Notes

Naamidine A Is an Antagonist of the Epidermal Growth Factor Receptor and an in Vivo Active Antitumor Agent

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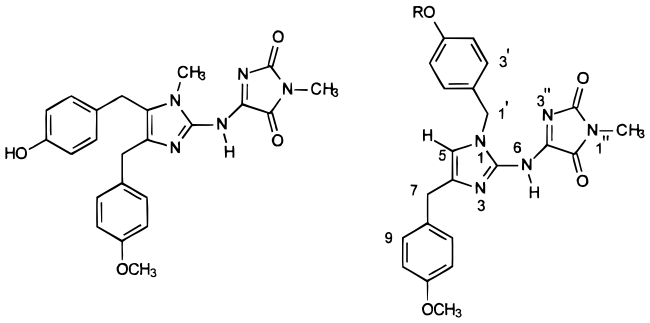
The known 2-aminoimidazole alkaloid naamidine A (**1**) was isolated from a Fijian *Leucetta* sp. sponge as an inhibitor of the epidermal growth factor (EGF) receptor. The compound exhibited potent ability to inhibit the EGF signaling pathway and is more specific for the EGF-mediated mitogenic response than for the insulin-mediated mitogenic response. Evaluation in an A431 xenograft tumor model in athymic mice indicated that naamidine A exhibited at least 85% growth inhibition at the maximal tolerated dose of 25 mg/kg. Preliminary mechanism of action studies indicate that the alkaloid fails to inhibit the binding of EGF to the receptor and has no effect on the catalytic activity of purified *c-src* tyrosine kinase.

The epidermal growth factor (EGF) receptor signal pathway is recognized as an important pathway in the development of some human tumors.[1] The amplification or overexpression of the EGF receptor in certain tumor types is related to cell growth and tumorigenicity.[2] It has been found that compounds which interfere with EGF binding to its receptor or at some point along the signal pathway have utility as antiproliferative agents in tumors which are dependent on EGF for growth.[3] Screening of extracts of marine invertebrates for the ability to inhibit EGF-dependent DNA synthesis and cell proliferation showed that the EtOH extract of a bright-yellow *Leucetta* sp. sponge was moderately active and exhibited selective inhibition of the EGF mitogen versus the nonspecific control mitogen insulin.[4] Subsequent EGF mitogenic bioassay-directed fractionation of the chloroform−methanol extract of the frozen sponge led to the isolation of three bright-yellow alkaloids: naamidine A (**1**), isonaamidine B (**2**), and isonaamidine C (**3**) (Chart 1). Naamidine A was solely responsible for the biological activity of the crude extract.

Chart 1. Structures of Naamidine A (**1**), Isonaamidine B (**2**), and Isonaamidine C (**3**)



1 **2** R = H **3** R = CH_3

Chemistry

Isolation and Structure. Bioassay-directed fractionation of the chloroform−methanol extract of the Fijian sponge *Leucetta* sp. led to the isolation of three bright-yellow alkaloids, one of which was solely responsible for the biological activity of the crude extract. The active compound exhibited spectroscopic properties identical to those reported for naamidine A (**1**), a dibenzylated 2-aminoimidazole alkaloid previously reported from a Red Sea *Leucetta* sp. sponge.[5] The second major alkaloid component of the sponge was found to be the known alkaloid isonaamidine B (**2**),[5] while the third was a novel closely related compound, isonaamidine C (**3**).

The new metabolite, isonaamidine C (**3**), was deduced to be structurally related to isonaamidine B (**2**) due to the near equivalence of their respective spectral data. Crucial differences observed included the presence of an additional aromatic *O*-methyl signal (δ_H 3.76 (3H, s), δ_C 55.24) in the NMR spectra of **3**, as well as the presence of an extra 14 mass units (CH_2) in the parent ion observed in the EI mass spectrum. These data allowed us to conclude that the structure of isonaamidine C (**3**) was the *O*-methyl derivative of isonaamidine B (**2**). Complete structural assignment of **3** was achieved by $^1H-^1H$ COSY,[6] HMQC,[7] and HMBC[8] NMR experiments and also by comparison with the data reported for isonaamidines A and B (**2**).[5] Isonaamidine C has previously been reported as the naturally occurring bis-

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Table 1. Inhibition of EGF and Insulin Receptor-Mediated Mitogenesis

	IC_{50} (μM)		
compd	EGF	insulin	selectivity ratio
1	11.3	242	21
2	22.7	9.8	0.43
3	36.9	6.7	0.18

Table 2. In Vivo Evaluation of Naamidine A (**1**) against A431 Tumor Implanted in Athymic Mice ($N = 6$)

dose (mg/kg)	% inhibition of tumor growth (number of deaths)
50	96.4 (2)
25	87.4 (1)
12.5	52 (0)
3.13	35 (0)

Zn^{2+} metal complex.[9] We found no evidence for the presence of such a metal complex in our specimens of *Leucetta* sp. and so this represents the first report of isonaamidine C as the naturally occurring free ligand.

