J|A|C|S COMMUNICATIONS

Published on Web 02/02/2009

# Carbohydrate Dependent Targeting of Cancer Cells by Bleomycin−Microbubble Conjugates

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The bleomycins (BLM $A_5$, **1**) are clinically used antitumor agents efficacious in the treatment of squamous cell carcinomas and malignant lymphomas.[1] Their antitumor activity is believed to result from their ability to mediate the selective oxidative cleavage of DNA[2] and possibly also RNA.[3]

One property of bleomycin that has been recognized for decades is its ability to target tumors; this has been documented in numerous tumor imaging studies that employed BLMs bound to radionuclides.[4,5] The importance of tumor targeting to the therapeutic efficacy of BLM is underscored by the therapeutic dose of BLM (~5 $\mu$mol), which is quite low relative to the doses of many clinically used agents. Understanding the molecular nature of tumor targeting by BLM would facilitate the synthesis of analogues with improved properties and might also enable the selective delivery of other probes and drugs to tumor cells.

Microbubbles, consisting of a shell enclosing a gas core, are used in ultrasonography as contrast agents and intravascular blood flow tracers. The shell is usually composed of albumin, carbohydrates, or lipids. The gas core is usually air, nitrogen, or a perfluorocarbon.[6] While individual microbubbles may exist in varying sizes, they are usually smaller than red blood cells and their mean diameter is typically within the range 1−4 $\mu$m. This small size permits free flow of the microbubbles through the (micro)circulation. In recent years, microbubbles have been modified with ligands that bind specific receptors expressed by cell types of interest, including inflamed and cancer cells.[7] The majority of ligands to date have been monoclonal antibodies. Presently we describe the conjugation of BLM $A_5$ to microbubbles, demonstrate that the conjugate adheres selectively to cancer cells, and report that the carbohydrate moiety of BLM $A_5$ is required for tumor cell targeting.

The C-terminus of BLM $A_5$ (**1**) was acylated with biotin, as shown in Figure 1, to afford BLM $A_5$−biotin conjugate **2a**.[8] The preparation of BLM $A_5$-conjugated microbubbles was then accomplished by admixture of BLM-biotin **2a** to commercially available microbubbles derivatized with streptavidin.[9] A schematic representation of the resulting targeted microbubble is shown in Figure 2.

MCF-7 human breast carcinoma cells were grown on sterile glass coverslips (40 mm) and incubated at 37 °C until they reached 40−60% confluency in a 5% fetal bovine serum/RPMI 1640 medium. They were then assembled into a parallel plate flow chamber with a constant temperature maintained at 37 °C. The suspension containing the BLM−microbubble conjugate was introduced into the flow chamber at a controlled rate of 0.01 mL/min, and possible attachment of the microbubbles to the cultured MCF-7 cells was imaged using an inverted microscope fitted with a camera. As shown in Figure 3, the BLM−microbubble conjugate adhered to the cultured MCF-7 cells, but microbubbles lacking attached BLM **2a** failed to adhere to the cultured MCF-7 cells.

To judge the ability of the BLM $A_5$−microbubble conjugate to bind selectively to tumor cells, the experiment was repeated using

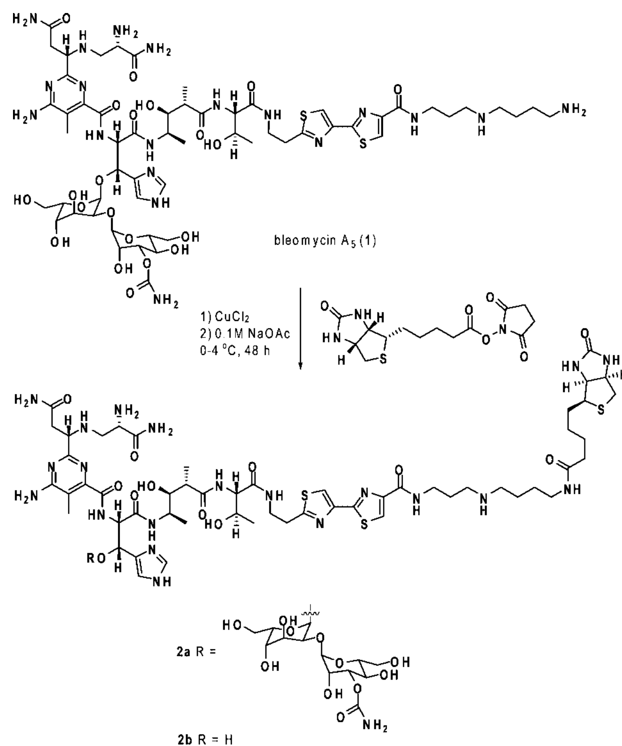


***Figure 1.*** Synthesis of biotinylated derivatives of bleomycin $A_5$ and deglycobleomycin $A_5$.

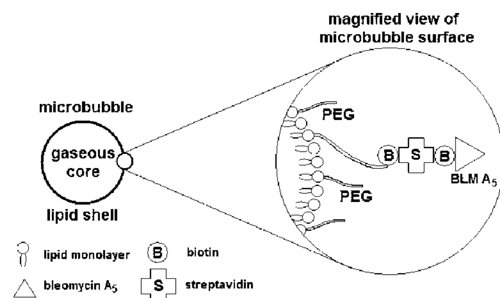


***Figure 2.*** Constitution of the bleomycin $A_5$−microbubble conjugate.

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10.1021/ja8091104 CCC: $40.75 © 2009 American Chemical Society

