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Monoclonal antibody mediated intracellular targeting of tallysomycin S_{10b}

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Abstract—The potency of tallysomycin S_{10b} (TLM S_{10b}) an analogue bleomycin was enhanced by up to 875-fold when it was conjugated to the internalizing antibody BR96. Attachment to the antibody is achieved via a Cathepsin B cleavable linker. The enhancement in potency is believed to be a result of cellular uptake of the conjugate upon antigen binding followed by rapid release of the drug inside the lysosome. This method provides a novel approach for increasing the potency and therapeutic index of nominally moderately-active cytotoxic agents.
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Monoclonal antibodies (mAbs) hold great promise as drug delivery agents in the treatment of cancer.[1] The goals in this area are twofold, reduce exposure of sensitive organs and tissue to the drug (referred to as negative targeting) and enhance the exposure of the tumor and metastatic foci (referred to as positive targeting). The negative and positive aspects of antibody targeting combined yield an increase in the apparent therapeutic index of the targeted drug. This is an especially useful technique for reducing the toxicity associated with potent cytotoxic agents. An example of the state of the art in this area is the immunoconjugate BMS-182248. This agent is composed of the anticancer agent doxorubicin (Dox) linked to the tumor selective antibody BR96. BR96 recognizes a Le^y related antigen primarily associated with carcinomas of the colon, breast, ovary, and lung but not normal tissues.[2] As such BMS-182248 is capable of completely curing xenotransplanted tumors in mice and rats, syngenic tumors in rats and is active against various models of disseminated cancer.[3] More importantly, animal models show that the heart, which is sensitive to doxorubicin, is spared the full dose of the drug when delivered as the conjugate.

In the current disclosure we describe one additional feature of certain antibody based antitumor agents beyond the negative and positive targeting aspects mentioned above that has yet to be exploited namely, mAb mediated intracellular targeting.[4] The idea is to use an internalizing antibody to increase the potency of a drug, which is poorly taken up by the cell but, which has high intrinsic activity against an intracellular target. In this case the drug by itself has a high maximum tolerated dose due to poor uptake by normal cells so that negative targeting is built in a priori provided that the carrier is not taken up by normal cells.

The cornerstone of this approach is BR96, which is endocytosed upon binding its antigen unlike most antibodies, which normally remain bound to the cell surface.[5] This has allowed us to devise a method for selective drug release at the tumor by taking advantage of the proteolytic environment of the lysosome (or endosome) to effect hydrolysis of the linker. Thus we have found that dipeptide based linkers allow for selective release of the drug in the lysosomal environment and that an optimal linker is the dipeptide, Phe-Lys, which is recognized and cleaved by Cathepsin B.[6] In order to insure that the drug does not interfere with enzyme cleavage of the linker a *p*-aminobenzyl alcohol (PABC) spacer is inserted between the peptide linker and the drug. The PABC moiety is designed to hydrolytically decompose upon enzymatic deacylation releasing

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the free drug.[7] Cathepsin B is a cysteine protease that is present in all mammalian cell lysosomes, does not differ very much from species-to-species, and is rarely found extracellularly except in the vicinity of certain pathological conditions such as metastatic sites, or in the areas of tissue breakdown in rheumatoid arthritis.[8,9] BR96 conjugates employing this linker and cytotoxic drugs such as doxorubicin, mitomycin C, and camptothecin are potent and selective antitumor agents.[10,11]

The drug selected for this investigation is tallysomycin S_{10b} (TLM S_{10b}), which belongs to the same family of cytotoxic agents as bleomycin. The mechanism of action for these agents involves Fe(II) mediated cleavage of DNA.[12] In preclinical models TLM S_{10b} exhibited activity similar to that of bleomycin against a variety of murine tumors (P388 leukemia, B16 melanoma, Lewis lung carcinoma, and Madison 109 lung carcinoma).[13] Unfortunately, in two phase II clinical trials it yielded no response in previously treated head and neck cancer patients and colorectal cancer patients while at the same time it exhibited a toxicity spectrum similar to bleomycin.[14] Therefore, TLM S_{10b} represents a perfect candidate for intracellular targeting since an improvement in potency will likely increase its therapeutic index.[15]

