Reversal effects of hyaluronan oligosaccharides on adriamycin resistance of K562/A02 cells

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As the interaction of hyaluronan (HA) with its receptor CD44 contributes to multidrug resistance (MDR) of tumor cells, HA oligosaccharides (o-HAs), as HA antagonists, may be useful to reverse the MDR. The objective of this study was to investigate the reversal effects of four o-HAs, including 4 saccharide residue (o-HA4), 6 saccharide residue (o-HA6), 8 saccharide residue (o-HA8), and 10 saccharide residue (o-HA10) fragments, on adriamycin (ADR)-resistant K562/A02 cells. The four o-HAs were prepared by digesting the native high molecular weight HA with hyaluronidase and gel filtration chromatography. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess the cytotoxicity of the four o-HAs and/or ADR on K562/A02 and K562 cells. The intracellular accumulation of ADR in K562/A02 cells was measured by flow cytometry. By comparing the IC_{50} (concentration resulting in 50% inhibition of cell growth) of ADR with K562/A02 cells in the presence and absence of a series of different concentrations of o-HAs, the reversal folds of the four o-HAs were calculated. The reversal folds of o-HA4, o-HA6, o-HA8, and o-HA10 were 2.04, 2.05, 1.91, and 1.84, respectively. After o-HA4, o-HA6, o-HA8, and o-HA10 treatment, the intracellular amounts of ADR were increased to 3.90, 3.92, 3.76, and 3.39 times, respectively. Shorter o-HAs (o-HA4 and o-HA6) showed stronger reversal effects than longer o-HAs (o-HA8 and o-HA10). In conclusion, the results showed that the four o-HAs could effectively reverse the ADR resistance of K562/A02 cells by increasing the intracellular accumulation of ADR. O-HAs may be used as MDR reversal drugs to increase the effectiveness of chemotherapy. *Anti-Cancer Drugs* 20:800–806 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Hyaluronan (HA) is a linear glycosaminoglycan composed of regularly alternating units of *N*-acetyl glucosamine and D-glucuronic acid linked by β-(1→4) and β-(1→3) glycosidic bonds, with molecular weights ranging from 10^5 to 10^7 Da. HA is usually derived from animal body tissues or fluids, or from bacterial fermentation. In many adult tissues, the major function of HA is structural based on its unique hydrodynamic properties, but in developing and remodeling tissues it plays an instructive and signal-transducing role [1–3]. HA is also a major component of the pericellular matrices within tumors, and influences the activity of tumor cell signaling pathways, which are important in the development of malignant cellular properties [4].

Different degradation products of HA have been reported to have extremely different or even opposite biological activities [5]. For example, very large mass (approximately 10^{3-4}-mers) and very small mass (approximately 2–20-mers) HA molecules have opposite effects on cells with respect to wound healing responses [6,7], angiogenesis [8], and cancer cell growth and metastasis [9–11]. HA oligosaccharides (o-HAs) are small chains or oligomers of HA with molecular weights of less than 10^4 Da (2–40-mers). Generation of o-HAs from the naturally occurring HA polymer is usually mediated by the endoglycosidase, hyaluronidase (HAase). O-HAs may interact with various cells and initiate a program of gene expression leading to cell proliferation, migration, or activation [12–14], and exhibit biological functions, which are quite distinct from those of the native high molecular weight polymer.

Chemotherapy is the treatment choice for about 50% of all cancers. However, multidrug resistance (MDR) mediated by drug-efflux pumps, such as P-glycoprotein (P-gp), minimizes the effectiveness of such therapy in a large number of patients. A number of studies have showed that HA plays significant roles on MDR in cancer cells by binding to its receptor CD44 molecule [15,16]. As o-HAs are the antagonists of endogenous polymeric

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HA and compete with HA for binding CD44 [16], it may effectively reverse MDR. It was proved that o-HAs with 6–18 sugar units, but not large polymers could effectively reverse MDR [17,18]. However, the reversal effects of o-HAs of defined molecular weight on MDR have not been investigated. In this study, four o-HAs of defined sizes, including 4 saccharide residue (o-HA4), 6 saccharide residue (o-HA6), 8 saccharide residue (o-HA8), and 10 saccharide residue (o-HA10) fragments, were prepared by hydrolyzing HA with hyaluronidase (HAase) and gel filtration chromatography, and the reversal effects of the four o-HAs were investigated in adriamycin (ADR)-resistant K562/A02 cells.

