HYDROGEN BROMIDE ADDUCT OF NEOCARZINOSTATIN CHROMOPHORE: ONE OF THE STABLE DERIVATIVES OF NATIVE NEOCARZINOSTATIN CHROMOPHORE

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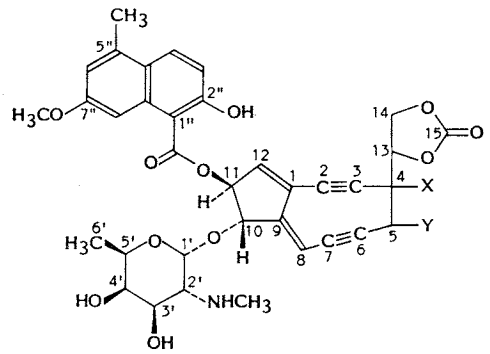
Neocarzinostatin (NCS) is a unique antibiotic protein with the antitumor activity isolated from the culture filtrate of *Streptomyces carzinostaticus* var. F-41[1]. NCS consists of apo-neocarzinostatin (apo-NCS) and neocarzinostatin chromophore (NCS-chr)[2~4]. NCS-chr, which alone is extremely unstable to UV light, heat treatment *etc.*, exhibits full biological activities of its parent NCS such as DNA strand scission[5] and inhibition of DNA synthesis[6]. Apo-NCS plays an important role as stabilizer and carrier of NCS-chr[7].

The total chemical structure of NCS-chr was determined by these authors as a bicyclo[7,3,0]-dodecadienediyne (BCD) derivative[8] having α-D-*N*-methylfucosamine (MF)[9], 2-hydroxy-7-methoxy-5-methyl-1-naphthalenecarboxylic acid (NA), ethylene carbonate (EC), and a highly strained epoxide (SE) (Fig. 1). It was surmised that the BCD moiety was the biologically active center of NCS-chr[10] and through comparative analysis of the extra-weak chemiluminescence of native NCS-chr (**1**) and the hydrogen chloride adduct form (**2a**)[11] it was shown that the SE was the most unstable functional group of NCS-chr.

In order to obtain more stable derivatives of NCS-chr retaining its biological activities and

Fig. 1. Chemical structures of NCS-chrs.



1 X, Y=O (epoxide)
2a X=OH Y=Cl
2b X=OH Y=Br

possessing improved chemotherapeutic properties over **1** and **2a**, a hydrogen bromide adduct of NCS-chr (**2b**) was prepared. In this paper, the stability and biological activities of **2b** are described in comparison with those of **1** and/or **2a**.

First, the antimicrobial activities against *Micrococcus luteus* of **1**, **2a** and **2b** were tested in the presence and absence of apo-NCS. In the absence of apo-NCS, the MICs of **1**, **2a** and **2b**, were 0.63, 0.63 and 1.25 μg/ml, respectively, while in the presence of apo-NCS (10 μg/ml) they were 0.08, 0.16 and 0.16 μg/ml, respectively. These results indicated that the three NCS-chrs had the almost same MICs in the presence and in the absence of apo-NCS, respectively and the antimicrobial activity of these NCS-chrs in the presence of apo-NCS were 4 to 8 times more potent.

Second, the cytocidal activities (IC_{50}) of the NCS-chrs against L1210 cells were examined in the presence and absence of apo-NCS. In the presence of apo-NCS (0.1 μg), IC_{50}s of **1**, **2a** and **2b** were 0.027, 0.060 and 0.058 μg/ml, respectively, while in the absence of apo-NCS those were 0.038, 0.072 and 0.056 μg/ml, respectively. Since the IC_{50} value of NCS was 4.9 ng/ml in the same experimental conditions, 100~200 times higher molar concentrations of NCS-chrs are required to get the same cytotoxic activity as the native NCS. *In vitro* antitumor activity was less affected by addition of apo-NCS although the antibacterial activitiy of NCS-chrs was enhanced in the presence of apo-NCS. These results suggested that different perme-

abilities of NCS-chrs between bacterial cell membrane and mammalian membrane may exist.

Next, the effect of NCS-chrs on ϕX174 DNA was studied. In the absence of apo-NCS, NCS-chr derivatives induced single strand scissions of ccc DNA (form I), converting to the open circular form (form II). NCS-chrs **1**, **2a** and **2b** induced DNA strand scission in the presence and absence of apo-NCS. The degree of conversion of form I to form II of **1**, **2a** and **2b** in the absence of apo-NCS were 26.4, 40.6 and 65.9%, respectively. In the presence of apo-NCS (20 μg/ml), **1**, **2a** and **2b** gave 9.4, 39.5 and 22.2% conversion of form I to form II, respectively. These results indicated that apo-NCS inhibited the DNA strand scission of NCS-chrs. Recently, MYERS[5] reported that the nucleophilic attack at C-12 and epoxide opening of NCS-chr (**1**) by methyl thioglycolate generate a cumulene, which cyclized to form the spontaneous biradical and this radical formation is a key step for DNA cleaving by NCS-chr (**1**). **2b** was more potent than **1** and **2a** in DNA cleaving activity. **2b** may easily be attacked at C-12 by the nucleophile of a thiol compound because bromine atom of **2b** is more potent as a leaving group than chlorine atom of **2a** and opening the epoxide of **1**.

Last, the stability of NCS-chrs to UV light and heat treatment was testing using antimicrobial activity for *M. luteus*. The residual activities of **2a** and **2b** after heat-treatment at 50°C for 2 hours was reduced less than 10%, while after for 5 hours they were 60 and 90% of the control, respectively. The residual activity of **1** was completely lost after 5 hours heat-treatment. On the other hand, after UV light treatment for 30 minutes, residual activities of **2a** and **2b** were 40 and 90%, respectively, whereas the residual activity of **1** after UV light treatment for 6 minutes was 20% of the control. These results indicated that **2b** was more stable to heat and UV light treatment than **1** and **2a**.