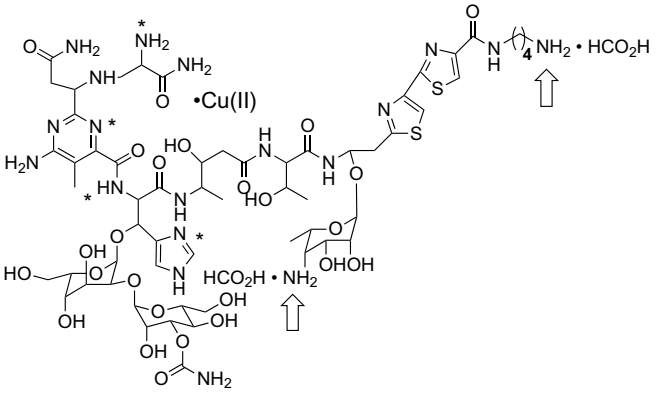
It is believed that the potency of this family of cytotoxic agents is attenuated by poor cell penetration thus restricting access of the drug to its target. A recent publication provides support for this notion by showing that the activity of bleomycin can be increased by up to 10,000 fold by using electroporation to enhance cellular uptake.[16] It is assumed that the activity of TLM S_{10b} would be enhanced to the same degree under similar conditions. One additional feature of TLM S_{10b} is that it is water soluble, thus reducing the possible formation of conjugate aggregation at high drug/mAb ratios.

In the current publication we illustrate the synthesis of a TLM S_{10b} BR96 conjugate and demonstrate that the resulting conjugate yields a substantial increase in the potency for the attached drug presumably by mAb mediated intracellular targeting (Fig. 1).

The peptide linker was assembled as shown in Scheme 1. The commercially available succinimide ester of Fmoc-protected phenylalanine (**2**) was coupled to Boc-protected lysine under basic conditions. The dipeptide **4** was then functionalized at the C- and N-termini with the *p*-aminobenzyl alcohol spacer and maleimidocaproic acid to yield **7**. Following this the linker was activated toward coupling with TLM S_{10b} as the corresponding *p*-nitrophenyl carbonate **8**.

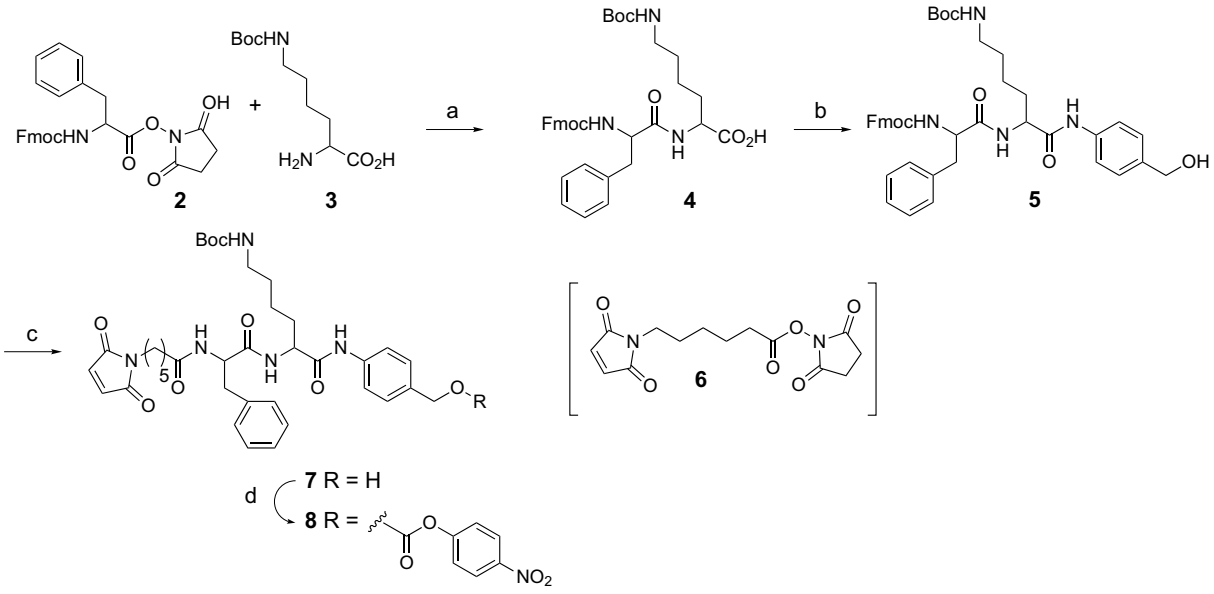
The Cu(II) complex of the bis-formate salt of TLM S_{10b} (TLM S_{10b}·Cu(II)·2HCO_2H) has two potential sites for linker attachment as indicated in structure **1**. The remaining amino groups, highlighted with asterisks, are involved in metal complexation and are thus unreactive toward acylating agents. In fact, previous reports have established that the amino group of the 1,4-diaminobutane side chain of this compound is the primary reaction site for coupling with acylating agents.[17] Nonetheless, regioselective linker attachment is not an issue for our purposes since this bond is cleaved at the tumor.

