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Development of a hyaluronan bioconjugate for the topical treatment of melanoma

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Hyaluronan (HA) is a glycosaminoglycan. Despite its high molecular weight, Brown *et al.* has shown that topically applied HA rapidly disseminated throughout the epidermis and reached the dermis in both mouse and human skin [1]. Importantly, the majority of cutaneous malignant melanomas are positive for the HA receptor, CD44 [2]. We envisioned that HA conjugated to anti-cancer drugs would be useful for: 1) Transport of the drug into the skin after topical application, and 2) Targeted delivery of the drug to CD44 positive malignant melanoma cells. To test this concept, we conjugated doxorubicin (DOX) to HA and tested its impact on B16-F10 melanoma cells *in vitro* and *in vivo*. We used DOX because of its intrinsic fluorescence and the availability of anti-DOX antibodies. Thus, DOX-HA could be evaluated *in vitro* and *in vivo*.

B16-F10 melanoma cells expressed CD44 as assessed by FACS (Fig. 1A). The binding of CD44 to HA and DOX-HA was evaluated using cell adhesion assays. As shown in Fig. 1B, panel 1, B16-F10 melanoma cells showed significantly better binding to wells coated with HA as compared to the wells lacking the HA substrate. An anti-CD44 monoclonal antibody significantly ($P<0.05$) blocked the adhesion of B16-F10 cells to HA showing that their binding to HA is CD44 mediated. Similarly, B16-F10 melanoma cells showed significantly better binding to wells coated with the DOX-HA as compared to wells lacking the DOX-HA (Fig. 1B, panel 2). Again, an anti-CD44 monoclonal antibody significantly ($P<0.05$) blocked the adhesion of B16-F10 cells to DOX-HA showing that their binding to DOX-HA is CD44 mediated.

To assess the uptake of DOX and DOX-HA by B16-F10 melanoma cells, we performed FACS. DOX and DOX-HA showed time dependent uptake in B16-F10 cells (Fig. 1C). However, the midpoints of the curves showed that the rate of DOX uptake was greater than the uptake for DOX-HA. DOX and DOX-HA both showed dose-dependent uptake by the B16-F10 cells but DOX accumulated to higher concentrations inside of the melanoma cells as compared with the DOX-HA (Fig. 1D).

Intracellular distributions of DOX and DOX-HA in melanoma cells were assessed by confocal microscopy. DOX and DOX-HA fluorescent patterns in B16-F10 cells were similar with cytoplasmic and punctate nuclear staining (Fig. 1E).

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Both DOX and DOX-HA inhibited the proliferation of melanoma cells in a dose dependent fashion (Fig. 1F). However, DOX was more potent than DOX-HA. We failed to detect any effect of HA alone on the proliferation of B16-F10 melanoma cells (Fig. 1G) showing that DOX-HA inhibited the proliferation of B16-F10 cells due to the attached DOX moieties. Finally, primary human keratinocytes were sensitive to DOX but were insensitive to DOX-HA (Fig. 1H). Decreased cytotoxicity to normal keratinocytes suggests that DOX-HA has improved selectivity as compared with DOX.

Next, we tested the penetration of DOX and DOX-HA into mouse skin using a transdermal delivery assay [3]. DOX and DOX-HA were recovered in the receptor solutions of Franz diffusion cells (Fig. 2A). The amount of recovered DOX was only modestly more than the amount of recovered DOX-HA but the difference between the two was significant ($P<0.01$).

To characterize the translocated DOX-HA, we assessed the recovered receptor solution using Western blotting and size exclusion chromatography. Results showed that an anti-DOX monoclonal antibody detected a single band. Treatment of the sample with hyaluronidase resulted in disappearance of the band, showing that the DOX detected in the sample recovered from the Franz cell was linked to HA (Fig. 2B). Therefore, DOX remained linked to the HA molecule as it transited the skin. On the other hand, the DOX-HA that crossed the skin was reduced in molecular weight (from $\sim 1 \times 10^6$ Da to $\sim 0.5 \times 10^6$ Da; Fig. 2C).

