation site has been modified such that it is only activated in the presence of proteinases such as matrix metalloproteinases which are involved in processes such as tissue remodeling during tumor development and the vascularization of these tumors. Mutants of alpha toxin in which the normal protease activation site has been replaced with a consensus site for matrix metalloproteinases (MMPs) have been generated.

Alpha toxin is produced as an inactive protoxin, and is normally activated on the surface of mammalian cells by various naturally occurring proteases at the RGKR site upstream of the propeptide. MMPs are only active during cell proliferation and therefore these toxin derivatives are only activated by cells which are proliferating. MMPs such as MMP1-MMP25 are known in the art and further discussion thereof is not deemed necessary herein. The

mutant toxins contemplated herein may also be engineered to be selectively activated by ADAMs (A Disintegrin and Metalloprotease) such as ADAMs 1-30, as are known in the art. Although a low level of tissue remodeling is ongoing in the human as well as other mammals, one of the major areas of cellular proliferation are tumors and the blood vessels which feed tumors. The growth of the vasculature that feeds tumors has been shown to be dependent on matrix metalloproteinases such as gelatinases A and B. The activity of these proteinases is tightly controlled and they are generally not active except under specific circumstances such as wound healing, tumorigenesis and menses.

Mutants of bacterial toxins such as alpha toxin have been generated *in vitro* by site specific mutagenesis of the native activation site wherein the site has been replaced by MMP-specific activation sites. The present invention contemplates specifically targeting the process of tumor neovascularization by engineering a bacterial toxin to be specifically switched on in the presence of a metalloproteinase such as gelatinase A and/or B. For example, the normal furin activation site of the proteolytically activated alpha toxin of *Clostridium septicum* has been replaced with the recognition site for the matrix metalloproteinases gelatinases A and B. A region of alpha toxin encoding the following residues, RGKRS, is the furin-dependent proteolytic activation site of alpha toxin. Once this region is cleaved by furin, alpha toxin monomers

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oligomerize into a heptameric, pore-forming complex which inserts into the cell membrane and kills the cell. The native furin cleavage site has been replaced with the gelatinase cleavage sites PLGIAG and PQGIAG to specifically target alpha toxin to vascular endothelial cells undergoing angiogenesis during the neovascularization of tumors. The results thus far suggest that we can alter this site such that alpha toxin can be cleaved and activated by gelatinase B.

The alpha toxin gene is cloned in the expression vector pET22b which adds a polyhistidine tail of 6 histidines at the carboxy terminus of alpha toxin. The mutants are generated by designing complementary oligonucleotide primers which code for the MMP target sequence (i.e., PLGIAG or others, for example, those described in Netzl-Arnett et al., Biochemistry, 32(25):6427-32, 1993; and Xia et al., Biochimica et Biophysica Acta, 1293:259-266, 1996., which are hereby both explicitly incorporated by reference herein in their entirety).

These primers are then used to mutate the cloned alpha toxin gene at the coding region for its proteolytic activation site. Mutation of the gene is accomplished by the PCR (polymerase chain reaction)-based overlap mutagenesis technique of Ho et al. (Ho, S.N.; Hunt, H.D.; Horton, R.M.; Pullen, J.K.; and Pease, L.R. (1989) Gene 77:51-59). These mutant alpha toxins are expressed in *Escherichia coli* BLR/DE3 and purified by affinity chromatography on

a cobalt affinity matrix (SEPHAROSE metal chelate resin loaded with cobalt) followed by a final purification step by cation exchange. Mutants of the alpha toxin protein have been purified which exhibit nearly total dependence on matrix MMPs for their activation, both
 5 by purified MMPs and in tissue culture on tumor cells producing active MMPs. These same mutant toxins have been found to lack toxicity towards at least one non-tumor cell line. Examples of mutants include those which have had the gelatinase-dependent recognition sequences DVANYNFF, PLGIAG or PQGIAG substituted in
 10 whole or in part for the native activation sequence of alpha toxin. These cleavage site mutants of alpha toxin can be administered at four times the LD50 of native toxin to a mouse and cause no visible effects whereas native toxin kills the mouse within minutes of its administration.