In order to avoid over acylation linker attachment conditions were first examined using di-*tert*-butyl dicarbonate (Boc_2O). Prior to reaction the formate salt was converted to the corresponding HCl salt by treatment with the ion exchange resin AG® 2-X8.[18] The resulting salt, TLM S_{10b}·Cu(II)·2HCl, was treated with 1.0 equiv of Boc_2O and 2.0 equiv of Hünigs base. Two products were observed by HPLC and MS consistent with mono- and bis-acylation compounds Boc-TLM S_{10b}·Cu(II)·HCl (**9a**) and $(Boc)_2$·TLM S_{10b}·Cu(II) (**9b**). In addition, ion exchange chromatography (Econo-Pac S, 0.5–1.0 M HCO_2NH_4) showed three peaks eluting in order and having the approximate statistical ratio one would expect for a mixture of mono-acylated, bis-acylated and unreacted TLM S_{10b}. Fortunately it was found that formation of the bis-acylated product could be suppressed by using 0.5 equiv of Boc_2O. Under these conditions the Boc-TLM S_{10b}·Cu(II)·HCl was favored by 5:1 over $(Boc)_2$·TLM S_{10b}·Cu(II) (Scheme 2).

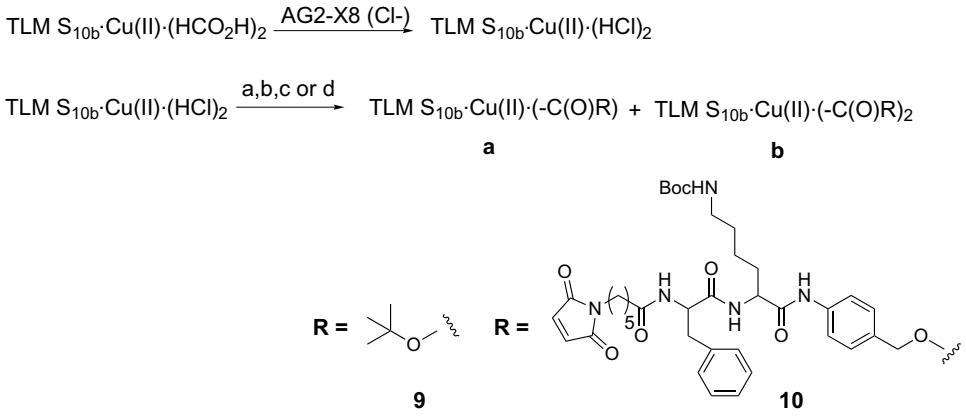


1 Tallysomycin S_{10b}, Cu(II), $(HCO_2H)_2$

Figure 1. Tallysomycin S_{10b} Cu(II)$(CHCO_2H)_2$ (*Cu(II) binding atoms).