To assess the potential utility of DOX-HA for the local treatment of malignant melanomas, we applied it to tumors of graded size using DOX and vehicle as comparators. DOX-HA significantly reduced the growth rates of B16-F10 melanoma tumors compared with vehicle alone ($P<0.01$; Fig. 2D). By contrast, DOX had an insignificant effect relative to the vehicle treated mice ($P>0.05$). Comparisons of the tumor sizes in the DOX treated groups *versus* the DOX-HA treated groups showed that the bioconjugate more significantly ($P<0.05$) impaired tumor growth. Thus, DOX-HA improved the pharmacological activity of DOX. Because *in vitro* experiments showed that B16-F10 melanoma cells expressed CD44, bound the DOX-HA biopolymer in a CD44 dependent fashion and cells actively endocytosed the DOX-HA, we concluded that DOX-HA is targeted to the B16-F10 melanoma cells after topical applications most likely due to molecular interaction with CD44.

Next, we compared the impact of topically applied DOX and DOX-HA on normal mouse skin. We failed to detect macroscopic (induration, erythema and ulceration, data not shown) and histological alterations in the skin of mice after applications of either DOX or DOX-HA (cumulative dose of 42 μ g; data not shown) suggesting that the dose and/or route of administration of DOX and DOX-HA did not induce local toxicities.

How can we explain the improved pharmacological activity of DOX-HA compared to DOX given that DOX showed higher cytotoxicity *in vitro* and that DOX showed better transdermal delivery? Previous *in vitro* tumor models have predicted that the rate of drug uptake into solid tumors is due to both protein binding and the diffusion gradient in the tumor interstitium [4]. Thus, the cytotoxicity of drugs on tumor cell monolayers may not correlate with their effects in the context of the tumor. We propose that a combination of CD44 targeting and interstitial diffusion gradients can account for the improved anti-tumor activity of DOX-HA as compared with DOX. In fact, as shown in Fig. 2E, we recovered higher concentrations of DOX-HA from B16-F10 tumors *in vivo* as compared with DOX which showed that HA improved drug localization.

In conclusion, we have described the preparation of a DOX-HA bioconjugate and have evaluated its impact on tumor growth after topical applications *in vivo*. Results described in this study provide the conceptual and technical foundations for further developing HA formulations for the topical treatment of cutaneous malignant melanoma.

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References

1. Brown TJ, Alcorn D, Fraser JR. Absorption of hyaluronan applied to the surface of intact skin. *J Invest Dermatol* 1999;113:740–6. [PubMed: 10571728]
2. Karjalainen JM, Tammi RH, Tammi MI, Eskelinen MJ, Agren UM, Parkkinen JJ, Alhava EM, Kosma VM. Reduced level of CD44 and hyaluronan associated with unfavorable prognosis in clinical stage I cutaneous melanoma. *Am J Pathol* 2000;157:957–65. [PubMed: 10980134]
3. Brown MB, Hanpanitcharoen M, Martin GP. An in vitro investigation into the effect of glycosaminoglycans on the skin partitioning and deposition of NSAIDs. *Int J Pharm* 2001;225:113–21. [PubMed: 11489559]
4. Heijn M, Roberge S, Jain RK. Cellular membrane permeability of anthracyclines does not correlate with their delivery in a tissue-isolated tumor. *Cancer Res* 1999;59:4458–63. [PubMed: 10485498]
5. Mummert ME, Mohamadzadeh M, Mummert DI, Mizumoto N, Takashima A. Development of a peptide inhibitor of hyaluronan-mediated leukocyte trafficking. *J Exp Med* 2000;192:769–79. [PubMed: 10993908]

Fig. 1.

Evaluation of DOX and DOX-HA *in vitro*. (A) B16-F10 melanoma cells were incubated with an anti-CD44 antibody (clone IM7) or an IgG isotype control. Bound antibodies were detected with a FITC-conjugated anti-rat antibody. Open histograms, cells without staining; dashed histograms, isotype control; dotted histograms, anti-CD44 antibody. These results show that B16-F10 melanoma cells expressed the HA receptor, CD44. (B) The CD44-dependent adhesion of B16-F10 melanoma cells to HA-coated plates (panel 1) or DOX-HA-coated plates (panel 2) was evaluated as previously described [5]. The CD44 specific binding of B16-F10 melanoma cells to HA were evaluated by pre-treating the cells with an anti-CD44 monoclonal antibody that antagonizes HA-binding or an irrelevant isotype control. Results are expressed as the percentage of bound cells per well and are presented as the means $\pm$ SD ($n = 4$). Statistically significant differences are indicated by an asterisk (* $P < 0.05$). (C) B16-F10 melanoma cells were treated with DOX (open circles) and DOX-HA (closed circles) for the