Materials and methods

Chemicals

HA was provided by Shandong Freda Biochem Co. Ltd. (Jinan, China). Sheep testicular HAase was from Shanghai No. 1 Biochemical Pharmaceutical Co. Ltd. (Shanghai, China). Injection of ADR was from Zhejiang Hisun Pharmaceutical Company (Taizhou, China). Verapamil was from Shenzhen Wanle Pharmaceutical Industry Co. Ltd. (Shenzhen, China). RPMI 1640 medium was from Invitrogen Corp., Carlsbad, California, USA. Fetal bovine serum was from Si Ji Qing Bioengineering Material Company (Hangzhou, China). 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) was from Amersco, Solon, Ohio USA. Sodium dodecyl sulfate was from Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, China).

Preparation of the four o-HAs

The preparation and purification methods for the four o-HAs including o-HA4, oHA6, o-HA8, and o-HA10 will be described in detail elsewhere (our unpublished data). Briefly, native HA was dissolved in acetic acid–sodium acetate buffer (pH 5.0) to a concentration of 10 g/l and treated with 1.5×10^5 U/l of sheep testicular HAase at 50°C for 20 h. Mixtures were then heated and centrifuged, and supernatants were lyophilized. The four o-HAs were fractionated and purified using Bio-gel P6 chromatography (Pharmacia, Piscataway, New Jersey, USA) after digestion with HAase, and then characterized by a combination of ultraviolet spectrometry, infrared spectra, mass spectrometry, and uronic acid content determination. These results showed the structures of o-HA4, o-HA6, o-HA8, and o-HA10 and are as shown in Fig. 1, and the purities were all more than 92% (data not shown).

Cells and cell culture conditions

The human chronic myeloid leukemia cell line K562 resistant to ADR (K562/A02) and sensitive to ADR (K562) were from the Department of Pharmacology, the Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). K562/A02 cells and K562 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in 5% CO_2. The cells were cultured for 2 weeks in drug-free medium before their use in the experiments.

MTT assays

After reaching 80% of confluence, cells were collected and seeded into 96-well plates at a density of 1.5×10^4/well. ADR of 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.39 μg/ml and ADR of 500, 250, 125, 62.5, 31.3, 15.6, 7.8, and 3.9 μg/ml were used to determine the inhibition effects of ADR on K562 and K562/A02 cells, respectively. The four o-HAs dissolved in RPMI 1640 medium were added to produce final concentrations of 400, 200, 100, 50, 25, and 12.5 μg/ml to determine the inhibition effects of o-HAs on K562/A02 cells. Cells treated with 30 μg/ml of ADR in the presence or absence of different concentrations of o-HAs and verapamil were used to investigate the reversal effects of o-HAs on ADR resistance in K562/A02 cells. After incubation for 24 h, cell viability was assessed by adding 10 μl of MTT reagent into each well. Then 100 μl of 10% sodium dodecyl sulfate solution was added into each well. The absorbance was then measured at 570 nm by a microplate reader (Model 550; Bio-Rad, Philadelphia, Pennsylvania, USA).

Inhibition ratios (IRs) of ADR and o-HAs were calculated as follows: IR (%) = (1–A_{570} of experimental wells/A_{570} of control wells) × 100%. IC_{50} values for ADR (concentration resulting in 50% inhibition of cell growth) were calculated from plotted results using untreated cells as 100%. The reversal folds (RF), as the potency of reversal, were calculated as RF = IC_{50} of ADR alone/IC_{50} of ADR in the presence of o-HAs. Verapamil was used as a positive control in the experiment. Each group was conducted in six individual wells.