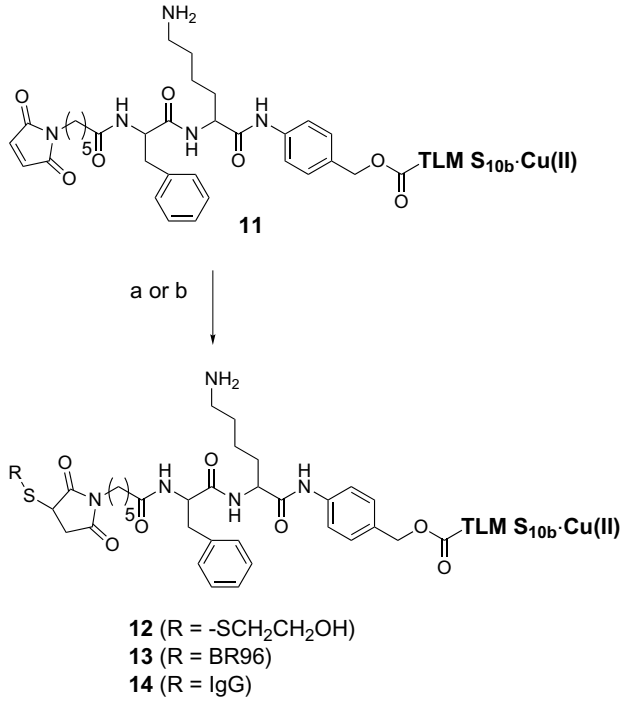
Scheme 1. Regents and conditions: (a) $NaHCO_3$, 1,2-dimethoxyethane, H_2O, (b) di-*tert*-butyl dicarbonate, *p*-aminobenzyl alcohol, pyridine, DMF, (c) (1) Et_2NH, CH_2Cl_2, (2) **6**, *i*-Pr_2NEt, CH_2Cl_2, (d) bis-(*p*-nitrophenyl)-carbonate, *i*-Pr_2NEt, DMF.



Scheme 2. Reagents and conditions: (a) 1.0 equiv $(Boc)_2O$, 2.0 equiv *i*-Pr_2NEt, (b) 0.5 equiv $(Boc)_2O$, 2.0 equiv *i*-Pr_2NEt, (c) 1.0 equiv **8**, 2.0 equiv *i*-Pr_2NEt, (d) 0.5 equiv **8**, 2.0 equiv *i*-Pr_2NEt, 1 h.

When peptide **8** was used in place of Boc_2O the results were qualitatively the same. Two products, **10a** (mono-acylated) and **10b** (bis-acylated), were obtained when 1.0 equiv of peptide was used in the reaction while mainly **10a** was produced when 0.5 equiv of **8** were used. It is important that the reaction time be restricted to 1 h, since longer reaction times lead to formation of a third product having a molecular weight consistent with the addition of two equiv of TLM S_{10b}·Cu(II). Nonetheless, after selective acylation, the crude reaction mixture consisted mainly of the mono-acylated product and unreacted TLM S_{10b}·Cu(II) as indicated by HPLC and MS. The desired product was easily isolated by preparative HPLC (C_{18}, 60:40 CH_3OH/H_2O 0.05 M HCO_2NH_4). Following isolation, **10a** was treated with TFA to effect protecting group removal to yield compound **11**.

