A-72363 A-1, A-2, and C, Novel Heparanase Inhibitors from *Streptomyces nobilis* SANK 60192

II. Biological Activities

YUMI KAWASE, MASAAKI TAKAHASHI, TOSHIO TAKATSU†, MASATOSHI ARAI†, MOTOWO NAKAJIMA†† and KAZUHIKO TANZAWA

Biological Research Laboratories, †Biomedical Research Laboratories, Sankyo Co., Ltd., 2-58 Hiromachi 1-chome, Shinagawa-ku, Tokyo 140, Japan
††Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Inhibitory activities of A-72363 A-1, A-2, and C, the diastereomers of a neuraminidase inhibitor siastain B, against various glycosidases were tested in comparison to siastatin B. Despite these compounds differing only in their configuration, each compound showed strikingly different specificities towards the various glycosidases tested. A-72363 C inhibited bovine liver β-glucuronidase and tumor cell heparanase with IC_{50} values of 1.6 μM and 12 μM, respectively.

Heparanase degrades heparan sulfate (HS), one of the major components of basement membranes. Recent studies have demonstrated that heparanase participates in angiogenesis, tumor invasion and metastasis.[1,2] Although heparin, heparin derivatives, sulfated chitin derivatives, and suramin have been reported to inhibit heparanase and suppress tumor metastasis,[3~5] a significant drawback is that they are limited in their application, probably because they are of a high-molecular weight and/or exert non-specific effects in other systems. Specific low-molecular weight heparanase inhibitors, therefore, are expected to find utility in further evaluating the pathophysiological significance of haparanase. During the course of screening for new haparanase inhibitors, A72363 A-1, A-2, and C were isolated from *Streptomyces nobilis* SANK 60192.[6] They represent diastereomers of siastatin B, a neuraminidase inhibitor.[7] In addition to these novel compounds, the strain was found to produce siastain B and an *N*-acetyl-β-D-glucosaminidase (NAGase) inhibitor, nagstatin.[8] In this study, we have examined the inhibitory activities of these compounds towards various glycosidases.

Materials and Methods

Materials

B16-BL6 melanoma cell line was kindly supplied by Dr. FIDLER, Univ. of Texas, M. D. Anderson Cancer Center. Heparin Sepharose was purchased from Pharmacia-LKB. HS was purchased from Seikagaku Kogyo, Japan. *Clostridium perfringens* neuraminidase, bovine liver β-glucuronidase and bovine kidney NAGase were purchased from Sigma.

Heparanase Assay Method

B16-BL6 cells were cultured in HAM's F12-DMEM 1:1 medium supplemented with 1 mM glutamine, antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin) and 10% heat-inactivated fetal calf serum. Heparanase was extracted and partially purified from B16-BL6 melanoma cells using heparin-Sepharose according to the method of NAKAJIMA *et al.*[4] HS was radiolabeled with [^{3}H] acetic anhydride.[9] The solid-phase radiolabeled substrate was prepared based on the method of NAKAJIMA *et al.*[9] with a slight modification, and was used for the enzyme assay. A suspension of the [^{3}H]acetyl HS immobilized to gel beads was mixed with the partially purified enzyme and incubated in a total volume of 400 μl assay mixture consisting of 0.2 M sodium acetate (pH 5.0) and 20 mM D-saccharic acid 1,4-lactone at 37°C for 3 hours with vigorous shaking. The enzyme reaction was terminated by adding 40 μl of 50% trichloroacetic acid. The mixture was then centrifuged at 9,800 $\times g$ for 5 minutes, and the radioactivity of a 200 μl aliquot of the supernatant was counted using a scintillation counter.

Other Glycosidases

Neuraminidase assay was performed by the method of UMEZAWA *et al.*[7] The enzyme reaction was carried out in a total volume of 0.5 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.2 mg bovine sialyllactose. After preincubation at 37°C for 3 minutes, 10 μl of neuraminidase from *C. perfringens* (1.0 unit) was added to the assay mixture. After 30 minutes of incubation at 37°C, the released *N*-acetylneuraminic acid was quantified by the method of WARREN.[10]

β-Glucuronidase activity was measured as described by STAHL & TOUSTER[11] with the following modifica-

tions. The assay was carried out in a total volume of 0.2 ml containing 0.5 mM phenolphthalein-glucuronic acid, 0.1 M sodium acetate (pH 5.0). The reaction mixture was preincubated at 37°C for 3 minutes and then 66.7 μg of β-glucuronidase was added. After 15 minutes at 37°C, the reaction was terminated by adding 0.5 ml of 0.5 M glycine-KOH buffer (pH 10.5) and the reaction mixture was centrifuged at 7,000 × g for 5 minutes. The concentration of released phenolphthalein in the supernatant was measured by the absorbance at 550 nm.

