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Selective Tumor Cell Targeting by the Disaccharide Moiety of Bleomycin

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Supporting Information

ABSTRACT: In a recent study, the well-documented tumor targeting properties of the antitumor agent bleomycin (BLM) were studied in cell culture using microbubbles that had been derivatized with multiple copies of BLM. It was shown that BLM selectively targeted MCF-7 human breast carcinoma cells but not the "normal" breast cell line MCF-10A. Furthermore, it was found that the BLM analogue deglycobleomycin, which lacks the disaccharide moiety of BLM, did not target either cell line, indicating that the BLM disaccharide moiety is necessary for tumor selectivity. Not resolved in the earlier study were the issues of whether the BLM disaccharide moiety alone is sufficient for tumor cell targeting and the possible cellular uptake of the disaccharide. In the present study, we conjugated BLM, deglycoBLM, and BLM disaccharide to the cyanine dye Cy5**. It was found that the BLM and BLM disaccharide conjugates, but not the deglycoBLM conjugate, bound selectively to MCF-7 cells and were internalized. The same was also true for the prostate cancer cell line DU-145 (but not for normal PZ-HPV-7 prostate cells) and for the pancreatic cancer cell line BxPC-3 (but not for normal SVR A221a pancreas cells). The targeting efficiency of the disaccharide was only slightly less than that of BLM in MCF-7 and DU-145 cells and comparable to that of BLM in BxPC-3 cells. These results establish that the BLM disaccharide is both necessary and sufficient for tumor cell targeting, a finding with obvious implications for the design of novel tumor imaging and therapeutic agents.

The bleomycins (BLMs) are a family of glycopeptide-derived antitumor antibiotics used clinically for the treatment of squamous cell carcinomas and malignant lymphomas (BLM A_5 is shown in Figure 1).[1,2] Their antitumor activity is thought to result from selective oxidative cleavage of 5′-GC-3′ and 5′-GT-3′ sequences in DNA and possibly also from oxidative degradation of RNA.[3] In addition to its antitumor activity, BLM has been recognized for its ability to target tumors and shown to act as a tumor-imaging agent.[4] Identification of the molecular elements in BLM responsible for tumor cell targeting would not only enable analogues with improved properties to be explored but might also allow for the selective delivery of other drugs to tumor cells.

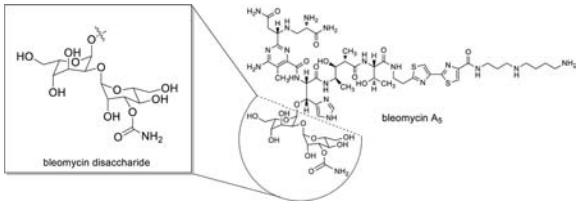

Figure 1. Structure of BLM A_5. The inset highlights the BLM disaccharide moiety.

We previously showed that BLM A_5 conjugated to microbubbles binds selectively to tumor cells.[5] Microbubbles, used traditionally as contrast agents for ultrasonography, have recently been modified with ligands that bind to specific receptors on cancer (and other) cell surfaces in an effort to probe ligand−cell-surface interactions.[6] In the previous study, the C-terminus of BLM was acylated with biotin and bound to commercially available streptavidin-derivatized microbubbles. It was shown that BLM-derivatized microbubbles (but not streptavidin-derivatized ones) bound to MCF-7 human breast carcinoma cells but not to the "normal" MCF-10A breast cell line.[5] To define the structural elements of BLM responsible for tumor targeting, the same experiment was performed using deglycoBLM, which lacks the disaccharide moiety. Cellular recognition was not observed for either MCF-7 or MCF-10A cells.[5] In the present work, we carried out analogous experiments using BLM and deglycoBLM conjugated to a cyanine dye (Cy5**). This permitted more facile quantification of the results as well as an investigation of internalization of the conjugates.