Since Cu(II) is capable of oxidizing thiols to disulfides we decided to model the thioether forming conjugation step prior to carrying out the conjugation to BR96. When mercaptoethanol was used as the nucleophilic reagent, thiol attack at the maleimide to form the corresponding succinimide could be selectively carried out, to yield **12** without interference from Cu(II). Conjugation to BR96 **11** was carried out using our standard method. Selective disulfide bond reduction of BR96 yielded 8 conjugatable thiol groups. Subsequent reaction with 8 equiv of peptide **11** (pH 6.0 PBS) gave the desired conjugate **13** having a TLM S_{10b}·Cu(II)/BR96 ratio of 9.5 as determined by second derivative UV-spectroscopy. Size exclusion chromatography (SEC) of the conjugate showed only one peak eluting at 9.7 min (1.0 mL/min, 0.1 M KH_2PO_4, SEC-250 (Biorad)) and having the same time as free BR96. UV analysis of this peak indicated an increase in the absorption at $\lambda = 293$ nm relative to $\lambda = 280$ for the conjugate compared to the same 293 nm/280 nm absorption ratio for the free mAb. Co-injection of BR96 and **11** gave two



Scheme 3. Reagents and conditions: (a) 1.2 equiv $HS(CH_2)_2OH$, CH_3OH, (b) (1) 8 equiv DTT, 1 equiv BR96 (or IgG), (2) 8 equiv **11**.

peaks one at 9.7 min and the other at 14.9 min suggesting that the conjugate, which shows only one peak is free of unreacted peptide **11**. Further analysis of the conjugate using size exclusion chromatography indicated $\leqslant 1\%$ protein aggregation. Peptide **11** was likewise conjugated with a nontumor cell recognizing IgG to provide a reference agent (**14**) for cell culture assays (Scheme 3).

Prior to evaluating the potential utility of **13** as an antitumor agent we set out to determine the Cathepsin B cleavage rate for the release of TLM S_{10b} from the Phe-Lys linker. Incubation of **12** with Cathepsin B (pH 5, 37 °C) resulted in rapid release of the drug ($t_{1/2} = 10$ min) while the no release of drug was observed when **13** was incubated at pH 5 (37 °C) for up to 24 h in the absence of Cathepsin B. This suggests that the resulting conjugate should be highly selective for Cathepsin B mediated drug release.

The activity of the BR96- and IgG-conjugates and TLM S_{10b} were evaluated against a panel of cell lines having varying levels of BR96 antigen expression with the corresponding results shown in Table 1. LS174T (human colon adenocarcinoma) and L2987 (human lung adenocarcinoma) show moderate expression of the BR96 antigen while H3396 (human breast adenocarcinoma) and MCF7 (human breast adenocarcinoma) are high expressers (cf. Ref. 2). The IC_{50}'s reported for the conjugates in the table are calculated based on the number of moles of TLM S_{10b} delivered in order to allow evaluation of the potency enhancing properties of BR96 conjugation. As can be seen, free TLM S_{10b} is only modestly active against the cell lines compared to doxorubicin ($IC_{50} = 0.3$, 0.3, 0.2, and 0.5 μM against

Table 1. Activity of TLM S_{10b} conjugates against cell lines with varying BR96 expression

Cell line	IC_{50} μM equivalent of TLM S_{10b}·Cu(II)		
	1	**13**	**14**
LS174T	2	0.04	3
L2987	0.5	0.04	4
H3396	7	0.008	2
MCF7	2	0.006	0.8

LS174t, L2987, H3396, and MCF7, respectively), which was used as a reference agent. In contrast the potency of the drug is enhanced by up to 875-fold when delivered as the corresponding BR96 conjugate. The data indicate that the potency enhancement is antigen dependent since the extent of the potency enhancement and the net IC_{50} are related to the antigen expression level. In addition, the corresponding IgG conjugate does not yield an increase in activity suggesting the antigen binding and internalization into the cell are required as well.