An NAGase assay was performed as described by AOYAGI *et al.*[8]

Results

Inhibition of Various Glycosidases by A-72363 Compounds

In the preceding paper,[6] we identified the compounds A72363 A-1, A-2, and C as being diastereomers of a neuraminidase inhibitor, siastatin B, and that an NAGase inhibitor, nagstatin is also produced by the same strain, *Streptomyces nobilis* SANK 60192. Therefore, the inhibitory activities of A-72363 group compounds against various glycosidases were compared with those of siastain B or nagstatin (Table 1). In spite of the structural similarities, these compounds exhibited inhibitory specificities which were markedly different from each other. A-72363 C inhibited heparanase with an IC_{50} value of 12 μM, whereas the other diastereomers did not inhibit the enzyme at all up to 350 μM. A-72363 C was also the most potent inhibitor for β-glucuronidase among the compounds tested. On the other hand, siastatin B was a inhibitor for neuraminidase and also inhibited β-glucuronidase. Nagstatin was a specific inhibitor for NAGase. A-72363 A-1 and A-2 were less effective in the glycosidases tested except that the A-2 component showed a weak inhibitory activity for neuraminidase. None of the five compounds inhibited other glycosidases such as maltase, isomaltase, sucrase, α-amylase, β-glucosidase, and α-mannosidase at 500 μM (data not shown). It is concluded that the configuration of the substituent groups on the piperizine ring determines the specificity of A-72363 components against various glycosidases.

Table 1. Inhibition of various glycosidases by the A72363 group compounds.

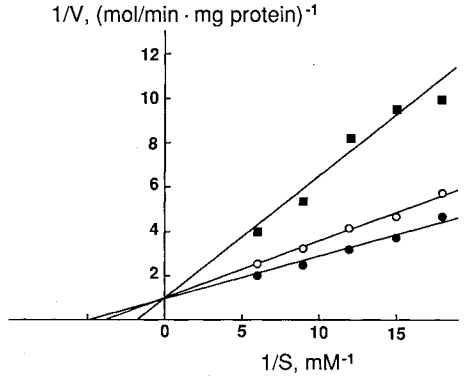
	IC50 (μM)			
	heparanase	neuraminidase	β-glucuronidase	NAGase
A-72363 A-1	>350	>350	50	>350
A-72363 A-2	>350	250	>350	>350
A-72363 B (siastatin B)	>350	150	39	>350
A-72363 C	12	>350	1.6	>350
nagstatin	>350	>350	>350	0.0066

The Mode of Inhibition of Glycosidases by A-72363 C and Siastatin B

The kinetics of the inhibition by A-72363 C was studied in β-glucuronidase. Lineweaver-Burk plots showed that A-72363 C inhibited β-glucuronidase in a competitive manner (Fig. 1). The *Km* value for phenolphthalein-glucuronic acid was 0.22 mM and the apparent *Ki* value for A-72363 C was 0.77 μM. As shown in Table 2, β-glucuronidase was preincubated with varying concentrations of A-72363 C at 37°C, and the concentrations of the compound were reduced to one-fifth in the enzyme assay. As indicated, the inhibition was potentiated by preincubation, and did not depend on the concentration of the inhibitor in the enzyme assay. Fig. 2A shows that the preincubation apparently inactivated the enzyme in a time-dependent manner. The IC_{50} value for β-glucuronidase after 120 minutes preincubation was 0.055 μM compared to 1.6 μM without preincubation. In contrast, the inhibition of heparanase by A-72363 C was reversible, and preincubation with the enzyme did not result in significant potentiation of the inhibitory activity (Fig. 2B). In the case of siastatin B, inhibition of neuraminidase was reversible and time-independent,

Fig. 1. Lineweaver-Burk plots for the inhibition of β-glucuronidase by A-72363 C.

● None, ○ 0.34 μM A-72363 C, ■ 1.38 μM A-72363 C.



The enzyme assay was carried out as described in "Materials and Methods", except that the concentration of the substrate was varied as indicated. The analysis was performed under the condition that the enzyme reaction was a linear function of time. The points in the reciprocal plots are the experimentally determined values, while the lines are calculated from the fits of these data to the rate equation for competitive inhibition.

