Characterization of a Reductively-Activated Elimination Pathway Relevant to the Biological Chemistry of the Kinamycins and Lomaiviticins

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The lomaiviticins (1 and 2) and kinamycins (3–5) are bacterial metabolites with potent antimicrobial and antiproliferative activities. Herein we establish that 1–5 are capable of generating electrophilic acylfulvene intermediates (6) under mildly reducing conditions. These acylfulvenes 6 are formed by a multistep process comprising two-electron reduction and loss of dinitrogen to form an ortho-quinone methide, followed by elimination. Based on these studies, the structure of the product formed from 1 in DNA-cleavage assays is proposed (26). We also show that the bis(hydroxynaphthoquinone) substructures of the lomaiviticins activate the metabolites toward reduction. Finally, based on COMPARE and time-dependent cell response profiling analyses, we show that kinamycin C (4) and the monomeric lomaiviticin aglycon (24) operate by a mechanism of action that is distinct from simple diazofluorenes, such as 23.

Introduction

The quinone methide occupies an eminent position in nature’s molecular arsenal. Frequently, this functional group behaves as a powerful electrophile, and this reactivity forms the basis for the cytotoxicity of many natural products and drugs. Here we present evidence that the diazobenzo[b]fluorene (diazofluorene) antitumor antibiotics the lomaiviticins (1 and 2) and the kinamycins (3–5) form quinone methides which do not react by nucleophilic addition but instead undergo an elimination reaction to produce electrophilic acylfulvene intermediates (see 6, Fig. 1a). This mode of reactivity was first postulated by Moore over 30 years ago for the kinamycins, but experimental evidence for its feasibility has not been reported, so far as we are aware. The cellular target(s) of the lomaiviticins and kinamycins are currently not known. Members of both classes of metabolites have been reported to cleave dsDNA in the presence of a reducing co-factor in vitro2,6 and in tissue culture. Hasinoff and Dmitrienko have also provided evidence that kinamycin F

Fig. 1 (a) Acylfulvene intermediate (6) implicated by this work. (b) Intermediates (7–9) previously proposed to form from the kinamycins and lomaiviticins. (c) Structure of a model diazofluorene previously studied (10) and the diazofluorenes used in this work (11, 12).
(5) targets a protein in the cyclic D3 production pathway. The structural dissimilarities between 1, 2 and 3–5 also raise the possibility that different mechanisms of action are operative.

Several studies aimed at elucidating the chemical behaviour of diazofluorenes have been described. These findings established the high reactivity of this functional group toward reducing agents and nucleophiles. Based on these prior results, several reaction manifolds have been proposed to underlie the cytotoxic effects of 1–5. These include intracellular reduction to form vinyl radicals (7, Fig. 1b) and quinone methide intermediates (8) nucleophilic attack to produce covalent adducts such as 9 and generation of reactive oxygen species by redox cycling of the quinone. All in vitro mechanistic studies have employed substrates which contain an aromatic D-ring, such as dimethylprekinamycin (10, Fig. 1c).

Results

Our studies have focused on the diazofluorenes 11 and 12, which contain an ether substituent on the D-ring (Fig. 1c). The diazofluorene 11 was prepared according to the sequence outlined in Scheme 1. The synthesis began with 4-((S)-(4-methoxybenzyl)oxy)cyclohex-2-ene-1-one (13), which was homologated to the β-(trimethylsilylmethyl)cyclohexenone 14 by a three-step sequence (49% overall). Fluoride-mediated coupling of 14 with 2,3-dibromo-5,8-dimethoxybenzophenone then formed the γ-alkylation product 15 (87%). Cyclization of 15, mediated by palladium acetate in the presence of silver carbonate, generated the ortho-quinone methide 16 (62%). Finally, diazo transfer to the ortho-quinone methide 16 produced the synthetic diazofluorene 11 in 97% yield.