Our previous experiments established that the disaccharide moiety of BLM is necessary for tumor cell recognition but did not address the issue of its possible sufficiency. Thus, the BLM disaccharide, consisting of L-gulose linked to 3-carbamoylmannose, was synthesized using a procedure similar to that described previously.[7] This disaccharide was coupled to a commercially available linker that had been protected as the benzyloxycarbonyl (CBz) derivative (**1**) to afford **2** in 66% yield (Scheme 1). Deprotection of the primary amine in **2** followed by deacetylation and subsequent conjugation to the cyanine dye Cy5**[8,9] via treatment with the *N*-hydroxysuccinimide (NHS) ester of Cy5** (**3**) provided the BLM disaccharide-Cy5**

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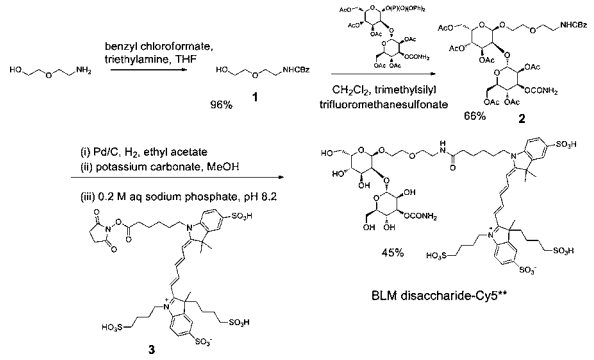
Scheme 1. Synthetic Route Employed for the Preparation of BLM Disaccharide-Cy5**



conjugate [2-*O*-(3-*O*-carbamoyl-α-D-mannopyranosyl)-L-gulopyranose linked to Cy5**] in 45% overall yield for the last three steps. The Cy5** conjugates of BLM A_5 and deglycoBLM A_5 (Figure 2) were also prepared in analogy with a published procedure.[5,10]

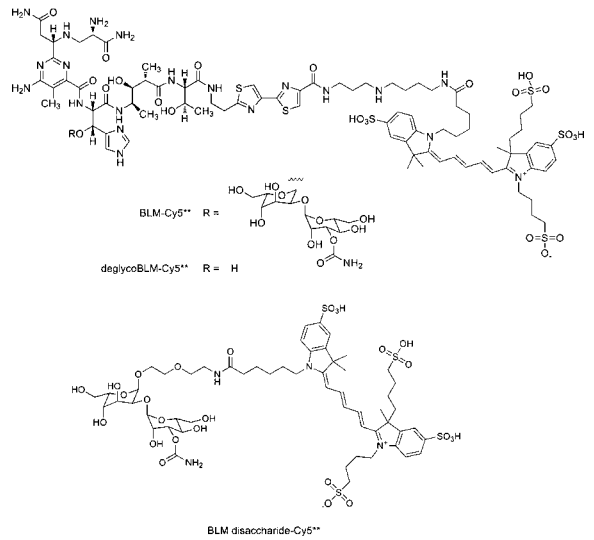


Figure 2. Structures of BLM-Cy5**, deglycoBLM-Cy5**, and BLM disaccharide-Cy5**.

MCF-7 human breast carcinoma cells and MCF-10A "normal" breast cells were cultured on 16-well glass chamber slides for 48 h and then treated with 50 μM BLM-Cy5** at 37 °C for 1 h to allow interaction with the cell surface. The cells were then washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde, after which fluoresence microscopy imaging (Zeiss Axiovert 200 M inverted microscope, 40× oil objective) was carried out. As shown in Figure S2 in the Supporting Information, treatment of MCF-7 cells with BLM-Cy5** resulted in significant cell binding and uptake, while no interaction was apparent for the MCF-10A cells. The cells were also treated with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) in order to stain the cell nuclei, permitting preliminary evaluation of the localization of the BLM-Cy5** conjugate in the MCF-7 cells relative to the cell nuclei. With these results in hand, an experiment was carried out in which the MCF-7 and MCF-10A cells were treated with 50 μM BLM-Cy5**, deglycoBLM-Cy5**, or BLM disaccharide-Cy5** and then fixed with paraformaldehyde.

After irradiation for 3 s, the images shown in Figure 3 were recorded. They clearly show that both BLM-Cy5** and BLM disaccharide-Cy5** were associated with the MCF-7 cells but deglycoBLM-Cy5** was not. None of the conjugates bound to the normal breast (MCF-10A) cells. The dye itself exhibited no affinity for either cell line. Figure 4 shows the results obtained by similar treatment of the prostate cancer cell line DU-145 and the normal prostate cell line PZ-HPV-7, which were qualitatively identical to those obtained for the MCF-7 and MCF-10A cell