Flow cytometry study

Intracellular ADR accumulation was measured by flow cytometry using a standard procedure. K562/A02 cells were incubated for 24 h at 37°C in the presence of ADR (30 μg/ml) alone or in combination with different concentrations of o-HAs or verapamil. Cells were then harvested and washed twice with ice-cold PBS to block the reaction until analysis. After washing, the cells were resuspended in PBS and then analyzed by a FACS Calibur flow cytometry (Becton Dickinson, Franklin Lakes, New Jersey, USA).

Statistical analysis

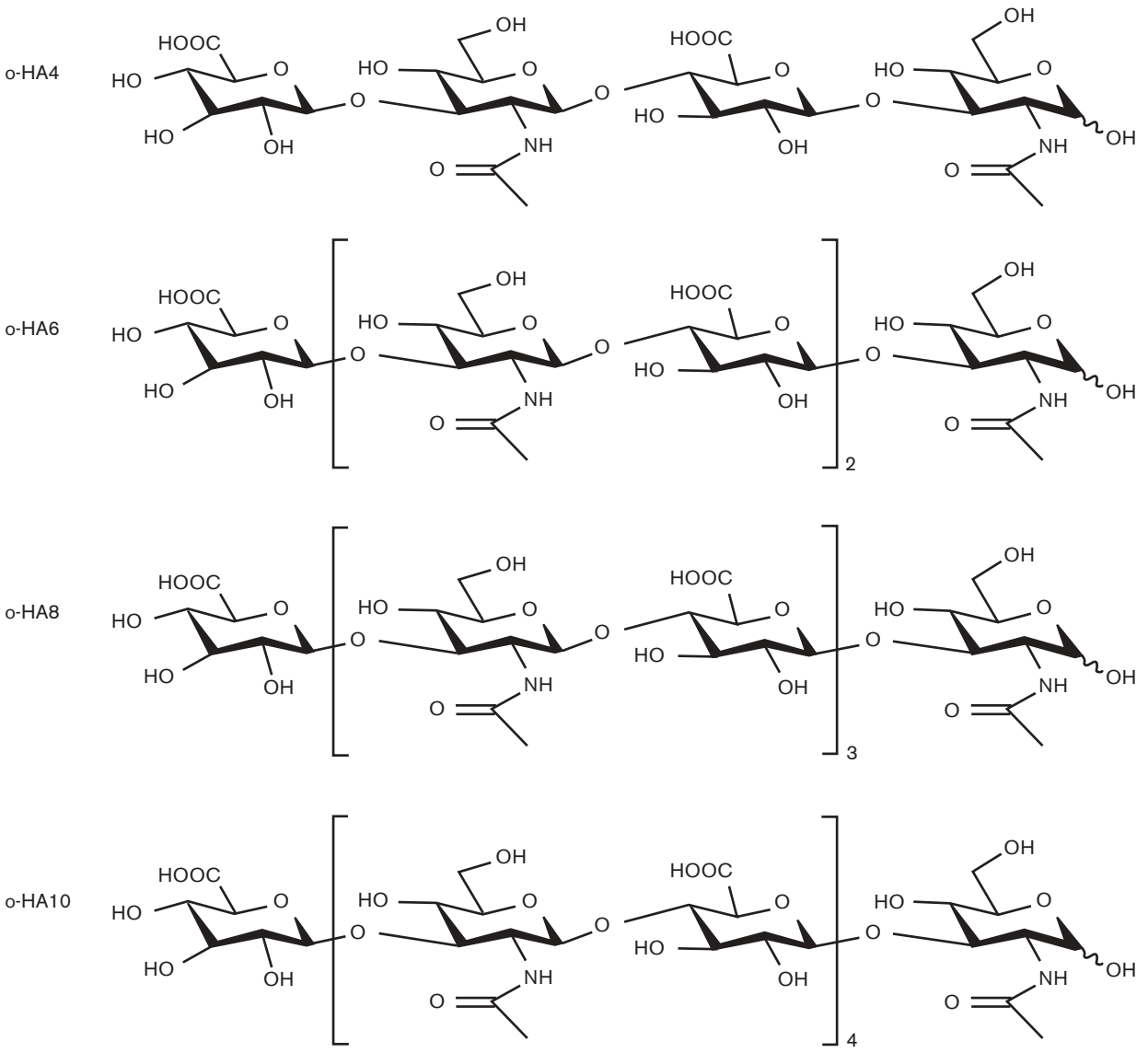
Data were described as the mean ± SD, and analyzed by Student's *t*-test. *P* values below 0.05 were considered as statistically significant.

