The ability of hyaluronan fragments to reverse the resistance of C6 rat glioma cell line to temozolomide and carmustine

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Aim of the study: Hyaluronan (HA) is an extracellular matrix (ECM) polymer that may contribute to the emergence of anti-cancer drug resistance. Attempts to reverse drug resistance using small hyaluronan oligomers (oHA) are being made. The initial reports suggest that the oHA fraction may effectively reverse anti-cancer drug resistance in glioma models. However, the reversal effects of oHA of defined molecular length on glioma cells have not been investigated yet. In this study, we examined HA fragments containing 2 disaccharide units (oHA-2), 5 disaccharide units (oHA-5), and 68 kDa hyaluronan polymer (HA-68k) as agents possibly reversing the resistance of a C6 rat glioma cell line to temozolomide (TMZ) and carmustine (BCNU).
Material and methods: A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) viability assay was used to assess the cytotoxicity of TMZ and BCNU in the presence or absence of the hyaluronan fragments. By comparing viability of the cells, the reversal effects of HA fragments on TMZ and BCNU resistance in C6 glioma cells were assessed.
Results: We found statistically significant decreases in the viability of cells in the presence of TMZ+oHA-5 as compared to TMZ alone (51.2 ±4.5 vs. 74.2 ±5.8, $p = 0.0031$), BCNU+o-HA5 as compared to BCNU alone (49.3 ±4.4 vs. 65.6 ±5.7, $p = 0.0119$), and BCNU+HA-68k as compared to BCNU alone (55.2 ±2.3 vs. 65.6 ±5.7, $p = 0.0496$).
Conclusions: Hyaluronan oligomers of 5 disaccharide units (oHA-5) significantly reversed the resistance of C6 cells to TMZ and BCNU. The results are only preliminary and a more thorough follow-up investigation is required to assess their actual role.

Key words: hyaluronan oligomers, CD44, glioma, drug resistance, temozolomide, carmustine.

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Introduction

Resistance to anti-cancer drugs

In recent decades, significant progress has been made in understanding cancer biology and inventing new therapeutic strategies. Despite these achievements, we are still far from successful management of cancer. Resistance to anti-cancer therapies, both intrinsic and acquired, constitutes one of the main causes of an inadequate response to treatment and diminished survival of cancer patients [1–3]. Drug resistance arises in a wide variety of mechanisms. It may develop due to decreased uptake of a drug into a cancer cell [4, 5], enhanced efflux of a drug out of a cancer cell, which is commonly mediated by ATP-binding cassette (ABC) transporters [6, 7], decreased activation of an anti-cancer pro-drug or inactivation of an active drug [8, 9], altered CYP-mediated metabolism [10], mutation or amplification of the targeted protein [11, 12], activation of alternative survival pathways [13], activation of repair mechanisms [14], and increased activity of anti-apoptotic signals [15, 16].

Resistance to anti-cancer drugs is primarily initiated by genetic factors [17]. However, the environment-mediated drug resistance model (EMDR) has been proposed as an alternative hypothesis explaining the origin of cancer cell resistance [18] – these hypotheses are not mutually exclusive. According to the EMDR model, resistance emerges as a result of an intimate relationship and subsequent signaling dialogue between cancer cells and the surrounding microenvironment [19, 20]. EMDR is mediated by either soluble factors present in the tumor microenvironment or cell adhesion-related factors [19]. Soluble factor-mediated drug resistance (SFM-DR) occurs through cytokines, chemokines and growth factors secreted by neighboring fibroblast-like tumor stroma [21, 22]. Cell adhesion-mediated drug resistance (CAM-DR) results from adhesion of tumor cell integrins to stromal fibroblasts [23] or to components of the extracellular matrix (ECM) [24].

Hyaluronan and drug resistance

One of the main components of the extracellular matrix is hyaluronan (HA). It is a linear high-molecular-weight polymer composed of repeating units of D-glucuronic acid residues and N-acetyl-D-glucosamine numbering up to 25,000 disaccharide units, which gives a molecular mass of up to 10 MDa. Hyaluronan is distributed ubiquitously in human tissues. It abundantly occurs in the vitreous body of the eye, synovial fluid, and connective tissue. Over half of the total body hyaluronan is present in the skin. Hyaluronan clearly plays a struc-

tural role in the organism. It maintains appropriate tissue hydration and tension, provides necessary lubrication in joint cavities, and decreases friction in tendon sheaths [25–27].

