Young's Modulus of tumour tissue from an *in vivo* breast cancer model- effect of treatment with guluronate oligosaccharides

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ABSTRACT

Increased tissue stiffness is a classic characteristic of solid tumours, and breast tumours are often first identified as a palpable lump. Increased tissue stiffness in tumours is more than just a typical, and often easily identifiable, consequence of tumour development. Rather, elevated tissue stiffness activates tumourigenic pathways, directly contributing to increased proliferation and survival, and even altering the tumour response to therapy. As such therapeutic interventions that reduce tumour stiffness may be of benefit in treating solid tumours.

We have utilized texture analysis to determine the Young's modulus of tumour tissue from two orthotopic syngeneic *in vivo* mouse models of breast cancer (67NR and 4T1) and the effect of treatment with guluronate oligosaccharides at a dose of 25mg/kg IP on tumour stiffness in these models.

4T1 is a more aggressive tumour type than 67NR and showed a significantly higher Young's modulus. Treatment with guluronate oligomers resulted in a significant reduction in Young's modulus in the 67NR model and a reduction that did not reach significance in the 4T1 model, alongside significantly reduced tumour growth in both models suggesting guluronate oligosaccharides may have potential in cancer therapy.

INTRODUCTION

A tumour can be considered a complex ecosystem of multiple cell types embedded within an extracellular matrix. The composition and properties of this matrix are dynamic, evolving as the tumour develops, and may contribute directly or indirectly to tumour growth and progression. One of the ways in which the extracellular matrix may contribute to tumour progression is through changes in matrix stiffness. Increasing matrix stiffness can alter signalling cascades and thereby the behaviour of cells within the tumour, which can drive malignancy.

The extracellular matrix is a complex composite matrix containing large amounts of carbohydrate, primarily in proteoglycans but also in glycosylated proteins. Carbohydrates are structurally complex molecules, and information is beginning to emerge indicating they play a central role in extracellular matrices not only by providing structural support to but also through complex contributions to maintaining tissue homeostasis.

It has previously been shown that guluronate oligosaccharides are able to modify complex biological matrices such as mucus and bacterial biofilms, and we have recently shown that guluronate oligosaccharides also have an anti-tumour effect. Given the matrix modifying

potential of these oligosaccharides it was of interest to investigate whether their anti-tumour effect was associated with changes in bulk tumour stiffness.

METHODS

Preparation of Guluronate oligomers

The low molecular weight guluronate oligomer samples were obtained by acid hydrolysis of high molecular weight alginates with a high content of guluronic acid residues. Briefly, pre-hydrolyzed high-G Na-alginate was dissolved in MQ water (20 g/l) and the high-G oligomers were obtained by slowly adjusting the pH to 2.8. The precipitate was washed 2x in 0.01 M HCl, resuspended in MQ water, neutralized with 0.5 M NaOH, filtered through a Watman GF/A glass microfiber filter (pore size1.6 μm) and finally freeze dried. Chemical composition and sequence, as well as the number average degree of polymerization (DPn) were determined by 1H NMR spectroscopy. The fractions of guluronate containing monad (F_G), diad (F_{GG}) and triad (F_{GGG}) were 0.95, 0.92 and 0.90, respectively. The number-average degree of polymerization (DPn) of the present guluronate oligomer sample was determined by 1H NMR end-group signals and determined to be 11. No high molecular weight tail was present. Active coal filtration removed endotoxins to a level of 17.6 EU/g as determined by the LAL method.

In vivo study

The mouse cell lines 67NR and 4T1, were kindly provided by Dr. Fred Miller (Wayne State University, Detroit, MI). The cells were sub cultured and maintained into a continuous cell culture in DMEM (GIBCO) supplied with 10% FBS, 250 μg/ml amphotericin B (Sigma-Aldrich), 100 μg/ml gentamycin (Invitrogen).

Female 7-8 week-old BALb/c mice were purchased and housed in a pathogen-free barrier room in the Animal Care Facility at the Norwegian University of Science and Technology. This study was approved by The Norwegian Food Safety Authorities (FOTS number 7875), and the experiments were performed in accordance with the institutional ethical guidelines at the Comparative Medicine Core Facility (CoMed) at NTNU. All mice were housed in wire-top cages with aspen wool chip bedding. Room temperature was 24±1°C with a relative humidity of 45-50% and a 12-H light/dark cycle. A total of 4×105 cells were injected orthotopically into the left tertiary fat pad of anesthetized mice (Isoflurane) G-blocks was administered 25mg/kg (100μl) intraperitoneally every alternate day. Mice were randomized into two groups when all of them had palpable tumours. The tumour volumes were measured, by electronic vernier calipers three times per week. After 22 days the mice were euthanized by cervical dislocation under isoflurane anesthesia. The tumours were harvested and a bisected a one hemisphere from each tumour was immediately transported (chilled) for mechanical testing.

Ex vivo mechanical testing of tumour tissue

Tumours were placed cut side down on a piece of tissue (to avoid slippage) on the stage of Stable Micro Systems TA.XT plus texture analyser fitted with a 2mm cylindrical probe. The probe was chosen to minimise the influence of irregular tumour shape and size on the data generated. In the absence of obvious inhomogeneities in the tumour surface the tumour was centred under the probe. If necessary, the placement was adjusted to avoid penetration in areas of obvious inhomogeneity. Tumours were tested in compression mode with a single compression to 75% strain. Results were plotted as force strain curves. Young's modulus (YM) was calculated from the initial gradient of the stress-strain curve.