Results

Effects of o-HAs on the growth of K562/A02 cells

As shown in Fig. 2, the IRs of the four o-HAs on the growth of K562/A02 cells were not in a dose-dependent

Fig. 1



Structures of four o-HAs we prepared. o-HAs, hyaluronan oligosaccharides; o-HA4, 4 saccharide residue; o-HA6, 6 saccharide residue; o-HA8, 8 saccharide residue; o-HA10, 10 saccharide residue.

manner. The result showed that all of the four o-HAs had no inhibition effects on the growth of K562/A02 cells in the concentration range of 12.5–400 μg/ml.

Inhibition effects of ADR on the growth of K562/A02 and K562 cells

Treatment of K562 cells with 0.39, 0.78, 1.56, 3.13, and 6.52 μg/ml of ADR produced IRs of 41.60, 49.38, 60.65, 71.76, and 91.72%, respectively (Table 1), and treatment of K562/A02 cells with 31.3, 62.5, 125, 250, and 500 μg/ml of ADR produced IRs of 42.51, 50.77, 59.45, 70.81, and 91.68%, respectively (Table 1). The regression equations of inhibition effects of ADR on the growth of K562 and K562/A02 cells were $y = 0.081x + 0.4341$ ($r = 0.980$) and $y = 0.001x + 0.4384$ ($r = 0.987$), with the IC_{50} of 0.81 μg/ml for K562 and 61.6 μg/ml for K562/A02, respectively.

Reversal effects of o-HAs on K562/A02 cells

The K562/A02 cells were incubated with 30 μg/ml ADR in the presence or absence of different concentrations of the four o-HAs to examine the reversal effects of o-HAs on K562/A02 cells. As shown in Fig. 3, all of the four o-HAs in the concentrations ≥ 100 μg/ml could significantly increase the cytotoxicity of ADR to K562/A02 cells ($P < 0.05$). It was worth noting that the reversal effects of o-HA4 and o-HA6 were a little higher than those of

o-HA8 and o-HA10, which implies the probable advantages of o-HAs with low molecular weight on the drug resistance reversal effects in cancer cells. The cytotoxicity of ADR to K562/A02 cells increased with the increase of o-HAs concentrations, though o-HAs could not entirely reverse the ADR resistance of K562/A02 cells at concentrations as high as 400 μg/ml. At the concentrations above 1.25 μg/ml, verapamil can effectively reverse the ADR resistance of K562/A02 cells.