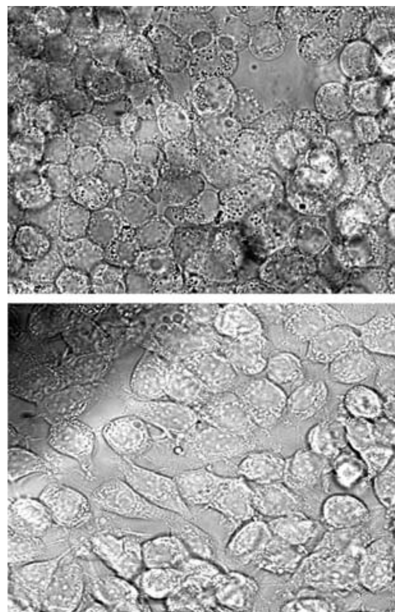

***Figure 3.*** Monolayers of cultured MCF-7 breast cancer cells treated with the BLM $A_5$−microbubble conjugate (top) or with underivatized microbubbles (bottom).

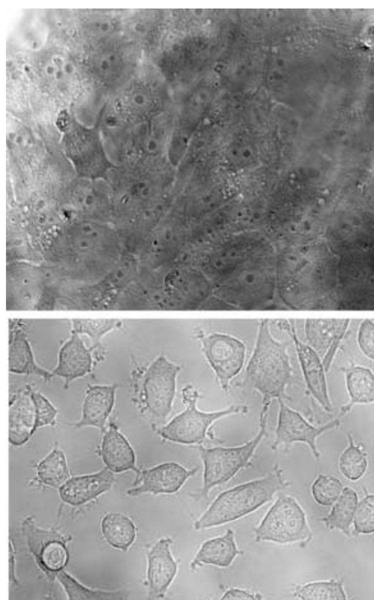

***Figure 4.*** Monolayers of cultured MCF-10A breast cells treated with the BLM $A_5$−microbubble conjugate (top) and of cultured MCF-7 cells treated with the deglyco BLM $A_5$−microbubble conjugate (bottom).

the "normal" breast cell line MCF-10A. As shown in Figure 4, there was no adhesion of the BLM $A_5$ microbubble−conjugate to the cultured MCF-10A cells. Thus the BLM $A_5$−microbubble conjugate adhered selectively to the cancer cell line.[10]

The realization of a system for monitoring the selective interaction of BLM $A_5$ with tumor cells provides a vehicle for evaluating those structural elements of BLM that contribute to tumor cell targeting. Accordingly, biotinylated deglyco BLM $A_5$ (**2b**) was prepared to permit a direct assessment of the role of the carbohydrate moiety of BLM in tumor cell targeting. BLM **2b** was conjugated to the same microbubbles and employed in experiments designed to test adhesion to cultured MCF-7 and MCF-10A cells. As shown in Figure 4 for the cultured MCF-7 cells, no adhesion of the deglyco BLM $A_5$−microbubble conjugate to either cell line was observed.

The foregoing experiments enable direct, visual observation of the binding of a BLM conjugate to cultured tumor cells and demonstrate that BLM adheres selectively to tumor cells, as compared with the same "normal" cells. Also established is the requirement for the carbohydrate moiety of BLM to support tumor cell targeting. Not yet resolved by these experiments is the related question of the sufficiency of the BLM carbohydrate moiety to support tumor cell targeting, a finding that could enable novel strategies for selective drug delivery and antitumor therapy.

**Acknowledgment.** K.S.T. was supported by R25GM071798 from the National Institute of General Medical Sciences.

**Supporting Information Available:** Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Cu(II)·BLM $A_5$ was treated with the *N*-hydroxysuccinmide ester of biotin, as outlined in Figure 1, in analogy with attachment of BLM $A_5$ to a solid support (Abraham, A. T.; Zhou, X.; Hecht, S. M. *J. Am. Chem. Soc.* **2001**, *123*, 5167). Following the removal of $Cu^{2+}$ by treatment with EDTA, the BLM $A_5$−biotin conjugate (**2a**) was purified by $C_{18}$ reversed phase HPLC.

(9) Targestar$^B$ Ultrasound Contrast Agent, containing streptavidin attached through a PEG spacer, was used for attachment of BLM **2a** and deglyco BLM **2b**. These microbubbles have a consistent mean diameter of ~2.5 $\mu$m with $\sim 1.5 \times 10^6$ streptavidin molecules/microbubble. The density of streptavidin was about 30 times greater than that of antibody in antibody-derivatized microbubbles (Talkalkar, A. M.; Klibanov, A. L.; Rychak, J. J.; Lindner, J. R.; Ley, K. *J. Controlled Release* **2004**, *96*, 473). A 1-mL aqueous suspension of $1.5 \times 10^8$ streptavidin-derivatized microbubbles was treated with 50 $\mu$L of aq. 500 $\mu$M BLM **2a** or **2b**. The combined solution was agitated gently for 20 min, then free BLM was removed by repeated centrifugation (400 × *g*, 3 min, 10 °C) and washing with buffer.

(10) Analogous experiments were carried out using other sets of cultured cancer and normal cells. Selective adhesion of the BLM $A_5$−microbubble conjugate was observed using SW480 colon carcinoma cells (but not CRL-1541 colon cells), DU145 prostate carcinoma cells (but not CRL-2221 prostate cells), and A549 lung carcinoma cells (but not CCL-75 lung cells).

JA8091104