15 The invention contemplates a mutant *Clostridium septicum* alpha toxin protein or mutant aerolysin protein or other toxin comprising a cleavage site of a metalloproteinase wherein the cleavage site is in substitution of all of or a portion of the normal activation site of the alpha toxin, aerolysin or other toxin and wherein the
 20 alpha toxin, aerolysin or other toxin is activated substantially only by a metalloproteinase. The cleavage site is preferably selected from the group consisting of PLGIAG, PQGIAG, and DVANYNFF, but may be any appropriate MP site.

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This invention is directed to a unique and innovative therapy for eliminating tumors. Unlike other tumor therapies in which the tumor itself is the primary target, the gelatinase-activated toxins developed herein are primarily intended to impact tumor vasculature, but with the added benefit of attacking invading tumor cells. Since tumor types vary widely in their susceptibility to cancer therapies, treatments based on tumor cell recognition often lead to the development of resistant tumor cells. This is particularly true when the treatment consists of drugs that are susceptible to cellular detoxifying mechanisms. Other therapies based on immunological approaches often require that a unique target be identified on the tumor cell that distinguishes it from a normal cell. The search for tumor-specific markers has been difficult since often times it is not a question of the absence or presence of a marker, but the relative concentration of the marker on tumor cells versus the level on normal cells.

In the approach contemplated herein, the need to target a unique aspect of the tumor itself is bypassed, and the neovascularization of the tumor is targeted instead. The process of angiogenesis (the production of new capillaries) is necessary for the growth of breast tumors and other solid tumors. Targeting the angiogenic process enables us to target a wide variety of tumor types rather than just a single or few tumor types. In addition, tumors cannot become resistant to this form of therapy since it is

not a tumor cell antigen or receptor that is the target. A fundamental requirement for angiogenesis is the dissolution of basement membranes, and possibly other extracellular matrix proteins, by the action of metalloproteinases. MMP inhibitors are potent anti-angiogenic agents and are uniquely positioned to target the neovascularization of tumors such as breast tumors because (1) Clostridial toxin has a high cytolytic activity towards the vascular endothelium, but is also cytolytic to most other cell types, (2) the toxin requires proteolytic activation, and (3) the protease activation site of this toxin can be altered without affecting the toxin's structure or activity. Neovascularization of tumors requires the localized presence and activation of MMPs, and most likely gelatinase A, to break down the basement membrane for the development of new capillary vessels. Therefore, this process can be affected by alpha toxin or aerolysin having gelatinase A target sequences in the activation site. Other, more general, pan-MMP activated toxins with optimal substrate characteristic for cleavage by gelatinase A, but with some susceptibility to other MMPs are also contemplated.

Based on studies with broad specificity MMP inhibitors used in several animal models, and based on a large number of localization studies, MMPs are typically not expressed in normal tissues, and, when expressed, appear to be latent. In other words, MMP activity is highly controlled, and occurs during developmental and repair

processes, as well as pathologically. The few tissues known to express MMPs normally, including cycling uterus, may be susceptible to MMP-activated toxins. Because the mechanisms utilized by tumor cells to invade and attract a vasculature are common to those used by normal tissues, the problem of absolute tumor specificity is shared by all therapies. The modified toxins described herein offer a unique and effective means of combating tumor growth. They combine the specificity and efficacy of active MMPs with the lethality of a very potent toxin. Treatments contemplated herein eliminate the need for a lifetime of therapy with its attendant complications and expense. In particular, tumors cannot become resistant to this therapy, the toxins can be used against a broad spectrum of tumor types, they can be used against non-cancer angiogenesis-dependent diseases, and cancer therapy based on the use of MMP-activated toxins can rapidly kill tumors, eliminating the need for long term therapy.

Derivatives of alpha toxin described herein appear to be completely dependent on MMPs for activation. These toxins efficiently kill gelatinase expressing fibrous sarcoma cells but do not kill normal human fibroblasts. In one therapeutic treatment protocol, a therapeutic dose of the purified mutant toxin, for example between 40 and 400 $\mu\text{g/kg}$ body weight of the subject, is administered daily (e.g., intravenously) for a period of days or weeks.

