European Journal of Medicinal Chemistry 85 (2014) 139–146

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

"Switch off/switch on" regulation of drug cytotoxicity by conjugation to a cell targeting peptide

Yossi Gilad [a, c], Michael A. Firer [b], Alex Rozovsky [a, c], Elena Ragozin [a], Boris Redko [a, c], Amnon Albeck [c], Gary Gellerman [a, *]

[a] Department of Biological Chemistry, Ariel University, Ariel, 40700, Israel
[b] Department of Chemical Engineering, Ariel University, Ariel, 40700, Israel
[c] The Julius Spokojny Bioorganic Chemistry Laboratory, Department of Chemistry, Bar Ilan University, Ramat Gan, 52900, Israel

ARTICLE INFO

Article history:
Received 26 May 2014
Received in revised form
19 July 2014
Accepted 21 July 2014
Available online 22 July 2014

Keywords:
Amino acid platform
Controlled drug release
Peptide–drug conjugates
Prodrug
Solid phase organic synthesis

ABSTRACT

Bi-nuclear amino acid platforms loaded with various drugs for conjugation to a peptide carrier were synthesized using simple and convenient orthogonally protective solid-phase organic synthesis (SPOS). Each arm of the platform carries a different anticancer agent linked through the same or different functional group, providing discrete chemo- and bio-release profiles for each drug, and also enabling "switch off/switch on" regulation of drug cytotoxicity by conjugation to the platform and to a cell targeting peptide. The versatility of this approach enables efficient production of drug-loaded platforms and determination of favorable drug combinations/modes of linkage for subsequent conjugation to a carrier moiety for targeted cancer cell therapy. The results presented here potentiate the application of amino acid platforms for targeted drug delivery (TDD).

1. Introduction

Targeting drugs through biomolecular carriers with high affinity to receptors on cancer cells can overcome two major problems in anticancer therapy: the lack of target cell specificity of most anticancer drugs and improvement of their toxicology [1]. Although many targeted drug delivery approaches are being tested, the linkage of several and different drugs to a single carrier molecule might further enhance their therapeutic efficacy, particularly if the drugs are engineered for variable time release.

Over the past two decades carrier–drug conjugates have been developed for target cell delivery of potent anticancer drugs with the aim of eliminating the morbidity-causing non-specific side effects common to conventional chemotherapy. Usually the carriers are macromolecules such as monoclonal antibodies and other proteins, or smaller molecular carriers like polynucleotide segments and peptides [2].

Despite advances in these areas, the biomolecular–drug conjugates reported so far are limited to carry one drug type, although drug conjugation chemistry is well elaborated [3]. This point is exemplified by antibody–drug conjugates (ADC): (a) Gemtuzumab ozogamicin (Mylotag), contains Gemtuzumab, a recombinant humanized IgG4 monoclonal antibody (mAb) targeting the cell membrane protein CD33, which is linked to several copies of calicheamycin; (b) Vedotin (Adcetris), a chimeric antibody directed to CD30, is conjugated to the antimitotic compound monomethyl auristatin E; (c) Trastuzumab emtansine (T-DM1) is a conjugate of the well-studied antibody Trastuzumab (Herceptin) that targets the HER-2 cell surface protein, and maytansine derivative DM1; (d) Inotuzumab ozogamicin, a conjugate of an IgG4 monoclonal antibody targeting the CD22 antigen linked to calicheamicin. Despite these advances, ADCs still have several important limitations including target cell specificity, conjugation chemistry, tumor penetration and product heterogeneity [4]. Peptide–drug conjugates (PDCs) could overcome many, if not all, of these shortcomings as we have described elsewhere [5], although they are yet to reach the clinic. Several recent publications presented biologically active peptide–drug conjugates, manifesting improvement of drug-like features of the linked drugs. Sherz and coworkers reported on selective accumulation and prolonged retention of the RGD analog c(RGDfK) linked to fluorescent bacteriochlorophyll derivative in the tumor necrotic domain in MDA-MB-231-RFP bearing mice. This construct enables early detection of tumor growth and foster prognosis and the development of novel modes of treatment [6].