These NCS-chr derivatives showed almost equal levels of biological activity to native NCS-chr **1**. In addition **2b** was more stable than **1**. If more stable and easy-to-handle NCS-chr is obtained, the NCS-chr alone could be used clinically. This may decrease the risks inherent in using a foreign peptide in clinical use. Further investigation of stable NCS-chr derivatives possessing antitumor activity is now in progress.

Materials and Methods

Chemicals

NCS and apo-NCS were generously provided by Kayaku Co., Ltd., Tokyo, Japan. All other chemicals were of the highest grade commercially available.

Preparation of NCS-chrs

Native NCS-chr (**1**) and its hydrogen chloride adduct (**2a**) were prepared by the method previously reported[8,12]. Hydrogen bromide adduct of native NCS-chr (**2b**) was prepared by treating NCS powder (500 mg) with a mixture of 25 ml of acetic acid and 0.25 ml of 47% hydrobromic acid at 4°C for 30 minutes. The resulting suspension was centrifuged at 3,000 rpm for 15 minutes and the supernatant was lyophilized to give **2b** (yield *ca.* 30 mg). The hydrogen bromide adduct (**2b**) showed a single peak on HPLC analysis with Zorbax ODS column (4.6×150 mm, DuPont) using MeOH - H_2O - HCOOH (90 : 10 : 2) as solvent. Physico-chemical properties of **2b** were as follows: Amorphous powder; mp 122°C (dec); fast atom bombardment (FAB)-MS *m/z* 740 ($C_{35}H_{34}BrNO_{12}$, MH^+); $[\alpha]_D^{20}$ +5.1° (*c* 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 228 (20,300), 262 (5,840), 271 (5,800), 296 (4,940), 330 (3,810); IR ν_{max}^{KBr} cm^{-1} 1805, 1780, 1729; ^1H NMR (300 MHz, CD_3OD) 1.26 (3H, d, *J*=6.6 Hz), 2.56 (3H, s), 2.91 (3H, s), 3.42 (1H, dd, *J*=3.8 and 11.0 Hz), 3.69 (1H, d, *J*=3.0 Hz), 3.82 (3H, s), 4.00 (1H, dd, *J*=11.0 and 3.0 Hz), 4.07 (1H, q, *J*=6.6 Hz), 4.65 (1H, dd, *J*=5.2 and 8.7 Hz), 4.68 (1H, dd, *J*=8.7 and 7.7 Hz), 4.97 (1H, br s), 5.13 (1H, br s), 5.32 (1H, dd, *J*=7.7 and 5.2 Hz), 5.68 (1H, d, *J*=3.8 Hz), 5.92 (1H, br s), 6.16 (1H, br s), 6.62 (1H, br s), 6.83 (1H, br s), 6.97 (1H, d, *J*=9.2 Hz), 7.47 (1H, br s), 7.99 (1H, d, *J*=9.2 Hz); ^{13}C NMR (75 MHz, CD_3OD) 16.6 (q), 20.3 (q), 32.6 (q), 46.2 (d), 56.1 (q), 59.5 (d), 67.4 (t), 68.6 (d), 69.3 (d), 72.7 (d), 79.8 (s), 80.2 (d), 83.0 (d), 83.1 (d), 90.5 (s), 95.0 (s), 96.1 (d), 100.0 (s), 101.8 (s), 103.5 (d), 108.4 (s), 108.4 (d), 116.4 (d), 118.1 (d), 124.4 (s), 131.6 (s), 132.9 (d), 135.3 (s), 137.2 (d), 138.5 (s), 157.2 (s), 157.5 (s), 160.9 (s), 162.3 (s), 172.3 (s).

Determination of Antibacterial Activity

MICs against *M. luteus* (1×10^7 cells/ml) were determined using agar plate dilution method with nutrient agar in the absence or the presence of apo-NCS (10 μg/ml).

Cytocidal Activity Tests *In Vitro*

Cell suspensions of L1210 murine leukemia (5×10^4 cells/ml), which contained various amounts of NCS-chrs in RPMI 1640 medium supplemented with 5% fetal calf serum (GIBCO) in the presence or the absence of apo-NCS (0.1 μg/ml), were incubated at 37°C for 2 days in CO_2 incubator. Cell numbers were counted by dye exclusion method with trypan blue to calculate the 50% inhibitory concentration (IC_{50}) values.

NCS-chr-induced DNA Damage

The extent of DNA strand damage induced by NCS-chrs was monitored by following the conversion of supercoiled DNA (form I) to its relaxed circular form (form II). The reaction was conducted with 50 mM Tris-HCl buffer (pH 7.5) solution into a mixture of 0.5 μg φX174 DNA (Bethesda Research Laboratories), 10 mM 2-mercaptoethanol and 2 μg/ml NCS-chr, in the presence or absence of 20 μg/ml apo-NCS. The incubation was started immediately after the addition of NCS-chrs and continued for 30 minutes at 37°C and stopped by the addition of 5 μl of 0.1% bromphenol blue in 50% glycerol.

Agarose gel (1%) electrophoreses were run at 4 volt/cm for 4 hours at room temp in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 20 mM sodium acetate, and 18 mM NaCl. After staining in 1 μg/ml of ethidium bromide, DNA bands were quantified with densitometer (Shimadzu CS-920 High speed TLC scanner).

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