Hyaluronan-evoked anti-cancer drug resistance may be of a physico-mechanical nature as a dense extracellular matrix limits the delivery and distribution of therapeutic agents [28]. That is why enzymatic depletion of HA may be explored as a means to improve drug delivery [29].

Although hyaluronan has been perceived for decades as being only a passive component of the tissues [30], now it is evident that HA also mediates various intracellular signaling pathways involved in embryonic morphogenesis [31], inflammation and immune regulation [32], wound repair [33], and cancer [34]. Hyaluronan exerts its function through the interaction with cell surface receptors. CD44 is a transmembrane protein that constitutes a major hyaluronan-binding receptor [35–37].

CD44 (mainly its alternative splice variants) has been observed to be up-regulated in many cancers, including cancer stem-like cells, and to correlate with malignant phenotype of the cells [37–41]. The relevance of hyaluronan-CD44 interaction in environment-mediated drug resistance has been explicitly confirmed in many studies [42–45]. Overproduction of hyaluronan stimulates drug resistance in drug-sensitive cancer cells [46], and the phenomenon is likely to be mediated in part through the apoptotic pathways [47]. However, the regulation of drug transporters, including P-glycoprotein (also known as multidrug resistance protein 1; P-gp/MDR1 encoded by the gene *ABCB1*) [48], multidrug resistance-associated protein 2 (MRP2 encoded by the gene *ABCC2*) [48, 49] and breast cancer resistance protein (ABC family drug transporter BCRP encoded by the gene *ABCG2*) [50] is a major contributor to HA-CD44-mediated drug resistance. Interestingly, P-gp has also been found to be localized in close molecular vicinity and to be functionally associated with CD44 [24, 51].

Tackling drug resistance

Research efforts in cancer biology and pharmacology are nowadays focused on developing therapeutic strategies to overcome resistance to anti-cancer drugs [7, 18, 52, 53]. Hyaluronan-CD44-dependent drug resistance may be defeated by disrupting HA-CD44 interaction. It may be achieved by using anti-CD44 antibodies, small interfering RNA (siRNA) or short hairpin RNA (shRNA), soluble HA-binding proteins, and small hyaluronan oligosaccharides (oHA) [24, 37].

Attempts to use small hyaluronan oligosaccharides as agents reversing resistance to anti-cancer drugs are being successfully made [50, 54–58]. As CD44 receptor co-localizes with P-glycoprotein, treating the cells with hyaluronan oligomers induces rapid internalization of both the efflux pump and CD44 into the cell [24]. Moreover, disruption of endogenous hyaluronan-induced signaling in mammary carcinoma cells using small hyaluronan oligomers (3–9 disaccharide units) suppresses resistance to vincristine by 10-fold, to paclitaxel by 12-fold, and to carmustine by 78-fold. It also reverses resistance to doxorubicin and methotrexate [46, 56].

Similar effects have been reported in malignant glioma models. The addition of the oHA fraction (3–10 disaccharide units) to methotrexate-resistant glioma cell culture significantly reduced cell viability in the presence of methotrexate. *In vivo*, the glioma growth rate was suppressed by oHA, possibly by decreasing recruitment of host-derived BCRP-positive progenitor cells into the engrafted gliomas [50]. CD44 depletion by shRNA-mediated knockdown also resulted in glioma cell lines' sensitization to temozolomide and carmustine, the first-line cytotoxic drugs for glioblastoma multiforme. However, in that study, HA oligomers were not tested [38].

Aim of the study

The findings presented above suggest that hyaluronan oligomers may reverse drug resistance of glioma cells. However, the reversal effects of oHA of defined molecular length on glioma cells have not been investigated yet. In this study, we examined three hyaluronan fragments – oligomers containing 2 disaccharide units, 5 disaccharide units, and hyaluronan of the weight of 68 (±5%) kDa (approximately 180 disaccharide units) – as agents possibly capable of reversing the resistance of a C6 rat glioma cell line to temozolomide and carmustine.