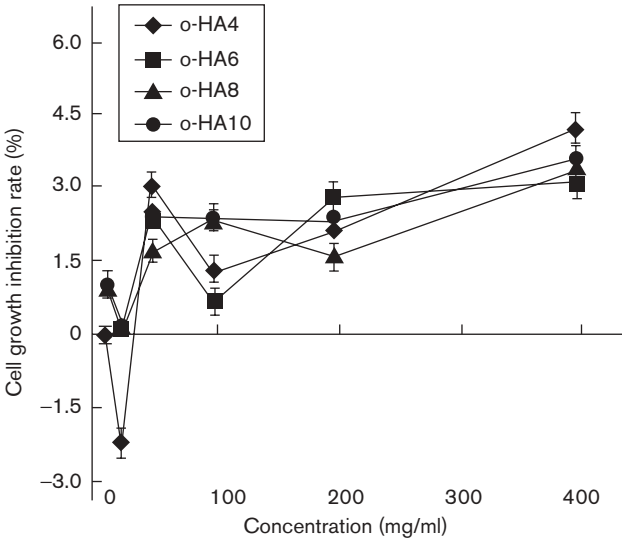
RF values of o-HAs on ADR resistance in K562/A02 cells

As the lowest concentration, which could significantly increase the cytotoxicity of ADR to K562/A02 cells, 100 μg/ml of o-HAs was chosen to calculate the RF values of o-HAs on the ADR resistance in K562/A02 cells. As shown in Fig. 4, the IRs of 62.5 μg/ml of ADR in the presence of 100 μg/ml o-HA4, o-HA6, o-HA8, o-HA10, and 2 μg/ml verapamil in K562/A02 cells were 82.94, 81.83, 78.88, 76.00, and 91.93%, respectively. The RF values of o-HAs and verapamil on ADR resistance in K562/A02 cells are shown in Table 2. The RF values of o-HA4, o-HA6, o-HA8, o-HA10, and verapamil were 2.04, 2.05, 1.91, 1.84, and 3.52, respectively. Similarly, the reversal effects of o-HA4 and o-HA6 were a little stronger than that of o-HA8 and o-HA10.

Effects of the four o-HAs on the intracellular ADR accumulation of K562/A02 cells

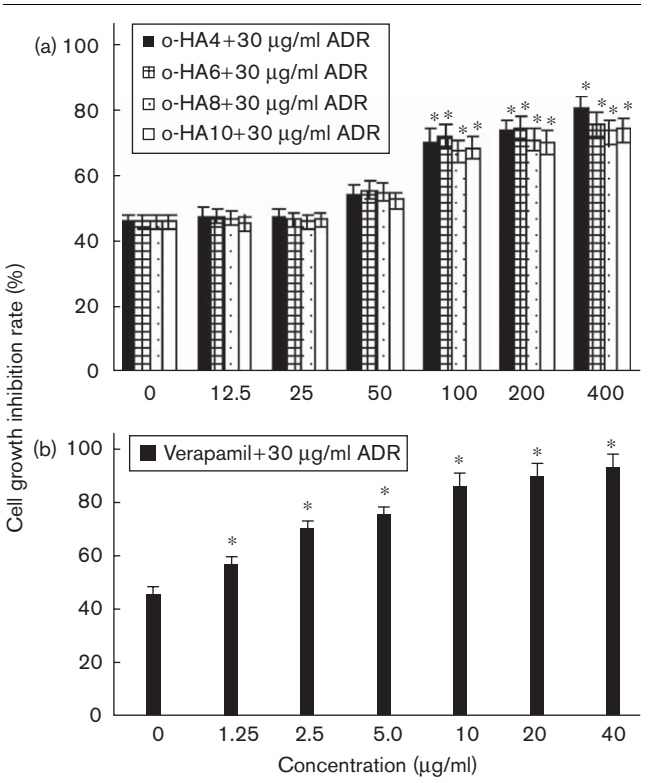
Intracellular ADR accumulation was determined by measuring fluorescence intensity with flow cytometry as the density of ADR in the cells (Fig. 5). The mean fluorescence intensities of K562/A02 cells before and after incubation with 50 μg/ml ADR were 2.74 and 19.38,

Fig. 2



Effects of hyaluronan oligosaccharides (o-HAs) on the growth of K562/A02 cells. The four o-HAs in the final concentrations of 400, 200, 100, 50, 25, and 12.5 μg/ml in the cell cultures were used to determine the inhibition effects of o-HAs on K562/A02 cells. After 24 h, cell viability was assessed by MTT assay. o-HA4, 4 saccharide residue; o-HA6, 6 saccharide residue; o-HA8, 8 saccharide residue; o-HA10, 10 saccharide residue.