RESULTS

Treatment with guluronate oligosaccharides resulted in a reduction in tumour growth in both the 67NR (55% lower tumour volume) 4T1 (33 % lower volume) models (see also).

The Youngs modulus of control 67NR and 4T1 tumour hemispheres (as calculated from the initial gradient of the force strain curves from the texture analysis) showed the 4T1 tumours to be significantly stiffer than the 67NR tumours (fig. 1) (unpaired t-test in Graphpad Prism software). The variance was similar in the two groups.

Comparing treated with control tumours for the 67NR allograft, the treated tumours show a significant reduction in Youngs modulus compared to the control (untreated) tumours (fig. 2A) with the variance being similar in the two groups. For the 4T1 allograft there was a similar trend towards reduced Youngs modulus in the treated group, but it did not reach statistical significance (fig. 2B). Interestingly, the distribution differed somewhat between the control and the treated group with the distribution in the treated group being less clustered around the mean.

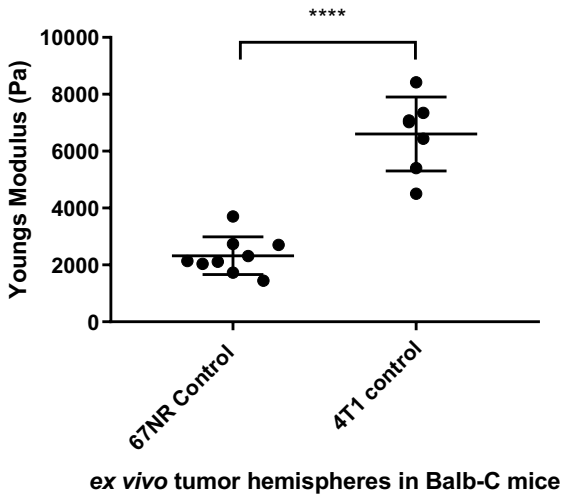

Figure 1 Youngs Modulus of tumour hemispheres from 67NR and 4T1 control tumours

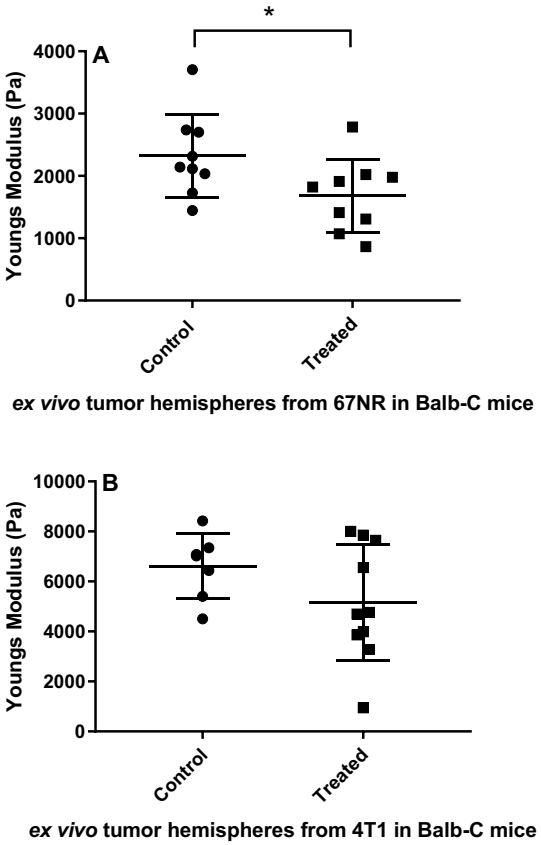

Figure 2 Youngs Modulus of tumour hemispheres from control and treated 67NR (A) and 4T1(B) tumours

DISCUSSION

Ex vivo mechanical tissue testing has previously been used to identify and characterize tumours from several tumour types including breast tumours from a mouse model. The method utilized in this study was able to distinguish clear mechanical differences between the tumours from the 67NR and the 4T1 models (fig. 1) in keeping with the known characteristics of 4T1 as a stiffer and more aggressive tumour type than 67NR, although it should be noted there was considerable variation within each group. This may reflect inter-tumour variability, intra-tumour variability or a combination of these. Given the heterogeneous nature of tumours and their

host interactions a degree of both intra-tumour and inter-tumour variation is certainly to be expected, for example faster growing tumours may exhibit a more necrotic core. Treatment with guluronate oligosaccharides resulted in a significant reduction in Youngs modulus for the less stiff 67NR tumours, and whilst it did not reach significance, a similar trend was seen for the relatively stiffer 4T1 tumours (fig 2). These results are interesting considering the role tumour stiffness can play in cancer progression however the current study cannot distinguish between a direct effect of guluronate oligosaccharides on matrix stiffness and an indirect effect as a result of biochemical or metabolic changes in the tumour as a result of the treatment. Regardless of the mechanisms at play here, this study gives further support to the potential of carbohydrates in pharmaceutical applications.

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