Material and methods

Reagents

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), temozolomide (TMZ) and carmustine (BCNU) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hyaluronan fragments – oligomers containing 2 disaccharide units (oHA-2), 5 disaccharide units (oHA-5), and hyaluronan of the weight of 68 (±5%) kDa (HA-68k) – were purchased from Lifecore Biomedical (Chaska, MN, USA).

Cell culture

The rat C6 glioma cell line was obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). Dulbecco's Modified Eagle's Medium with 1,000 mg/l glucose (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution (10,000 units/ml penicillin and 10 mg/ml streptomycin in normal saline), phosphate buffered saline (PBS; pH 7.4) and trypsin-EDTA (0.25% trypsin, 1 mM EDTA-4 Na) were purchased from Invitrogen (Carlsbad, CA, USA). L-glutamine was purchased from Sigma Chemical Co. (St. Louis, MO, USA). C6 glioma cells were grown in 60-mm Petri dishes in DMEM with 1,000 mg/l glucose, supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were maintained at 37°C in humidified atmosphere of 95% air and 5% CO_2. For subcultures, cells were harvested in trypsin–EDTA solution twice a week and seeded at a density of 1.0×10^6 cells per dish.

Cell viability assay

Cell viability and mitochondrial function were measured by MTT reduction to formazan derivative through cellular mitochondrial dehydrogenases. C6 glioma cells were seeded at a density of 1.5×10^4 cells per well in 96-

well plates and grown in standard culture conditions for twenty-four hours. Then, the culture medium was replaced with fresh serum-free medium and the cells were exposed to the tested chemicals for the next twenty-four hours.

Firstly, hyaluronan fragments were dissolved in sterile water. The cells were exposed to HA fragments of 10, 50, and 150 µg/ml to ensure their non-toxicity. Secondly, TMZ was dissolved in dimethyl sulfoxide (DMSO), and BCNU was dissolved in ethanol 96% to produce the final concentrations of 150 mM. The cells were exposed to TMZ of 125, 250, 500, 750, 1,000, and 1,500 µM, and BCNU of 31.25, 62.5, 125, 250, 500, and 1,000 µM in order to plot the calibration curves and to determine IC_{50} values. The cells were also exposed to solvents of TMZ and BCNU in corresponding concentrations to ensure their non-toxicity. Finally, in order to investigate the reversal effects of HA fragments on TMZ and BCNU resistance, the cells were treated with 500 µM of TMZ or 125 µM of BCNU in the presence or absence of hyaluronan fragments of 50 µg/ml.

After incubation for twenty-four hours, MTT in the final concentration of 1.0 mg/ml was added and the cells were incubated for 4 h at 37°C. The supernatants were carefully aspirated. Formazan crystals were solubilized in DMSO and absorbance, directly proportional to the number of viable cells, was measured at 570 nm using a microplate reader (BioTek, EL ×800). The results were expressed as the relative absorbance, i.e. (A570 of experimental wells) / (A570 of control wells) × 100%.

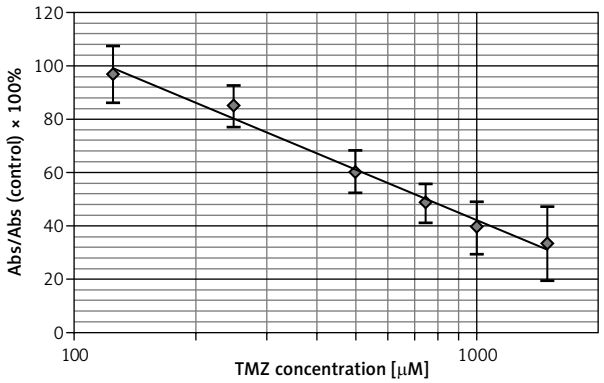

Fig. 1. Inhibitory effect of TMZ on the growth of C6 cells. The regression equation is: $y = -27.42 \times \ln(x) + 231.56$ ($r = 0.9934$)