We found that the diazofluorene 11 was indefinitely stable as a solid or in solutions of aprotic, non-nucleophilic solvents at ambient temperature. In contrast, under mildly reducing conditions in protic solvents 11 reacted rapidly (Scheme 2). Thus, treatment of a methanolic solution of 11 with dithiothreitol (DTT; [11] = [DTT] = 20 mM) and warming to 37 °C produced the methoxy-substituted ortho-quinone methide 17. In a preparative-scale experiment, 17 was obtained in 80% isolated yield after 24 h. The experiments outlined below establish that the pathway from 11 to 17 comprises: (1) reduction of the diazo function of 11 to form the ortho-quinone methide 16; (2) elimination of para-methoxybenzyl alcohol from 16 to generate the acylfulvene intermediate 18; (3) 1,6-addition of methanol to 18 to provide the methyl ether product 17.

![Scheme 1 Synthesis of the diazofluorene 11: Reagents and conditions: (a) TMSCH₂MgCl, Cul, THF, −60 °C, then HMFA, Et₂N, TMSCl, −60 → 24 °C; (b) PhSeCl, THF, −78 °C, 78% (two steps); (c) H₂O₂, CH₂Cl₂, 24 °C, 63%; (d) 2,3-dibromo-5,8-dimethoxybenzophenone, TASF(Et), THF, −78 °C, 87%; (e) Pd(OAc)₂, polymer-supported triphenylphosphine, Ag₂CO₃, toluene, 80 °C, 62%; (f) TMSN₃, Et₂N, CH₂CN, 0 °C, 97%.](image)

![Scheme 2 Reduction–solvolysis of the diazofluorene 11.](image)

![Fig. 2 Reduction–solvolysis of the diazofluorene 11: (●) diazofluorene 11, (●) ortho-quinone methide 16, (○) methyl ether 17. Reagents and conditions: 11 (200 μM), DTT (200 μM), 1% CH₂Cl₂–CH₂OH, 37 °C.](image)
37 °C, the half-life for solvolysis was 10 h, and in a preparative-scale experiment, 17 was isolated in 96% yield (see ESI†). Addition of trichloroacetic acid (1 equiv) increased the half-life for solvolysis to 2.4 h, whereas addition of triethylamine (1 equiv) increased the half-life of 16 to approximately 51 h. Under strongly basic conditions (1 equiv sodium methoxide), the solvolysis occurred smoothly, albeit more slowly, with a half-life of 161 h.

To gain insight into the effects of the phenol substituents of the lomaiviticins on this reduction-elimination pathway, we prepared the bis(hydroxy)diazofluorene 12 (Scheme 3). Analogous to the bis(methyl ether) 11, treatment of 12 with DTT in methanol formed the expected ortho-quinone methide 20 and solvolysis products (Scheme 21). Although the intermediates in this series showed partial decomposition after 4 h in solution, this experiment revealed that 12 is reduced three-fold faster than 11 (60% conversion of 12 and 21% conversion of 11 after 30 min, see ESI†). This enhanced reactivity suggests that the phenol substituents of the lomaiviticins activate the metabolites for reduction.

To translate our findings to phenotype-based assays, we assessed the antiproliferative properties of diazofluorenes 11 and 12, the simpler constructs 22 and 23, the monomeric lomaiviticin aglycon 24, the ortho-quinone methide 16, and kinamycin C (4, Table 1).45 Our results establish that analogs bearing free phenols are more potent than those with aryl methyl ethers (compare 12 to 11), possibly due to the increased rate of reduction of the former. Furthermore, the presence of a reductively-labile oxygen substituent on the D-ring leads to an approximately two-fold increase in activity (12 vs. 22). Surprisingly, however, certain analogs possessing a D-ring with a quaternary center (23) exhibited high potency, suggesting that mechanisms other than reductive activation may contribute to these molecules’ cytotoxic effects. These assays also show that isolated ortho-quinone methides such as 16 are inactive. This may be due to rapid degradation in cell culture; less than 40% of 16 remained after warming to 37 °C for 15 h in LNCaP cell lysate.

We generated time-dependent cell response profiles (TCRPs) by measuring cell culture impedance as a function of time for kinamycin C (4), the diazofluorene 23, and the monomeric lomaiviticin aglycon 24, employing the A172 cell line. This technique has previously been used to assess similarities between natural products’ mechanisms of action.106 As shown in Fig. 3, the TCRPs of kinamycin C (4) and the monomeric lomaiviticin aglycon 24 are similar but distinct from the diazofluorene 23. Moreover, a COMPARE analysis of the anticancer activities of 2317 and kinamycin C (4) in the NCI 60-cell line panel18 reveals a poor correlation (r = 0.527).19 These data show that these diazofluorenes operate by distinct or multiple mechanisms of action.