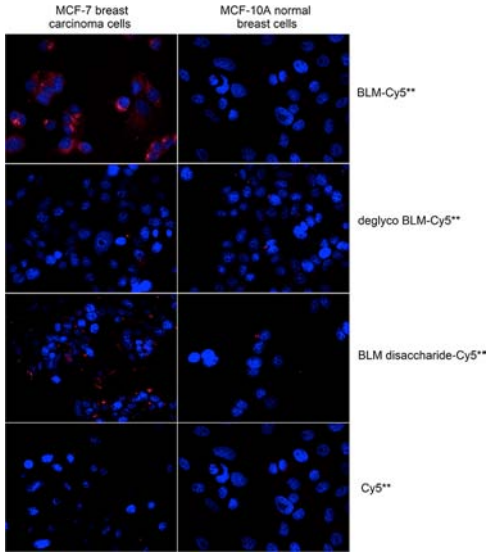


Figure 3. Binding/uptake of Cy5** conjugates in MCF-7 breast carcinoma cells and MCF-10A normal breast cells. The cells were treated with 50 μM BLM-Cy5**, deglycoBLM-Cy5**, BLM disaccharide-Cy5**, or Cy5** at 37 °C for 1 h, washed with PBS, and fixed with 4% paraformaldehyde. The cell nuclei were stained with DAPI. Fluorescence imaging was carried out after a 3 s exposure.

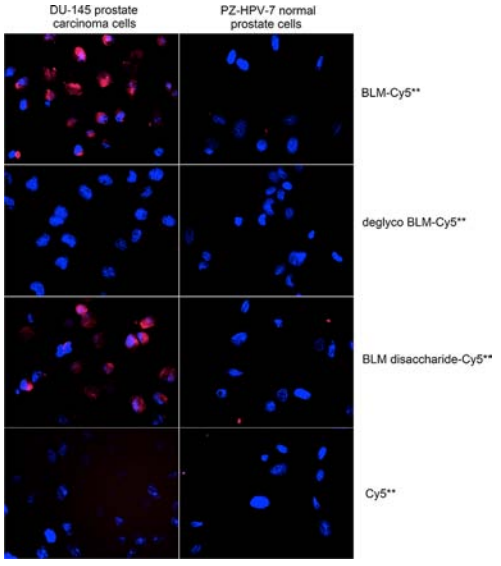


Figure 4. Binding/uptake of Cy5** conjugates in DU-145 prostate carcinoma cells and PZ-HPV-7 normal prostate cells. The cells were treated with 25 μM BLM-Cy5**, deglycoBLM-Cy5**, BLM disaccharide-Cy5**, or Cy5** at 37 °C for 1 h, washed with PBS, and fixed with 4% paraformaldehyde. The cell nuclei were stained with DAPI. Fluorescence imaging was carried out after a 1 s exposure.

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lines, although more efficient binding of BLM-Cy5** and BLM disaccharide-Cy5** was observed for DU-145 and PZ-HPV-7 cells. The results shown in Figures 3 and 4 are quantified in Figure 5. The binding/uptake in MCF-7 cells was shown not to vary significantly with the extent of cell confluence (Figure S3).

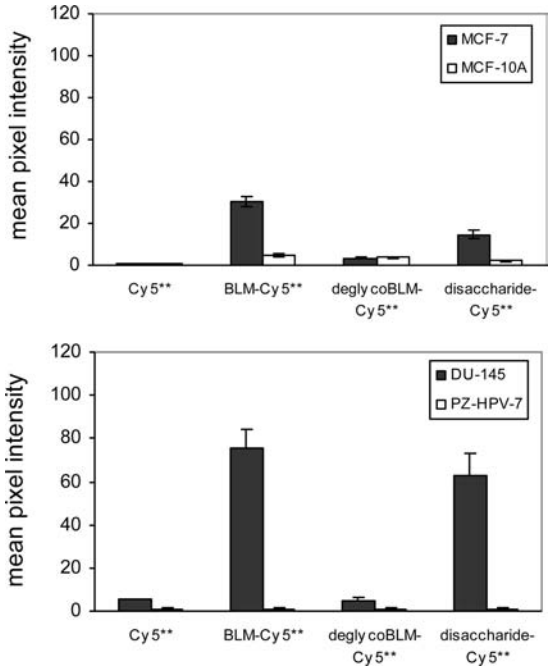

Figure 5. Quantification of the binding/uptake of BLM-Cy5**, deglycoBLM-Cy5**, BLM disaccharide-Cy5**, and Cy5** by MCF-7 and MCF-10A cells (upper panel) or DU-145 and PZ-HPV-7 cells (lower panel). The MCF-7 and MCF-10A cells were treated with 50 μM dye conjugates and irradiated for 3 s prior to fluorescence imaging. The DU-145 and PZ-HPV-7 cells were treated with 25 μM dye conjugates and irradiated for 1 s prior to imaging.