* Corresponding author.
E-mail address: garyg@ariel.ac.il (G. Gellerman).

http://dx.doi.org/10.1016/j.ejmech.2014.07.073
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Kostenich et al. developed accurate detection of small-cell lung cancer (SCLC) with fluorescence-based imaging by preparing novel backbone cyclic somatostatin analogs conjugated with fluorescein and rhodamine [7]. We recently reported an increased efficacy of multidose chlorambucil–peptide conjugates on murine leukemic B cells through receptor-mediated endocytosis [8]. The most clinically advanced peptide–drug-conjugate is GRN1005, an angiopeptin-2-paclitaxol conjugate that targets lipoprotein receptor protein-1, a cell surface molecule overexpressed on solid tumor cells. The conjugate is under clinical assessment for treatment of advanced solid tumors, in particular in patients with brain metastases [9]. Although diverse, the above examples share one common feature: they all describe conjugation of just one drug molecule (or a few copies of the same drug) to the carrier. Nanoparticle systems represent an approach that could be utilized for multiple drug delivery [10]. Liposomes were demonstrated to be efficient vehicles for the delivery of two drugs with very different solubility properties [11]. However, they suffer various limitations such as stability and loading efficiency [12]. Cross-linked multilamellar liposomal vesicles were studied in order to address those limitations [13]. Thus, there are no examples yet of well-defined unimolecular platforms containing a few different drugs – a cocktail.

The chemistry of carrier-drug attachment has received much attention. Important parameters include selection of a linker attachment site that retains carrier activity, linker length and composition, and the design of drug analogs for attachment to the linker [14]. For ADCs, two methods are now commonly used for conjugating drugs to antibodies: alkylation of reduced inter chain cysteine disulfides through a non-cleavable maleimido linker and acylation of lysine residues by cleavable linear amino acids [15]. Cathepsin-cleavable linkers are also utilized (for example Val-Cit, or Phe-Lys) bound to self-emulative moiety PABA (p-aminobenzyl alcohol), enabling selective drug release in cancer cells [14c]. Spacers are usually essential extensions of the drug linkage and are responsible for avoiding the shielding of the active site of the antibody as well as improving solubility properties of ADCs (for example by the use of polyethylene glycol [16]).

We have previously demonstrated that Ligand Drug Conjugates (LDCs) can be successfully employed for the targeted delivery of drugs and toxins to receptor-positive murine leukemic cells [8,17]. In particular, the use of multifunctional dendrone linkers that bear several covalently bound DNA alkylating Chlorambucil molecules to one peptide carrier have enhanced efficacy of inhibition of target cancer cell growth [8]. Based on these results, we envision substantial therapeutic potential for drug–carrier conjugates that consist of several different cytotoxic compounds linked via biodegradable linkages to Multifunctional Amino Acid Platforms (MAAP, Fig. 1). The rationale behind this concept is based on the following: (1) Amino acids are not toxic *in vivo*, (2) Prodrugs can be prepared with enzymatically cleavable moieties such as esters, carbamates, carbonates; (3) Drugs can be linked to MAAPs through these same types of moieties; (4) Linker-tagged drugs will be enzymatically cleaved *in vivo* in the same manner as prodrugs; (5) The release profile of the drugs will depend on the nature of the linker, linking moiety and drug itself. By optimizing these parameters in the design of loaded MAAPs that are subsequently conjugated to a target specific carrier, we hope to release the payload specifically in the target cancer cells and thereby avoid exposure of benign tissues to the cytotoxic treatment [18]. We anticipate that MAAP technology can be integrated into the rational design and application of targeted drug delivery strategies and ultimately to a broader basket of more effective therapies for cancer patients.

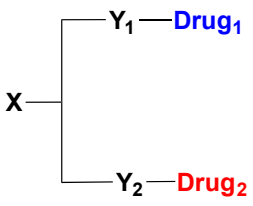


Fig. 1. General structure of multifunctional amino acid platform (MAAP) for loading of two drugs.

In this communication we describe the synthesis and initial evaluation of first generation (G1) MAAPs (Fig. 1), linked to known chemotherapy drugs, as well as the controllable release of the payload. Several fundamental tasks were achieved during this research: (1) Development of a novel synthetic protocol of orthogonally protective SPOS for the facile synthesis of heterogeneous L-Lys, L-Ser and L-Tyr based G_1 MAAPs with selective drug loading capabilities; (2) Measurements of chemostability at various pHs and bio-stability in mouse liver homogenate for linker-containing drugs and loaded MAAPs; (3) Screening the activity of free drugs *vs* peptide carrier-conjugated drugs on cancer cells *in vitro*.

2. Results and discussion

2.1. Synthesis

Initially, we loaded MAAPs with four known anticancer drugs and drug candidates and one previously reported 9-aminoacridine anticancer compound YG-42 [19] (Fig. 2). These agents act through different cellular pathways, therefore their combination on MAAPs present a model for delivery of chemotherapeutic "cocktails".