The mutant toxins described herein can be used in other treatments for pathologies or diseases involving angiogenesis, cell proliferation, matrix turnover, cell motility, cell invasion or migration, keloids, or abnormal wound healing. Other toxins which may be reengineered as contemplated herein are diphtheria toxin, anthrax toxin and exotoxin A (from *Pseudomonas aeruginosa*) for example, and in general any toxin comprising a known consensus, furin-like target activation site.

Examples

Figure 1 shows the DNA and amino acid sequence of the preprotoxin form of alpha toxin (signal peptide + protoxin). The native furin activation site is RGKRS at amino acid residues 395-399 (of the preprotoxin). The native alpha toxin is cleaved at this site by furin and other cellular proteases, and by other proteases at other amino acids from position 391 to 417.

Preferably, the MMP activation site (e.g., PLGIAG, PQGIAG, or DVANYNFF) is inserted, beginning at position 398 wherein the same number of amino acids are deleted as are inserted (e.g., 6 for PLGIAG or 8 for DVANYNFF).

The MMP activation site can be inserted into the toxin at any position such that the activation of the toxin by furin or related cellular proteases is disrupted. One or more other substitutions within positions 391-417 can be made (in addition to insertion of

the MMP site) wherein other proteolytic sites are altered thereby decreasing non-MMP protease activation and optimally causing the mutant toxin to be MMP-specific thereby enhancing its specificity during treatment.

- 5 Examples of positions 391-407 of various alpha toxin mutants of the present invention are shown below:

391-DKPLGIAGSVDSLDARL-407	(Native)
DKKPLGIAGVDSLDARL	(PLGIAG394)
DKKRRPLGIAGSLDARL	(PLGIAG396)
DKKRRGKPLGIAGDARL	(PLGIAG398)
DKKRRPQGIAGSLDARL	(PQIIAG396)
DKKRRGKPQGIAGDARL	(PQGIAG398)

Examples of other substitutions for increasing the MMP-specificity of the toxins include replacing remaining R or K residues with other residues, e.g., serine or threonine, which decrease non-MP protease activation. Preferably, the inserted MP activation site begins at a position from 395-404, and most preferably from 397-399, and wherein any or all of the R and/or K residues are replaced with an amino acid such as serine, or threonine, (but which may be any appropriate amino acid which inhibits non-MP-protease activation).

In aerolysin, the MP-activation site is inserted preferably between positions 430 to 438, and substitutions of other residues to increase MP-specificity are made from residues 425-448 of the mature aerolysin protein.

Cell Culture Studies

Mutant and wild-type toxins were introduced to cultured human HT-1080 fibrosarcoma cells grown in culture. These cells are known to constitutively activate gelatinase A (MMP-2), and are thus a good model for developing gelatinase A-specific targets. Wild-type toxin killed these cells with a LD50 of 0.02 µg/ml, and MMP-specific inhibitors were unable to block any of this toxicity. The PQGIAG398 mutant with a K397S substitution (a serine substituted for the wild-type lysine at position 397) killed cells with a LD50 of 0.25 µg/ml. MMP specificity was determined using the hydroxamic acid-based inhibitor GM6001 at 1 µM, which completely blocked toxin-mediated cell death up to a toxin concentration of 1.25 µg/ml. These data indicate a toxin that is entirely metalloproteinase-specific at the concentrations tested.

Normal human fibroblasts (strain 5387), which do not constitutively activate gelatinase A, were significantly less susceptible to MMP-specific, toxin-mediated cell death than were HT-1080 cells. No MMP-mediated cell death could be detected using PLGIAG398 or PQGIAG398, and further analysis using the more MMP-

Proprietary Data

Specific toxins should eliminate the ability of toxin mutants to kill these normal cells.

PLGIAG393, 394, 395, 396, 397, and 402 have less MMP-specificity than PLGIAG398 or PQGIAG398. An alternative sequence, DVANYNFFP, when substituted at position 395, and which represents a potentially gelatinase A-specific site, was able to kill HT-1080 cells in a partially MMP-specific manner.



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