Evidence for the Rapid Conversion of Stephacidin B into the Electrophilic Monomer Avrainvillamide in Cell Culture

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Here we report that the dimeric alkaloid stephacidin B (1) and the monomeric alkaloid avrainvillamide (2) function equivalently within experimental error to inhibit the growth of four different cultured human cancer cell lines. We also show that the proportion of the monomer greatly outweighs that of the dimer in the medium of incubation, and that the half-life for the transformation of 1 to 2 is short relative to the half-life of cell division. Finally, using a monomer-based affinity reagent, we provide evidence that the monomer (2) interacts with intracellular thiol-containing proteins, likely by covalent modification.

Table 1. Measured GI₅₀ Values for 1 and 2

<table>
<thead>
<tr>
<th>cell line</th>
<th>natural enantiomer</th>
<th>unnatural enantiomer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>LNCaP</td>
<td>135 nM (40–231)</td>
<td>241 nM (159–323)</td>
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* Values in parentheses reflect the upper and lower limits for a 90% confidence interval. The XLfit software package was used for curve-fitting and estimation of confidence intervals.

Figure 1. (A) Retrodimerization of (−)-stephacidin B (ent-1) to form (−)-avrainvillamide (ent-2) in cell culture medium containing 6% DMSO at 23 °C; (B) structures and activities of simplified avrainvillamide analogues.

We followed the transformation of the dimeric alkaloid (−)-1 to the monomeric alkaloid (−)-2 in cell culture medium containing 6% DMSO at 23 °C; (B) structures and activities of simplified avrainvillamide analogues.

Further evidence that the growth-inhibitory activity of the dimeric alkaloid stephacidin B (1) arises from the monomeric alkaloid avrainvillamide (2) comes from our discovery that a number of structurally simpler analogues, incapable of dimerization, exhibit considerable potency in antiproliferative assays. Two representative examples from several that we have identified are the 3-alkylidene-3H-indole-1-oxides 3 and 4, both micromolar inhibitors of LNCaP and T-47D cell lines (Figure 1B). Compound 3, first prepared in a
Figure 2. Western-blot detection following affinity–isolation of four cysteine-containing proteins with probe 5, in the absence and presence of competitors (+)-avrainvillamide (2) and structural analogue 3.

The results of our study suggest that both (+)-avrainvillamide (2) and the analogue 3 interact with a variety of cysteine-containing proteins in the cell lysate, including heat-shock protein 60 (HSP60), exportin 1 (XPO1), glutathione reductase (GR), and peroxiredoxin 1 (PRX1). HSP60 and XPO1 do not have cysteine in their active sites, but both have accessible, nucleophilic cysteine between two active sites. HSP60 and XPO1 do not have cysteine either the biotin moiety (2) or the 3-alkylidene-3H-indole 1-oxide function of these compounds.

Interestingly, while co-incubation with 2 or 3 led to a reduction in the amounts of HSP60 and XPO1 bound to probe 5 (compare lane 3 of Figure 2 with lane 2, or compare lane 5 with lane 4) the same conditions led to a marked increase in the binding of GR and PRX1 to 5. This presumably relates to the fact that GR and PRX1 have dual active sites, which may exhibit cooperative binding. Protein binding to 5 was blocked in the presence of iodoacetamide (10 mM), lending support to the hypothesis that 2–5 may function by alkylating cysteine residues. In enzymatic assays (+)-avrainvillamide (2) reversibly inhibited human GR activity in LNCaP cell lysate with an IC50 value of ~125 μM (see Supporting Information). The inhibitory activity decreased with the addition of glutathione disulfide, the natural substrate for GR, consistent with active-site inhibition by 2. (+)-Avrainvillamide (2) was a poor inhibitor of yeast GR.

Our results suggest that the antiproliferative activity of stephacidin B (1) arises from its prior dissociation to form avrainvillamide (2), which may then bind to one or more proteins with nucleophilic cysteine residues, likely by covalent modification. The four specific proteins identified here may or may not be important cellular targets for 2 (the relatively weak inhibition of GR by 2 makes this protein an unlikely candidate); further experiments are necessary. It is interesting to note that while our early model studies showed that both alcohols and thiols can add reversibly to the unsaturated nitroene function of 2 and 3, thus far only cysteine-based nucleophilic proteins have been identified as binding partners in our affinity isolation studies.

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Supporting Information Available: Preparation of 3–6 and details of affinity–isolation and enzymatic assays. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(9) Roswell Park Memorial Institute culture medium, series 1640, was purchased from ATCC. For formulation, see: Moore, G. E.; Gerner, R. E.; Franklin, H. A. J. Am. Med. Assoc. 1967, 199, 519–524.
(10) This experiment employed the unnatural enantiomers (+)-1 and (-)-2, which we had synthesized first. (11) Myers, A. G.; Herzon, S. B. J. Am. Chem. Soc. 2003, 125, 12800–12801.
(12) Structural analogues were evaluated using a slightly different method than that used for the data in Table 1 (see Supporting Information). In this assay, the GI50 for (+)-2 was 330 nM vs. LNCaP and 420 nM vs. T-47D. (b) Bogyo, M.; Barnuch, A.; Jeffery, D. A.; Greenbaum, D.; Borodovsky, A.; Ovaa, H.; Kessler, B. Curr. Protoc. Protein Sci. 2004, 21.17.1.
(18) Control experiments (lanes 6–8 of Figure 2) with compounds lacking either the biotin moiety (2 or 3) or the 3-alkylidene-3H-indole 1-oxide function (6) did not affect detectable quantities of protein in subsequent Western-blot analyses.