Fig. 3



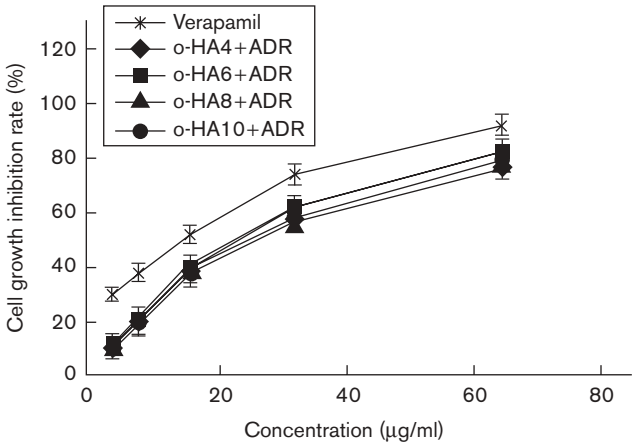
Reversal effects of hyaluronan oligosaccharides (o-HAs) (a) and verapamil (b) on adriamycin (ADR) resistance of K562/A02 cells in the presence of 30 μg/ml ADR. Cells were treated with 30 μg/ml of ADR in the presence or absence of different concentrations of o-HAs and verapamil for 24 h. Cell viability was assessed by MTT assay. o-HA4, 4 saccharide residue; o-HA6, 6 saccharide residue; o-HA8, 8 saccharide residue; o-HA10, 10 saccharide residue.

Table 1 **Inhibition effects of ADR on the growth of K562 and K562/A02 cells**

	K562 cells					K562/A02 cells				
ADR (μg/ml)	0.39	0.78	1.56	3.13	6.52	31.3	62.5	125	250	500
Inhibition rate (%)	41.60	49.38	60.65	71.76	91.72	42.51	50.77	59.45	70.81	91.68
Regression equations	$y=0.081x+0.4341$ ($r=0.980$)					$y=0.001x+0.4384$ ($r=0.987$)				
IC_{50} (μg/ml)	0.81					61.6				

ADR, adriamycin; IC_{50}, concentration resulting in 50% inhibition of cell growth.

Fig. 4



Reversal effects of hyaluronan oligosaccharides (o-HAs) and verapamil on adriamycin (ADR) resistance of K562/A02 cells. Cells were treated with 3.9, 7.8, 15.6, 31.3 and 62.5 μg/ml of ADR in the presence of 100 μg/ml 4 saccharide residue (o-HA4), 6 saccharide residue (o-HA6), 8 saccharide residue (o-HA8), 10 saccharide residue (o-HA10), and 2 μg/ml verapamil for 24 h.

Table 2 RF values of o-HAs on MDR in K562/A02 cells

Group	IC_{50} (μg/ml)	RF
o-HA4	30.17	2.04
o-HA6	30.04	2.05
o-HA8	32.29	1.91
o-HA10	33.33	1.84
Verapamil	17.48	3.52

IC_{50}, concentration resulting in 50% inhibition of cell growth; MDR, multidrug resistance; o-HAs, hyaluronan oligosaccharides; o-HA4, 4 saccharide residue; o-HA6, 6 saccharide residue; o-HA8, 8 saccharide residue; o-HA10, 10 saccharide residue; RF, reversal folds.

and the mean fluorescence intensities of K562/A02 cells incubated with 50 μg/ml of ADR in combination with 100 μg/ml of o-HA4, o-HA6, o-HA8, o-HA10, and 2 μg/ml of verapamil were 75.67, 75.97, 72.95, 65.78, and 93.44, respectively. Accordingly, the amounts of ADR in K562/A02 cells were increased to 3.90, 3.92, 3.76, 3.39, and 4.82 times, respectively, after the treatment with o-HA4, o-HA6, o-HA8, o-HA10, and verapamil.