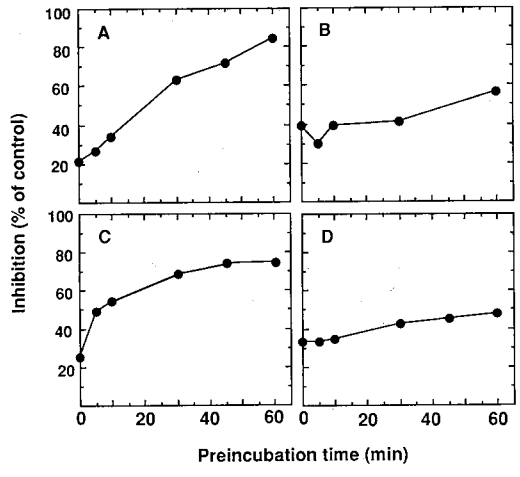
while preincubation with β-glucuronidase significantly potentiated the inhibitory activity in a time-dependent manner (Fig. 2C, D). Although the A-72363 group compounds inhibit various glycosidases, the mechanisms of their inhibitions are different in respect to the enzyme they are targetting. Lineweaver-Burk plots on the inhibition of heparanase by A-72363 C were not performed since difficulty was encountered in varying the substrate concentration in the solid-phase assay empoyed.

Table 2. Irreversible inhibition of β-glucuronidase by A-72363 C.

concentration (μM)		relative activity (%)
at preincubation	at enzyme assay	
0	0	100
0	0.23	82
0	0.46	64
0	0.69	49
0	1.2	35
1.2	0.23	0.4
2.3	0.46	1.8
3.4	0.69	0
5.7	1.2	0.4

Enzyme assay was carried out as described in Materials and Methods, except that the enzyme was preincubated with A-72363 C at 37°C for 30 minutes and the concentration of A-72363 C was lowered to one-fifth of the preincubated level.

Fig. 2. The effects of preincubation on the inhibitory activities of A-72363 and siastatin B for various glycosidases.



β-Glucuronidase and 0.23 μM of A72363 C (A), heparanase and 9.2 μM of A72363 C (B), β-glucuronidase and 4.6 μM of siastatin B (C), or neuraminidase and 68.8 μM of siastatin B (D), were preincubated for 0~60 minutes, and the remaining enzyme activities were determined after 30 minutes of incubation at 37°C.

Discussion

A structure-activity relationship is revealed in this study on the diastereomers of siastatin B, and there are two major findings. Firstly, it has become evident that the axial orientation of the *N*-acetyl substituent at the C-6 position on the piperidine ring is critical for the inhibition of both bovine liver β-glucuronidase and tumor cell heparanase. Secondly, it should also be noted that both A-72363 C and siastatin B irreversibly inhibited only β-glucuronidase, and the effect of both compounds on other enzymes were reversible. As it is difficult to consider compounds that bind covalently only in the case of β-glucuronidase, it is reasonably assumed that tight binding inhibition occurred. Therefore, we speculate that the inhibition mechanism depends on the target enzymes rather than on the chemical structure of the compounds. Recently, NISHIMURA *et al.* reported the synthesis of trifluoroacetamide analogues of siastatin B that are potent inhibitors of β-glucuronidase and have anti-metastatic activity against murine B16 melanoma.[12] They speculated that the anti-metastatic effect of these compounds could be attributed to the inhibition of tumor cell heparanase, although they did not have direct data on it. It should be noted that the orientation of the *N*-acyl substituent at the C-6 position on the piperidine ring of these trifluoroacetamide analogues is not axial but equatorial, as in the case of siastain B. It will be of interest to study the effect on inhibitory activity of the orientation of the trifluoroacetamide group.

Glycosidases are involved in various diseases, such as neuraminidase in virus infection. It is reported that some derivatives of siastatin B inhibit influenza virus neuraminidase and its infection *in vitro*.[13] From studies on the structure-activity relationship of siastatin B derivatives, it has been suggested that the axially oriented carboxyl group is important for the influenza virus neuraminidase inhibition.[13] The derivatives having equatorial carboxyl group, however, also inhibit other glycosidases.[13,14] Structural comparison of siastatin B and its reported derivatives[12~14] with A-72363 A-1, A-2, and C revealed that the orientations of the two hydroxyl and *N*-acetyl group are also important for neuraminidase inhibition. The present results will be also informative for the design of more specific inhibitors of neuraminidase together with other glycosidases.