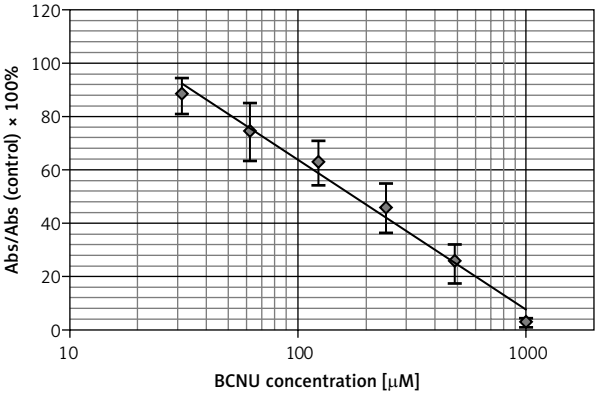

Fig. 2. Inhibitory effect of BCNU on the growth of C6 cells. The regression equations is: $y = -24.35 \times \ln(x) + 175.39$ ($r = 0.9922$)

Statistical analysis

The normal distribution of parameters was confirmed by the Shapiro-Wilks test; therefore data were described as the mean ± SD (standard deviation), and analyzed by Student's *t*-test. *P*-values below 0.05 were considered statistically significant. The analysis was performed using STATISTICA 10 StatSoft software.

Results

Effects of HA fragments on the growth of C6 glioma cells

We did not find any statistically significant differences between the viability of the cells treated with oHA-2, oHA-5, and HA-68k in the concentration range of 10–150 µg/ml and the control wells. The relative absorbance in the viability assay is presented in Table 1. The results suggest that none of the HA fragments had any inhibitory effects on the growth of C6 glioma cells.

Inhibitory effects of TMZ and BCNU on the growth of C6 cells

The solvents of TMZ and BCNU in corresponding concentrations, i.e. DMSO in the concentration range of 0.083–1.0% and ethanol in the concentration range of 0.021–0.67%, respectively, had no inhibitory effects on the growth of C6 glioma cells. Treatment of C6 cells with 125, 250, 500, 750, 1,000, and 1,500 µM of TMZ produced the relative absorbance in MTT viability assay of 96.9, 84.9, 60.4, 48.6, 39.4, and 33.4, respectively (Fig. 1), while treatment of C6 cells with 31.25, 62.5, 125, 250, 500, and 1,000 µM of BCNU produced the relative absorbance of 87.6, 74.0, 62.4, 45.3, 24.7, and 2.4, respectively (Fig. 2). The regression equation of the inhibitory effect of TMZ was $y = -27.42 \times \ln(x) + 231.56$ ($r = 0.9934$), and that of BCNU was $y = -24.35 \times \ln(x) + 175.39$ ($r = 0.9922$), with the IC_{50} = 751 µM for TMZ, and 172 µM for BCNU.

Table 1. Effects of hyaluronan fragments on the growth of the C6 glioma cells presented as the relative absorbance in MTT viability test ± SD (*p*-value). The C6 cells were incubated with or without hyaluronan fragments in the final concentrations of 10, 50, 150 µg/ml. After 24 h, cell viability was assessed by MTT assay

	Concentration of HA fragments		
	10 µg/ml	**50 µg/ml**	**150 µg/ml**
oHA-2	107.0 ±15.6 ($p = 0.4069$)	104.5 ±11.0 ($p = 0.6343$)	107.9 ±15.4 ($p = 0.3340$)
oHA-5	94.9 ±8.7 ($p = 0.5218$)	103.9 ±15.5 ($p = 0.6423$)	103.5 ±14.0 ($p = 0.6977$)
HA-68k	95.5 ±5.1 ($p = 0.6074$)	100.7 ±11.2 ($p = 0.9172$)	96.5 ±9.7 ($p = 0.7001$)