It is interesting to note that for TLM S_{10b} conjugation to the nonbonding mAb (IgG) yielded activity equivalent to the free drug. This is in contrast to our previous work with other drugs, which showed a significant reduction in activity when bound to IgG. In order to explain this apparent discrepancy we offer three possible explanations. First, the conjugate might contain free drug, but this seems unlikely since the conjugates were extensively purified in order to remove free TLM S_{10b} and no free drug was detected prior to assaying the conjugate. Second, the activity of the IgG-conjugate and TLM S_{10b} (at 2 h) might be due to extracellular activation of the redox pathway associated with the activity of the parent drug, which somehow harms the cellular membrane. Literature reports have provided evidence for cell membrane destruction mediated by BLM conjugated to albumin or nonbinding IgG.[19] Last, the conjugate might be internalized into the cell through a TLM S_{10b} binding site located on the outside of the cell. Some evidence exists to suggest that such a site exists for bleomycin.[20] Regardless, of the mechanism the results achieved in the current study show that the activity of BR96-TLM S_{10b} compared to IgG-TLM S_{10b} is sufficiently large to yield a desirable therapeuetic index in animals.

Table 2 compares the key activity parameters of BR96-TLM S_{10b} with our previously published BR96 based immunoconjugates. As can be seen, the TLM S_{10b} conjugate is an order of magnitude more potent than BMS-182248 and 2–3-times more active than our previous peptide linked immunoconjugates despite the fact that the apparent activity of parent drug is an order a magnitude weaker than doxorubicin and camptothecin (CPT). This suggests that the intrinsic activity of the TLM S_{10b} is actually higher than doxorubicin and camptothecin and is consistent with its proposed mechanism of cytotoxicity, which involves catalytic generation of radical species at the site of action. Moreover the potency enhancing effects of conjugation are higher for TLM S_{10b} than for doxorubicin and CPT as expected for a drug, which poorly internalized in the free state. The immunoselective activity of BR96-TLM

Table 2. Summary of BR96-TLM S_{10b} conjugate (**13**) compared to conjugates of Dox and CPT

Experiment[a]	Conjugate[b]			
	BMS-182248	BR96-F-K-Dox	BR96-F-K-CPT	BR96-F-K-TLM S_{10b} (**13**)
IC_{50} L2987 (μM)	0.63	0.15	0.1	0.04
IC_{50} drug/conj.	0.1	1	4	13
IC_{50} IgG/BR96	17	80	30	100

[a] Experiments: IC_{50} L2987; IC_{50} of the immunoconjugate against the L2987 cell line; IC_{50} drug/conjugate; ratio of the parent drug IC_{50} divided by the IC_{50} of the immunoconjugate; IC_{50} IgG/BR96; ratio of the IC_{50} obtained for the drug conjugated to the nonspecific IgG antibody divided by the IC_{50} of the drug conjugated to BR96.

[b] Conjugates: BMS-182248; doxorubicin conjugated to BR96 via a hydrazone linker; BR96-F-K-Dox; doxorubicin conjugated to BR96 via the cathepsin B cleavable Phe-Lys-PABC peptide linker; BR96-F-K-96; camptothecin conjugated to BR96 via the cathepsin B cleavable Phe-Lys-PABC peptide linker; BR96-F-K-TLM S_{10b}; compound **13** described in the text.

S_{10b} is higher than that of BMS-182248 and is approximately equivalent to that observed for our other peptide linked immunoconjugates. This window is large enough to expect that the immunoconjugate could be dosed at concentrations much higher than the IC_{50} while maintaining TLM S_{10b} exposure below toxic levels.

We have successfully synthesized an immunoconjugate of TLM S_{10b} employing the mAb BR96 and a dipeptide linker designed to release the drug by Cathepsin B catalyzed proteolysis. The parent drug is only moderately active against a panel of tumor cell lines in contrast to the conjugate (**13**), which results in a 13–875-fold increase in potency. This is presumably due to the internalizing properties of BR96, which allows for more efficient uptake of the drug and exposure to its primary target. This provides support for using internalizing mAbs as a vehicle to increase the potency of drugs, which exhibit poor cellular penetration in the free solution.

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