First, the DNA-Topo II intercalating inhibitor Azatoxin (AZA) [20], the DNA-Topo I intercalating inhibitor Camptothecin (CAMP) and the synthetic corticosteroid drug Prednisone (PRED) were functionalized for linkage to MAAP according to Scheme 1, forming activated carbonate functionalities through the free phenolic or aliphatic OH. Chlorambucil (CLB), a well known DNA mustard alkylator, and YG-42 were used without modification due to the presence of an existing carboxylic group suitable for linkage. The reactions of CAMP, AZA and PRED with $pNO_2PhOCOCl$ [21], when carried out under standard conditions in heated pyridine or in DCM at room temperature in the presence of excess of DMAP, afforded the preactivated drugs **1**–**3** respectively. These building blocks were used after standard workup procedure without any further purification.

Acid-sensitive Cl-Trt polystyrene resin was chosen as solid support for the synthesis of loaded G_1 MAAPs for two reasons: (1) The resin allows utilization of mild acidic cleavage conditions for avoiding decomposition of the linked drugs; (2) The system yields cleavage products of loaded MAAPs with a free carboxylic group that can act as an anchor point for conjugation chemistry to a carrier.

Next, orthogonally protected Fmoc-(L)-Lys(Alloc)-OH was loaded on to the resin using standard methods (DIEA, DMF then MeOH for capping) [22]. After Fmoc release (20% piperidine/DMF, 2 × 20 min), the pre-made active ester of CLB (PyBoP, DMF) was reacted with the deprotected L-Lys(Alloc) peptidyl residue at rt for 90 min, yielding the adduct **6**. This compound was further deprotected to **5** by homogenous catalysis using $Pd(PPh_3)_4$ in the presence of barbituric acid ligand. At this point, adduct **5** was divided into three parts, and each one was reacted with preactivated YG-42, AZA or CAMP (see Scheme 2), yielding, after mild acidic cleavage (3%TFA in DCM), the corresponding **4a**–**c** compounds in good yields and purity (see Supplementary Information). In particular,

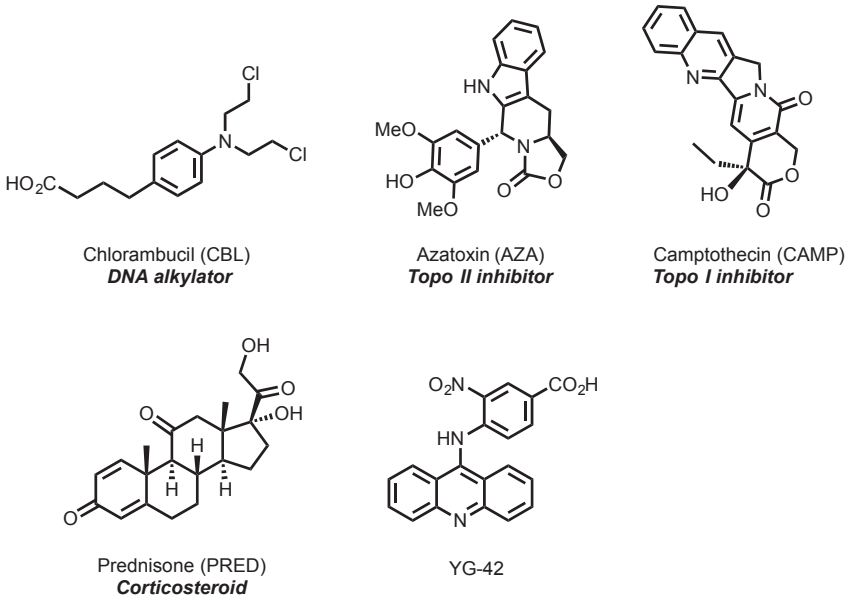
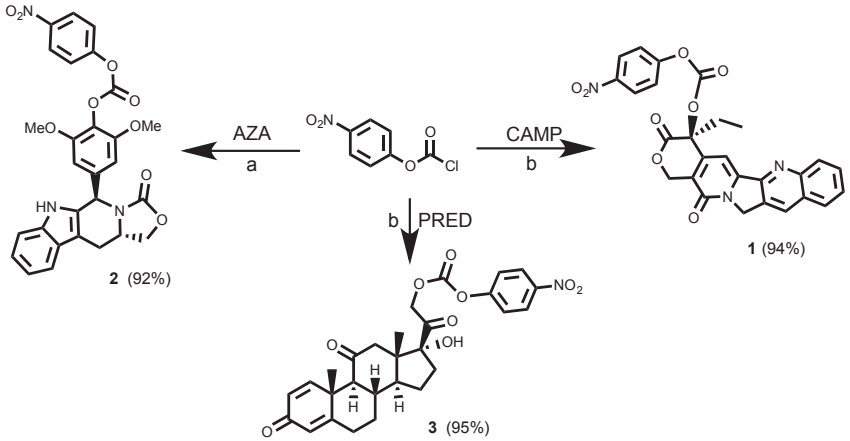


Fig. 2. Anticancer substances acting through different oncogenic mechanisms.