Discussion

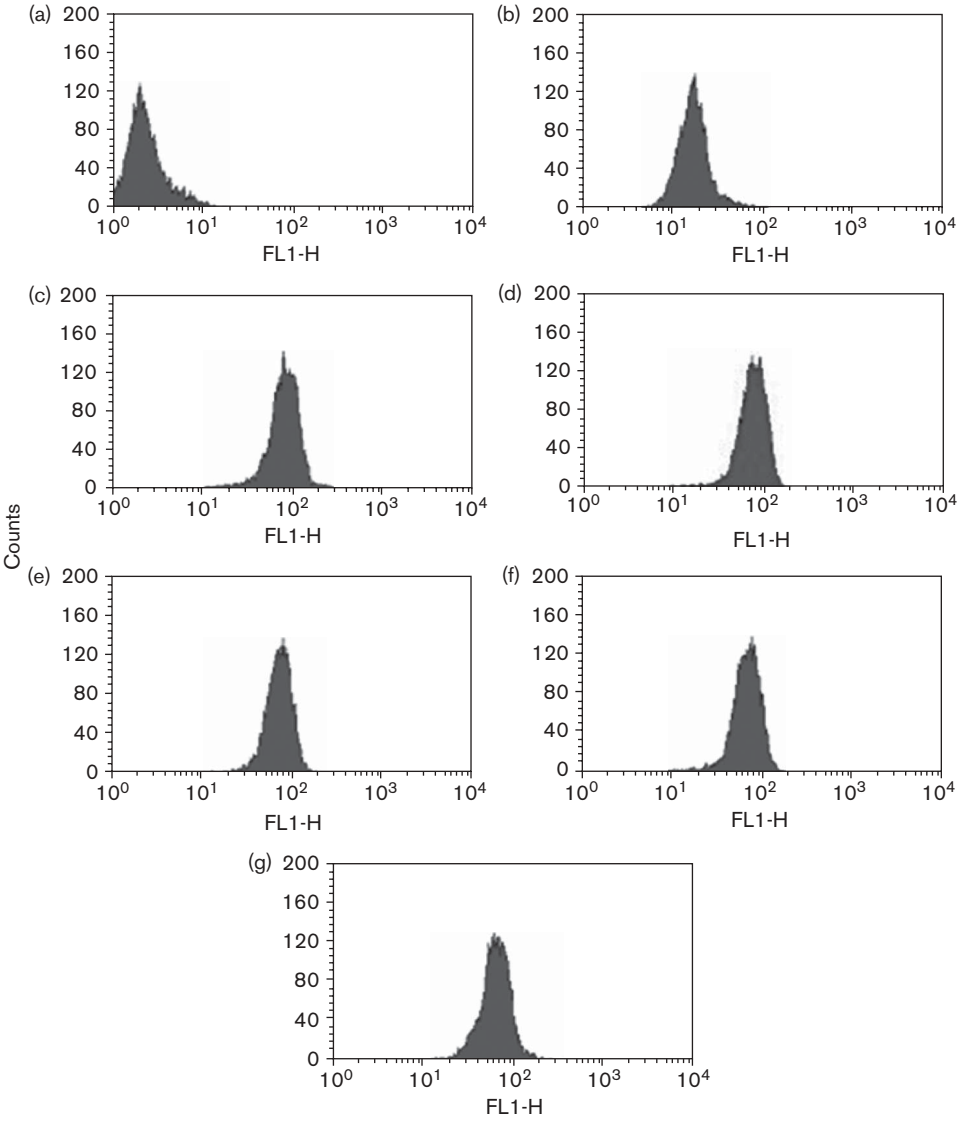
MDR is a major obstacle to cancer treatment and leads to poor prognosis for many of the patients. A variety of approaches have been pursued to inhibit or reverse MDR. It was suggested that MDR was frequently associated with upregulation of the P-gp and MDR-related proteins (MRP), which are members of a large family of ABC trafficking proteins that couple ATP hydrolysis to ligand transport across a lipid membrane [19,20]. Although P-gp binds directly to unmodified drugs and mediates their efflux across the cell membrane in an ATP-dependent manner, the mechanism of MRP-mediated MDR is not clear [21,22]. HA, a very large polysaccharide, is a ubiquitous component of extracellular matrices. Recent studies suggest that HA may interact directly with drug transporters as well as influence their expression. It was reported that the interaction of HA and its receptor CD44 stimulates phosphoinositide 3-kinase activity, and phosphoinositide 3-kinase activates Akt and downstream antiapoptotic events, which contribute to drug resistance by amplifying MDR1 expression and increasing the synthesis of P-gp in tumor cells [15]. These results illustrated the potential importance of HA as a therapeutic target in multidrug-resistant carcinomas.