Also studied was the targeting of BxPC-3 pancreas cancer cells by BLM-Cy5**, deglycoBLM-Cy5**, and BLM disaccharide-Cy5**. Unlike the results obtained with the MCF-7 and DU-145 cell lines, the binding/uptake of BLM disaccharide-Cy5** and BLM-Cy5** were essentially equivalent in BxPC-3 cells. As shown in Figure 6, BLM-Cy5** and BLM disaccharide-Cy5** were associated with the BxPC-3 cells, but deglycoBLM-Cy5** was not. None of the dye conjugates exhibited binding/uptake by the SVR A221a normal pancreas cell line. Two additional cancer

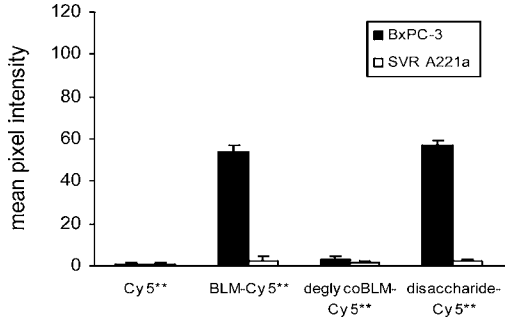

Figure 6. Quantification of the binding/uptake of BLM-Cy5**, deglycoBLM-Cy5**, BLM disaccharide-Cy5**, and Cy5** by BxPC-3 and SVR A221a cells. The cells were treated with 25 μM dye conjugates and irradiated for 1 s prior to imaging.

cell lines have also been observed to exhibit similar selectivities.[11] While the scope of tumor cell lines targeted by BLM and its disaccharide are presently the subject of ongoing investigations, the reports of targeting with radionuclides of BLM have included numerous tumor types, suggesting that the phenomenon observed here may prove to be reasonably general.

The process by which the BLM-Cy5** and BLM disaccharide-Cy5** conjugates are internalized presumably involves initial binding to the cell surface (as demonstrated for BLM in our earlier report involving microbubbles[5]) followed by internalization.[12] The apparent lack of any dye conjugate bound to the cell surface in Figures 3 and 4 presumably reflects facile internalization following binding to the cell surface. This assumption was tested directly utilizing biotinylated BLM A_5, deglycoBLM A_5, and BLM disaccharide bound to commercially available streptavidin-derivatized microbubbles as described previously[5] (Figure 7). The BLM A_5- and BLM disaccharide-

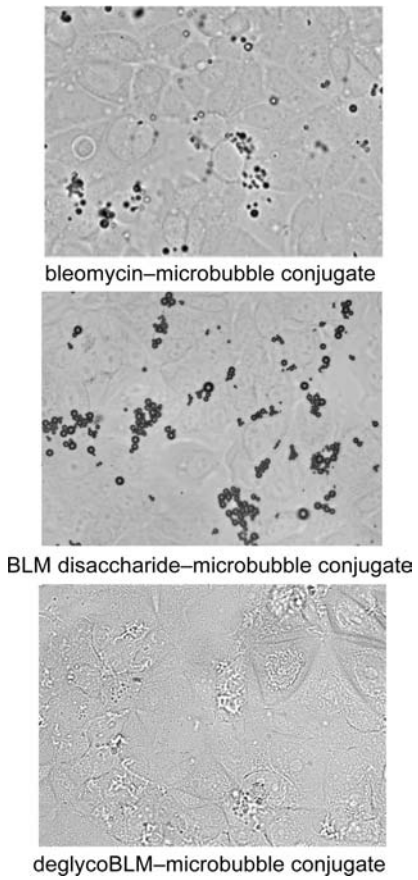

Figure 7. Monolayers of cultured MCF-7 breast cancer cells treated with microbubbles derivatized with BLM (top), BLM disaccharide (center), or deglycoBLM (bottom).

derivatized microbubbles bound to MCF-7 cells, but the deglycoBLM A_5-derivatized microbubbles did not. The large size of the microbubbles (~2.5 μm, each estimated to contain ~1.5 × 10^6 ligands[5]) precluded facile internalization following cell-surface binding. As anticipated, under the same conditions, none of the derivatized microbubbles bound to MCF-10A cells (Figure S5).