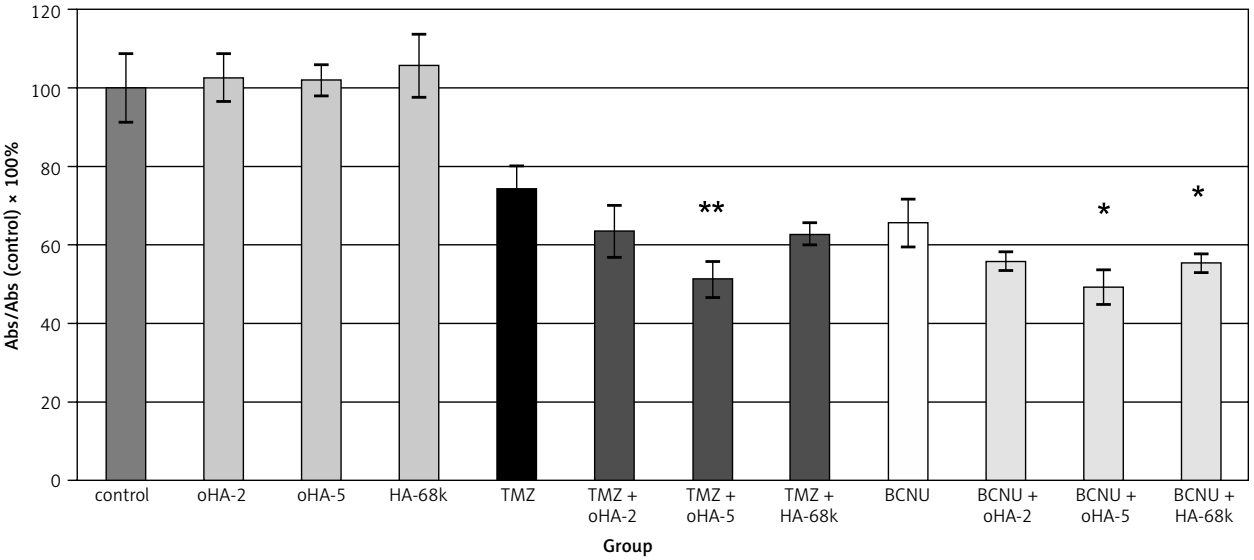

Fig. 3. Reversal effects of hyaluronan fragments on temozolomide (TMZ) and carmustine (BCNU) resistance in the C6 glioma cells. The significance level of the comparison of cell viability in the presence of a cytotoxic drug with a HA fragment compared to a drug alone is indicated above the bars by: * for $p < 0.05$, and ** for $p < 0.01$

Reversal effects of HA fragments on TMZ and BCNU resistance in C6 glioma cells

The C6 cells were incubated with 500 µM of TMZ or 125 µM of BCNU in the presence or absence of hyaluronan fragments of 50 µg/ml. In the MTT assay statistically significant decreases in the viability of cells occurred in the presence of TMZ+oHA-5 compared to TMZ alone (51.2 ±4.5 vs. 74.2 ±5.8, $p = 0.0031$), BCNU+o-HA5 compared to BCNU alone (49.3 ±4.4 vs. 65.6 ±5.7, $p = 0.0119$), and BCNU+HA-68k compared to BCNU alone (55.2 ±2.3 vs. 65.6 ±5.7, $p = 0.0496$). Data are presented in Fig. 3.

Discussion

Malignant gliomas, including particularly glioblastoma multiforme (GBM), are highly challenging to treat. Surgery is the first therapeutic approach to GBM, but infiltrative characteristics of the tumor make complete resection virtually impossible. Radiation therapy (RT) is the second mainstay of GBM treatment. RT is frequently combined with adjuvant chemotherapy, as it results in a survival benefit with minimal additional toxicity [59]. Most treatment protocols employ alkylating agents: temozolomide (TMZ), or nitrosoureas, e.g. carmustine (BCNU) [60].