Discussion

The experiments outlined above demonstrate that the lomaiviticins and kinamycins undergo reduction to ortho-quinone methide products under biologically-relevant conditions. Although such intermediates were generated in earlier studies,20,21 these experiments were conducted under abortive conditions (catalytic hydrogenation or tributyltin hydride/AIBN) and employed planar, aromatic substrates, such as prekinamycin dimethyl ether (10). By using the more functionalized substrates 11 and 12, we have revealed that the D-ring of these metabolites is reactive as well. The chemistry we have observed was first proposed by Moore in 1977,3 but has not been evaluated experimentally.

In the early 1990s, it was shown that various glycosylated anthracyclines undergo reduction following by expulsion of an aminosugar residue to form vinylogous ortho-quinone methide intermediates.20 The experiments described herein suggest that the lomaiviticins follow a similar pattern of reactivity. Moreover, ESI/MS analysis of DNA-cleavage assays, employing lomaiviticin A (1) and DTT as reductant, revealed a product consistent with loss of dinitrogen and an aminosugar residue, and incorporation of one equivalent each of dihydrogen and methanol.21 However, the complexity of lomaiviticin A (1) and small sample size prevented rigorous determination of the structure at that time. In light of our observations, we propose the structure of this product as the methyl ether 26 (Scheme 4), and suggest 26 is formed by reduction-elimination to the acylfulvene 25, followed by 1,6-addition of methanol.22

Our cell viability data suggest multiple mechanisms of action underlie the biological activity of synthetic diazofluorenes. Specifically, analogs such as 23, which do not contain a reductively-labile D-ring substituent, retain potent cytotoxic properties, but clearly operate by a mechanism of action that is distinct from those that do, such as kinamycin C (4) and the monomeric lomaiviticin aglycon 24. In retrospect this is not surprising: several quinone-based anticancer agents, such as the mitomycins and anthracyclines,20,23 manifest two or more chemical pathways that contribute to cytotoxicity, although in the case of the mitomycins, cross-linking of DNA is of the greatest biological significance. Finally, the inactivity of isolated ortho-quinone methides (e.g., 16) suggests reduction occurs after cellular entry and/or binding of the diazofluorenes to their target(s). An alternative interpretation of this result is that cytotoxicity arises directly from the diazofluorene itself.108 However, the different TCRPs of 4 and 24 compared to 23, as well as our COMPARE analysis, argue against this.
Conclusion

Our studies have established a reductively-activated elimination pathway for the lomaiviticins and kinamycins and allow us to provide a plausible structure for the product formed from lomaiviticin A (1) in DNA-cleavage assays. Although the primary mechanism of action of 1 and 2 has yet to be established, the mild conditions and time-scale within which reduction and solvolysis occur suggest that this pathway is viable in tissue culture. Additionally, our structure–function studies reveal an activating role for the hydroxy groups of the lomaiviticins and show that isolated ortho-quinone methides (e.g., 16) are unstable under cellular conditions. Delineating the significance of these various pathways and intermediates as they relate to the observed cytotoxic effects of the lomaiviticins will be the focus of future research.

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Notes and references


The glycoside residues and dimeric structures of the lomaiviticins may modify their mechanism of action, relative to the kinamycins. In a compelling study by Thorson and co-workers, synthetic colchicine glycoconjugates were shown to stabilize tubulin polymerization, while colchicine itself inhibits tubulin polymerization; see: (a) Ahmed, N. R. Peters, M. K. Fitzgerald, J. A. Watson, Jr., F. M. Hoffmann and J. S. Thorson, *J. Am. Chem. Soc.*, 2006, 128, 14224.


See ESI† for the syntheses of 22 and 23.

Kinamycin C (4, NSC 138425) was obtained from the NCI/DTP Open Chemical Repository (http://dtp.cancer.gov).


C. M. Woo and S. B. Herzon, unpublished results.


Generally, compounds with high Pearson coefficients operate by similar mechanisms of action, see ref. 18.


W. Ding, unpublished results.

The stereochemistry of the methyl ether function of 26 is unknown and is arbitrarily depicted.