Biological Results and Discussion

Purified alkaloids **1**−**3** were tested in the EGF mitogenic assay (Table 1). The mitogenic response due to EGF was quantitated by measuring DNA synthesis, indicated by the incorporation of [^{3}H]thymidine into NIH3T3 cells that have been transfected with the EGF receptor gene. Test samples were preincubated with the cells for 1 h, before addition of the mitogen EGF or insulin. Stimulation of these cells with insulin was used as a "nonspecific" mitogen control. After 16 h, mitogenic stimulation was assessed by a 1-h incorporation of [^{3}H]-thymidine. The concentration at which control EGF stimulation was inhibited 50% by the test sample was calculated (EGF IC_{50}) as was the corresponding value for insulin (insulin IC_{50}). Selectivity was defined as the ratio of the IC_{50} after insulin-stimulated growth versus the IC_{50} after EGF-stimulated growth. Of the three compounds tested, only naamidine A (**1**) exhibited selective inhibition of EGF-mediated mitogenesis. General cytotoxicity of the compounds was examined in human colon tumor (HCT116) cells using an XTT dye conversion assay[10] to measure cell proliferation and, in all cases, was found to be relatively weak. IC_{50} values observed for **1**−**3** were 72, 1154, and 288 μM, respectively.

In vivo testing of naamidine A was performed to measure the ability of the alkaloid to inhibit the growth of EGF-dependent tumors in nude mice. The model system used for these in vivo studies was a squamous cell carcinoma (A431) which overexpresses the EGF receptor, implanted under the kidney capsule of athymic mice.[11] Tumor-bearing mice were treated with naamidine A at various doses once a day for 5 days beginning on the first day after tumor implantation (Table 2). The effect of naamidine A on the tumor size was assessed 7 days after tumor implantation. As shown in Table 2, an active criteria of at least 85% growth inhibition was reached, at a dose of 25 mg/kg, which was considered the maximal tolerated dose. The highest dose used (50 mg/kg) resulted in the death of two mice and was considered a toxic dose.

To investigate the mechanism of action of naamidine A, two studies were undertaken. In the first of these, doses of **1** (up to 1 mM) were found to have no effect upon the binding of biotinylated EGF to the EGF receptor in A431 cells (Figure 1), while in the second study, naamidine A did not inhibit the catalytic function of *c-src* tyrosine kinase (Figure 2). These results suggest that naamidine A inhibits EGF-mediated DNA synthesis and cell proliferation downstream from the ligand binding and tyrosine kinase domains.

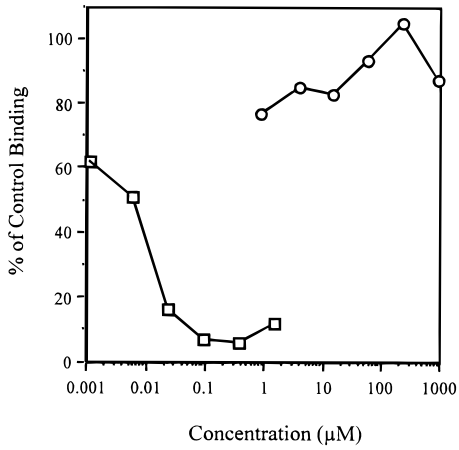

Figure 1. Lack of inhibition of EGF receptor binding by naamidine A in A431 cells. The cells were incubated in the presence of the EGF standard (□) or naamidine A (○) plus biotinylated EGF for 1 h, and the degree of biotinylated-EGF binding to the EGF receptor was quantitated using a peroxidase-linked anti-biotin antibody.

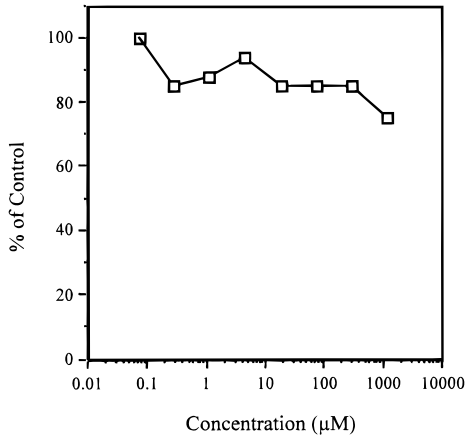

Figure 2. Lack of inhibition of *c-src* tyrosine kinase activity by naamidine A. The compound was incubated with the substrate RCM lysozyme, *c-src* enzyme, ATP for 30 min at which time the reaction was terminated and tyrosine phosphorylation was assessed using a peroxidase-linked anti-phosphotyrosine antibody.