Gliomas are known for their resistance to therapy [61]. The objective response rates of GBM to chemotherapy (except oligodendroglial subtypes) are approximately 30%, and time to progression (TTP) is short (3–6 months) [62]. The mechanisms of resistance to TMZ and BCNU in malignant gliomas are mediated by: (1) hyperactivity of methylguanine methyltransferase (MGMT) repair enzyme, which removes alkyl groups from the O6 position of guanine, (2) defects in the mismatch repair system (MMR), which makes the cells tolerant to mispairing of O6-methylguanine to thymine and stops apoptosis, (3) poly(ADP-ribose)polymerase (PARP) activity, which is involved in DNA adduct repair and contributes to cell survival, (4) dysregulation of apoptosis-regulating genes and the proteins *Bcl-2*, *Bcl-XL*, p53, and EGFR [63], or (5) ATP-binding cassette transporters' overexpression [64].

Small hyaluronan oligomers have been widely investigated as agents potentially reversing resistance to anti-cancer drugs. However, there is little evidence of effects of oHA of defined molecular length on reversing glioma resistance to alkylating agents. As it has been reported that HA degradation products had extremely different biological functions [37], we investigated HA fragments of various lengths: oligomers containing 2 disaccharide units (oHA-2), 5 disaccharide units (oHA-5), and hyaluronan of the weight of 68 (±5%) kDa (HA-68k), i.e. containing approximately 180 disaccharide units. The results showed that addition of oHA-5 to TMZ or BCNU treatment of the cells significantly reduced cell viability, effectively reversing both TMZ and BCNU resistance. No significant effect of TMZ+oHA-2 vs. TMZ alone, BCNU+oHA-2 vs. BCNU alone, or TMZ+HA-68k vs. TMZ alone on cell viability was found. The significance of the effect of HA-68k on BCNU resistance is uncertain, as the p-value approaches 0.05.

The positive effect of oHA-5 and non-significant effect of HA-68k on reversing resistance to TMZ and BCNU are in accordance with pharmacodynamic analysis of the quality of HA-CD44 interaction, assuming the reversal effect is CD44-mediated [38]. The analysis revealed that HA oligomers of 3–9 disaccharide units (including oHA-5), because of their shortness, exhibit only monovalent binding to CD44, displacing any hyaluronan polymer from membrane-bound receptors, serving as receptor antagonists. At approximately 10–19 disaccharide units, a progressive 3-fold increase in avidity was seen, suggesting that divalent binding and subsequent intracellular signaling occur. Larger polymers (including HA-68k) also induce CD44-mediated signaling [37, 58, 65].

The non-significant impact of the smallest hyaluronan oligomer, oHA-2, on anti-cancer drug resistance is not consistent with the research by Cui *et al*. [57]. They suggested

that the smaller the oHA, the stronger the reversal effect is. The lack of statistical significance may be due to inadequate oHA concentration, as Cui *et al*. reached the significance at oHA concentration of ≥ 100 μg/ml. On the other hand, oHA-2 fragments may produce a different effect than larger oligomers, as neither Misra *et al*. [46] Gilg *et al*. [50] nor Lesley *et al*. [65] included oHA-2 in their analyses.

To assess the reversal effects of hyaluronan fragments, TMZ and BCNU were used at concentrations of 500 μM and 125 μM, respectively. Concentrations somewhat lower than the IC_{50} values were deliberately chosen as it is unlikely that concentrations as high as IC_{50} values are reached in brain tissues during chemotherapeutic drug treatment in the clinical setting. Patients with brain tumors receiving a standard dose of oral temozolomide displayed plasma and cerebrospinal fluid (CSF) TMZ concentrations of only up to about 70 μM and 10 μM, respectively [66, 67]. Similarly, patients treated for advanced neoplasms receiving combination chemotherapy comprising cyclophosphamide, cisplatin, and carmustine were found to have a mean peak plasma concentration of carmustine of 7.8 μM [68]. The development of implantable carmustine wafers allowed drug concentrations of up to several hundred times the concentration achievable with intravenously administered doses in a brain tumor environment [69].

The C6 glioma cell line was chosen for this study because the cell line exhibits high CD44 expression [70]. Moreover, most of the C6 cells are cancer stem-like cells with *in vitro* characteristics of self-renewal [71–72]. That makes this cell line particularly suitable to study the reversal of resistance to anti-cancer drugs [61]. However, as cancer cell line models are continuously questioned [73], the possible clinical relevance of the obtained results requires further investigations.

The authors declare no conflict of interests.

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