The mechanism of uptake of the bound BLM disaccharide-Cy5** conjugate was studied by measuring the uptake by MCF-7 cells at 4 °C. The uptake measured at 4 °C after 1 h was less

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than that observed at 37 °C (Figure 8), suggesting that the uptake is ATP-dependent.[13] Thus internalization of the BLM

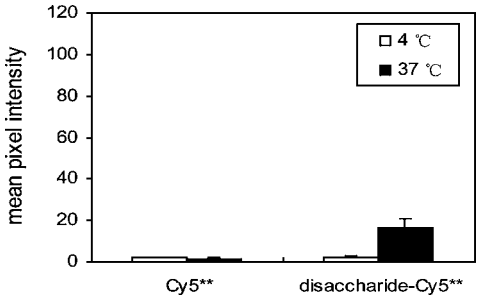

Figure 8. Effect of the incubation temperature on the internalization of BLM disaccharide-Cy5** conjugates in MCF-7 cells. The cells were treated with 50 μM BLM disaccharide-Cy5** or Cy5** at 4 or 37 °C for 1 h, washed with PBS, and fixed with 4% paraformaldehyde. Fluorescence imaging was carried out with a 3 s exposure time.

disaccharide-Cy5** conjugate apparently occurs by a specific uptake mechanism rather than by passive diffusion.

Current cancer therapies are limited by side effects resulting from a lack of tumor cell selectivity. Accordingly, there is great interest in molecules that can distinguish between healthy and cancerous tissue. A few such compounds, typically polypeptides or polysaccharides, have been described;[14] however, their sizes can limit their pharmacologic utility. The prospects for successful utilization in classic therapeutic strategies[15,16] would increase dramatically if the sizes of these tumor-targeting molecules could be reduced. For example, smaller compounds tend to be less immunogenic, easier and less expensive to prepare, and more readily amenable to use in prodrug conjugates or as diagnostic agents.

In summary, we have identified an uncomplicated disaccharide that selectively targets a variety of cultured tumor cells.[17] The unique character of this disaccharide at the levels of simplicity and selectivity argue for its potential utility. For example, the present findings should facilitate the identification of the cell-surface target for bleomycin and the mechanism(s) of cellular uptake. Whatever the cellular target, the implications of these findings for cancer diagnosis and therapy seem clear. Utilization of this sugar as a tumor-imaging agent in ultrasound or radionuclide imaging can be envisioned. Also, incorporation of this moiety into preexisting or novel cancer therapeutics could in principle increase drug delivery directly to the tumor cells, lowering the necessary dosage and potentially reducing side effects.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures for the synthesis and characterization of the BLM, deglycoBLM, and BLM disaccharide dye conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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(9) Although the scope of dyes that can support selective tumor cell targeting by BLM or its disaccharide is presently undefined, we found that conjugates prepared using Atto 647 (see: Kolmakov, K.; Belov, V. N.; Wurm, C. A.; Harke, B.; Leutenegger, M.; Eggeling, C.; Hell, S. W. *Eur. J. Org. Chem.***2010**, 3593) gave results comparable to those obtained with the Cy5** conjugates.

(10) Cu(II)·BLM A5 and Cu(II)·deglycoBLM A_5 were treated with the NHS ester of Cy5**, in analogy with attachment of BLM A_5 to a solid support (see: Abraham, A. T.; Zhou, X.; Hecht, S. M. *J. Am. Chem. Soc.* **2001**, *123*, 5167). The conjugates were purified by C_{18} reversed-phase HPLC (99:1 0.1% aq TFA/CH_3CN → 35:65 0.1% aq TFA/CH_3CN), treated with EDTA to remove Cu^{2+}, and then again purified by reversed-phase HPLC using the same method (Figure S1).

(11) Selective targeting of the BLM-Cy5** and BLM disaccharide-Cy5** conjugates was also observed using A498 human kidney cancer cells (but not CCD 1105 KIDTr kidney cells) and A549 lung carcinoma cells (but not WI-38 lung cells) (Figure S4).

(12) Alternatively, the selectivity may be due to a metabolic process specific to tumor cells.

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(17) Since the observation of tumor cell targeting/uptake was dependent on a conjugated dye, it is not currently possible to exclude the possibility that the dye was essential for these processes. However, the fact that structurally unrelated dyes conjugated to the disaccharide gave the same effect[9] argues against this possibility, as do the microbubble conjugate data (Figure 7).