Acknowledgments

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References

1) NAKAJIMA, M.; T. IRIMURA, D. DI FERRANTE, N. DI FERRANTE & G. L. NICOLSON: Heparan sulfate degradation: Relation to tumor invasive and metastatic properties of mouse B16 melanoma sublines. Science 220:

611～613, 1983

2) HOOGEWERF, A. J.; J. W. LEONE, I. M. REARDON, W. J. HOWE, D. ASA, R. L. HEINRIKSON & S. R. LEDBETTER: CXC chemokines connective tissue activating peptide-III and neutrophil activating peptide-2 are heparin/heparan sulfate-degrading enzymes. J. Biol. Chem. 270: 3268～3277, 1995
3) IRIMURA, T.; M. NAKAJIMA & G. L. NICOLSON: Chemically modified heparins as inhibitors of heparan sulfate specific endo-β-glucuronidase (heparanase) of metastatic tumor cells. Biochemistry 25: 5322～5328, 1986
4) SAIKI, I.; J. MURATA, M. NAKAJIMA, S. TOKURA & I. AZUMA: Inhibition by sulfated chitin derivatives of invasion through extracellular matrix and enzymatic degradation by metastatic melanoma cells. Cancer Res. 50: 3631～3637, 1990
5) NAKAJIMA, M.; A. DECHAVIGNY, C. E. JOHNSON, J. HAMADA, C. A. STEIN & G. L. NICOLSON: Suramin: a potent inhibitor of melanoma heparanase and invasion. J. Biol. Chem. 266: 9661～9666, 1991
6) TAKATSU, T.; M. TAKAHASHI, Y. KAWASE, R. ENOKITA, T. OKAZAKI, H. MATSUKAWA, K. OGAWA, Y. SAKAIDA, T. KAGASAKI, T. KINOSHITA, M. NAKAJIMA & K. TANZAWA: A72363 A-1, A-2, and C, novel heparanase inhibitors from *Streptomyces nobilis* SANK 60912. I. Taxonomy of producing organism, fermentation, isolation, and structure elucidation. J. Antibiotics 49: 54～60, 1996
7) UMEZAWA, H.; T. AOYAGI, T. KOMIYAMA, H. MORISHIMA, M. HAMADA & T. TAKEUCHI: Purification and characterization of a sialidase inhibitor, siastatin, produced by *Streptomyces*. J. Antibiotics 27: 963～969, 1974
8) AOYAGI, T.; H. SUDA, K. UOTANI, F. KOJIMA, T. AOYAMA, K. HORIGUCHI, M. HAMADA & T. TAKEUCHI: Nagstatin, a inhibitor of *N*-acetyl-β-D-glucosaminidase, produced by *Streptomyces amakusaensis* MG846-fF3. Taxonomy, production, isolation, physico-chemical properties and biological activities. J. Antibiotics 45: 1404～1408, 1992
9) NAKAJIMA, M.; T. IRIMURA & G. L. NICOLSON: A solid-phase substrate of heparanase: its application to assay of human melanoma for heparan sulfate degradative activity. Anal. Biochem. 157: 162～171, 1986
10) WARREN, L.: The thiobarbituric acid assay of sialic acid. J. Biol. Chem. 234: 1971～1975, 1959
11) STAHL, P. D. & O. TOUSTER: β-glucuronidase of rat liver lysosomes. J. Biol. Chem. 246: 5398～5406, 1971
12) NISHIMURA, Y.; T. KUDO, S. KONDO, T. TAKEUCHI, T. TSURUOKA, H. FUKUYASU & S. SHIBAHARA: Totally synthetic analogues of siastatin B. III. Trifluoroacetamide analogues having inhibitory activity for tumor matastasis. J. Antibiotics 47: 101～107, 1994
13) NISHIMURA, Y.; Y. UMEZAWA, S. KONDO & T. TAKEUCHI: Synthesis of 3-episiastatin B analogues having anti-influenza virus activity. J. Antibiotics 46: 1883～1889, 1993
14) KUDO, T.; Y. NISHIMURA, S. KONDO & T. TAKEUCHI: Synthesis of the potent inhibitors of neuraminidase, N-(1,2-dihydroxypropyl) derivatives of siastatin B and its 4-deoxy analogs. J. Antibiotics 46: 300～309, 1993