indicated periods of time and then subjected to FACS. The intrinsic fluorescence of DOX provided the signal. Results are presented as the geometric mean fluorescence intensities (MFI) *versus* time. The line is the curve fit to the data using non-linear regression implemented in Sigma Plot software, version 10.0. (D) Dose dependent uptake of DOX (open circles) and DOX-HA (closed circles) by B16-F10 melanoma cells was determined by treating the cells with different concentrations of DOX or DOX-HA for 4 h. Afterwards, the cells were harvested and then subjected to FACS. Results are presented as the geometric mean fluorescence intensities (MFI) *versus* the molar equivalents of the DOX concentration. The line is the curve fit to the data using nonlinear regression implemented in Sigma Plot software, version 10.0. (E) B16-F10 cells were treated with DOX and HA-DOX for 16 h. Cells were then harvested and subjected to confocal laser scanning microscopy (original magnification x63, scale bars = 20 μ m). (F and G) Impact of DOX, DOX-HA and HA on the proliferation of B16-F10 melanoma cells. B16-F10 melanoma cells were treated with graded doses of DOX (open circle), DOX-HA (filled circle) (F) and HA (G). Cultures were pulsed with 3 H-thymidine during the last 24 h of culture and then harvested onto glass fiber filter paper. The % inhibition of the 3 H-thymidine uptake at each dose relative to cultures without treatment was calculated. Data are presented as the means $\pm$ SD of triplicate cultures. The line in (F) is the curve fit to the data using non-linear regression implemented in Sigma Plot software, version 10.0. (H) Impact of DOX and DOX-HA on the proliferation of primary human keratinocytes (Cell Applications, Inc.). The keratinocytes were cultured in the presence of DOX or DOX-HA at 0.1 μ M drug equivalent and pulsed with 3 H-thymidine during the last 24 h of culture and then harvested onto glass fiber filter paper. The % inhibition of the 3 H-thymidine uptake relative to cultures without treatment was calculated. Data are presented as the means $\pm$ SD of triplicate cultures. Statistically significant differences are indicated by an asterisk (** $P < 0.01$).

Fig. 2.

Transdermal delivery of DOX and DOX-HA across intact skin and their impact on *in vivo* tumor growth. (A) The back skin of C57BL/6 mice was shaved, harvested and then mounted in a Franz diffusion cell with the stratum corneum side up. DOX and DOX-HA at equal DOX concentrations were applied to the surface of the skin. The receptor solution was collected 24 h later and the fluorescent intensities measured in a photon counter. The transdermal delivery was calculated as the percentage of the fluorescence intensity of the originally applied DOX or DOX-HA solutions with volume corrections. Values are expressed as means $\pm$ SD of triplicate measurements and statistically significant differences indicated by asterisks (** $P < 0.01$). (B) Receptor solutions from (A) were concentrated and then subjected to Western blotting using an anti-DOX antibody (clone MAD11). HA specificity was assessed by treating one half of the sample with bovine testes hyaluronidase (HA'se). (C) Concentrated receptor solutions from (A) were subjected to size exclusion chromatography (dashed line) and compared with the original DOX-HA bioconjugate (solid line). Fractions were monitored based on the DOX fluorescent intensities. Fluorescent signals were normalized and plotted relative to the fraction number. (D) Impact of DOX and DOX-HA on the growth of B16-F10

tumors. B16-F10 melanoma cells were inoculated into C57BL/6 mice. When tumors reached ~40 mm³ (time 0 in the Fig.) animals were treated with vehicle alone, DOX and DOX-HA every other day for the indicated time period starting on day 0 (cumulative dose of 42 µg drug equivalent). Tumors were measured daily and their volumes calculated from $V = (L \times W^2) \times \pi/6$. Data shown are as the means $\pm$ SD of the measured tumor volumes (n = 7). Statistically significant differences between groups of animals at each time point was assessed by Tukey's HSD test following the ANOVA (* P<0.05, ** P<0.01 compared with the vehicle treatment group; ‡ P<0.05 compared with the DOX treatment group). (E) Retention of DOX and DOX-HA in B16-F10 melanoma tumors. Solutions of the vehicle, DOX or DOX-HA were applied to tumors established in C57BL/6 mice essentially as described in (D). On day 5 following the start of the treatments the animals (n = 4) were sacrificed, the tumors were excised and dissected from the surrounding tissue and were then snap frozen in liquid N₂. The tumors were ground in a mortar with a pestle and extracted in PBS. The concentration of DOX in each of the samples was determined by the ELISA using an anti-DOX antibody (clone MAD11). Results are expressed relative to the total protein content of the tumors and show the means $\pm$ SD. Statistically significant differences are indicated by asterisks (**P<0.01).