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

Kiyoto Edo, Yuriko Akiyama-Murai, Kunihito Saito and Michinao Mizugaki

Department of Pharmaceutical Sciences,
Tohoku University Hospital,
1-1 Seiryo-machi, Sendai 980, Japan

Yoshio Koide
POLA Pharmaceutical R & D Laboratory,
560 Kashio-cho, Totsuka-ku,
Yokohama 244, Japan

Nakao Ishida
School of Medicine of Tohoku University,
2-1-1 Katahira-cho, Sendai 980, Japan

(Received for publication March 17, 1988)

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 \(\alpha\)-D-N-methylfucosamine (MF)\(^{9}\), 2-hydroxy-7-methoxy-5-methyl-1-naphthalene carboxylic 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 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 \(\mu g/ml\), respectively, while in the presence of apo-NCS \(10 \mu g/ml\) they were 0.08, 0.16 and 0.16 \(\mu g/ml\), 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 LI210 cells were examined in the presence and absence of apo-NCS. In the presence of apo-NCS (0.1 \(\mu g\)), IC\(_{50}\)s of 1, 2a and 2b were 0.027, 0.060 and 0.058 \(\mu g/ml\), respectively, while in the presence of apo-NCS (10 \(\mu g/ml\)) they were 0.08, 0.16 and 0.16 \(\mu g/ml\), respectively. Since the IC\(_{50}\) value of NCS was 4.9 \(\mu g/ml\) 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 activity 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, 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, Myers5) 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 anti-microbial 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 reported1,12). 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 - H2O - 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 (C25H24BrNO12, MH+); [α]D +5.1° (c 1.0, MeOH); UV λmax nm (ε) 228 (20,300), 262 (5,840), 271 (5,800), 296 (4,940), 330 (3,810); IR νmax cm−1 1805, 1780, 1729; 1H NMR (300 MHz, CD3OD) 1.26 (3H, d, J=6.6Hz), 2.56 (3H, s), 2.91 (3H, s), 3.42 (1H, dd, /=3.8 and 11.0Hz), 3.69 (1H, d, J=3.0Hz), 4.00 (1H, dd, J=11.0 and 3.0Hz), 4.07 (1H, q, J=6.6Hz), 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); 13C NMR (75 MHz, CD3OD) 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 (1x10⁵ 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 (5×10⁴ 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 CO₂ incubator. Cell numbers were counted by dye exclusion method with trypan blue to calculate the 50% inhibitory concentration (IC₅₀) 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).

Acknowledgment

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

References