It has been reported that HA degradation products had extremely different biological functions according to the length of the HA chains. The HA receptor has a small, well-like structure at the binding site, and such a receptor is probably more specific for o-HAs of a certain size. The exact size of o-HAs seems to be a reliable determinant of their resultant effects. The role of o-HAs on MDR may be extremely different from that of HA, by acting as the antagonists of endogenous polymeric HA [17]. MDR reversal effects of o-HAs of 6–18 sugar units have already been confirmed [18]. However, the reversal effects of o-HAs of defined molecular weight on MDR have not been investigated. In this study, four o-HAs of defined small size, including o-HA4, o-HA6, o-HA8 and o-HA10, were prepared and purified in our laboratory. The reversal effects of the four o-HAs on ADR resistance of K562/A02 cells were examined. It was shown that all of the four o-HAs can effectively reverse the ADR resistance of K562/A02 cells. In addition, compared with o-HA8 and o-HA10, o-HA4 and o-HA6 showed stronger reversal effects, probably because of their lower size. This result was a little different from earlier studies, which showed that o-HAs fractions of 6–18 sugar units but not large polymers could reverse MDR [18]. Further studies are needed to investigate the reversal effects of other o-HAs with defined length.

To determine whether the reversal effects of the four o-HAs were because of increased intracellular ADR accumulation in K652/A02 cells, intracellular ADR in K652/A02 cells was measured using flow cytometry. After incubation with the o-HAs, the amounts of ADR in K562/A02 cells were all significantly increased. Thus, reversal effects of the four o-HAs may be because of increased intracellular accumulation of ADR in K562/A02 cells. We are trying to investigate the detailed mechanisms of o-HAs on the increased accumulation of ADR in K652/A02 cells.

In this study, we obtained four o-HAs, which can effectively reverse the ADR resistance in K562/A02 cells, although the mechanisms are not clear. As oligosaccharides have better

Fig. 5



Intracellular adriamycin (ADR) accumulation of K562/A02 cells. (a) No drug; (b) ADR (50 μg/ml); (c) ADR (50 μg/ml) + verapamil (2 μg/ml); (d) ADR (50 μg/ml) + o-HA4 (100 μg/ml); (e) ADR (50 μg/ml) + o-HA6 (100 μg/ml); (f) ADR (50 μg/ml) + o-HA8 (100 μg/ml); (g) ADR (50 μg/ml) + o-HA10 (100 μg/ml). o-HA4, 4 saccharide residue; o-HA6, 6 saccharide residue; o-HA8, 8 saccharide residue; o-HA10, 10 saccharide residue.

biocompatibility and low toxicity, o-HA4, o-HA6, o-HA8, and o-HA10 may be used as candidate modulators to effectively potentiate the cytotoxicity of chemotherapeutic drugs towards resistant tumors.

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