Conclusions

Naamidine A (**1**) was isolated from a Fijian *Leucetta* sp. sponge and found to be an EGF-active agent. The alkaloid exhibits selective antagonism of the EGF-mediated mitogenic response. In vivo evaluation against EGF-dependent A431 tumors in athymic mice indicate the compound has modest antitumor activity.

Experimental Section

General Procedures. IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrophotometer. UV spectra were recorded on a Beckmann DU-8 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained at 500 and 125 MHz, respectively,

on a Varian Unity 500 spectrometer. Proton chemical shifts are reported in parts per million relative to residual undeuterated solvent. Low- and high-resolution EI mass spectral measurements were made on a Finnigan MAT 95 mass spectrometer.

Biological Assays. Procedures for the EGF mitogenic assay,[4] HCT116 cytotoxicity,[10] in vivo evaluation using the A431 tumor xenograft,[11] and determination of the ability of compounds to inhibit the function of protein tyrosine kinase[12] have been reported elsewhere.

Collection, Extraction, and Isolation Procedures. The bright-yellow sponge *Leucetta* sp. was collected by scuba (−10 m) from various shallow reef water sites off Dravuni Island in the Fiji Island Group in 1984 and kept frozen until used. Sponge specimens (400 g) were extracted repeatedly with methanol−chloroform solvent mixtures, and the resulting crude extract was partitioned between chloroform and water. The chloroform layer was then partitioned by column chromatography on isopropylamino support using a stepped gradient of hexane−chloroform and chloroform−methanol solvent mixtures. The chloroform eluting fraction contained crude isonaamidine C which was further purified by HPLC on amino support using $CHCl_3$, yielding **3** (40 mg). The 90% chloroform−10% methanol fraction from column chromatography contained a mixture of compounds which were resolved into the known compounds naamidine A[5] (**1**; 32 mg) and isonaamidine B[5] (**2**; 56 mg) by HPLC on amino support using 5% MeOH in $CHCl_3$.

Physical Properties. Isonaamidine C (3): yellow solid; MS (EI) *m*/*z* 433 (M^+, 64%), 121 (100); HR EI MS *m*/*z* 433.1746 (M^+), $C_{23}H_{23}N_5O_4$ requires 433.1750; IR (film) 3441, 2929, 1793, 1738, 1668, 1613, 1514, 1446, 1392, 1303, 1249, 1177, 1114, 1034 cm^{-1}; UV ($CHCl_3$) λ_{max} (ϵ) 241 (13 100), 275 (7 400), 380 (19 100); ^1H NMR ($CDCl_3$) δ 7.14 (2H, d, J = 8.5 Hz, H-3′), 7.11 (2H, d, J = 8.5 Hz, H-9), 6.82 (2H, d, J = 8.5 Hz, H-4′), 6.81 (2H, d, J = 8.5 Hz, H-10), 6.44 (1H, s, H-5), 5.16 (2H, s, H-1′), 3.78 (2H, s, H-7), 3.76* (3H, s, 5′-OCH_3), 3.75* (3H, s, 11-OCH_3), 3.16 (3H, s, 1″-NCH_3); ^{13}C NMR ($CDCl_3$) δ 161.98 (s, C-5″), 159.44 (s, C-5′), 158.22 (s, C-11), 155.10 (s, C-2″), 146.62 (s, C-2), 144.70 (s, C-4″), 139.89 (s, C-4), 130.79 (s, C-8), 129.67 (2C, d, J = 158 Hz, C-9), 129.39 (2C, d, J = 158 Hz, C-3′), 128.26 (s, C-2′), 115.93 (d, J = 194 Hz, C-5), 114.19 (2C, d, J = 161 Hz, C-4′), 113.96 (2C, d, J = 159 Hz, C-10), 55.24* (q, J = 143 Hz, 5′-OCH_3), 55.23* (q, J = 143 Hz, 11-OCH_3), 48.30 (t, J = 141 Hz, C-1′), 33.77 (t, J = 126 Hz, C-7), 24.66 (q, J = 143 Hz, 1″-NCH_3). NMR assignments marked by an asterisk (*) can be interchanged.

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