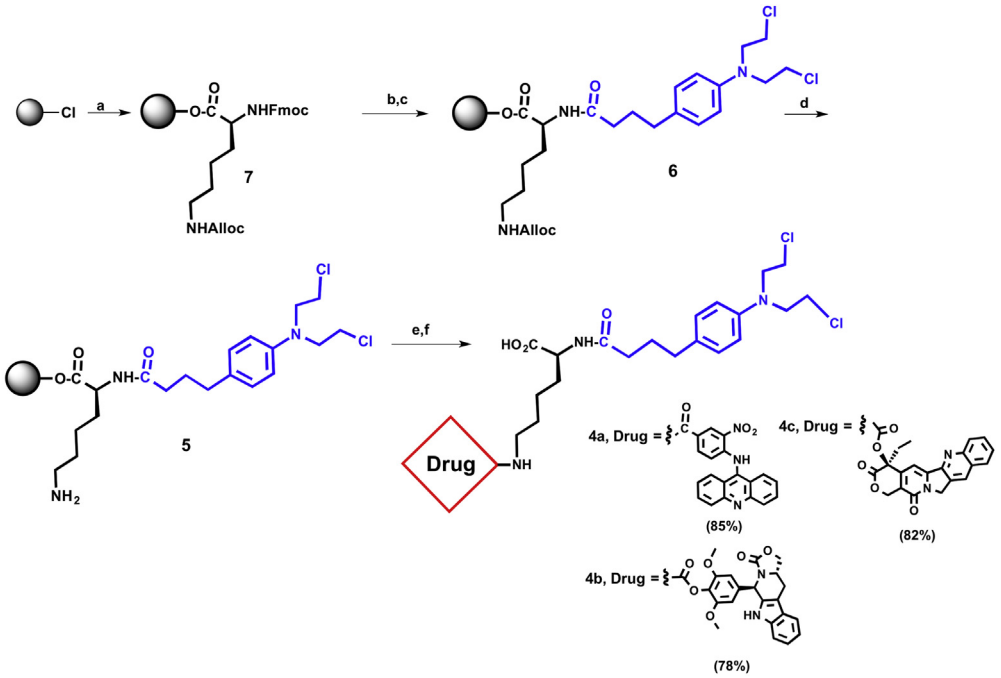
pharmaceutically active components YG-42 and CLB in **4a** are linked to the L-Lys platform through amide bonds, while AZA and CAMP in **4b**,**c** are linked through phenolic and aliphatic carbamates, respectively.

In the following step, L-serine and L-tyrosine were employed in the synthesis of G_1 MAAPs. These amino acids have two different functionalities for drug linkage: α-amine and a primary or a phenolic OH respectively on the side chain. The purpose of this stage was to extend the linkage repertoire for coupling of two bioagents, and to measure the drug release rate *vs* linkage mode. Initially, Fmoc-(L)-Ser-OH was loaded onto Cl-Trt resin (Scheme 3) using the same procedure as for **7**. Notably, reacting the α-amino protected Ser without side chain protection did not disrupt the regioselective loading toward **12a**, most probably due to the reduced nucleophilicity of OH under the coupling conditions. This significantly simplified the synthesis by avoiding the use of a second orthogonally protected group. Subsequently, after removal of Fmoc under standard conditions, the premade active ester of CLB (5 eq) was successfully coupled in two steps to the deprotected L-Ser peptidyl **11a** (EDC/DMAP), affording after cleavage the desired G_1 MAAP **8**, that bears two molecules of CLB linked to L-Ser by two different biodegradable moieties, namely amide at the α-amine and ester on the side chain hydroxyl. Being a hetero-tethering amino acid, L-Ser was found useful for loading of another combination of drugs through a different set of biodegradable moieties. For demonstrating this, **12a** first underwent esterification with active ester of CLB (EDC, DMAP) yielding **13**, and then Fmoc removal afforded deprotected Ser(CLB) residue on the solid support. Importantly, no intramolecular migration of CLB from the side chain to the α-amine was observed by HPLC and NMR analysis (data not shown). The resulting peptidyl was coupled with the activated carbonate derivative of PRED **3** (DIEA, DMF, rt, 90 min; twice) to give, after subsequent cleavage, the desired MAAP **10** in good yield and purity (see Supplementary Information). In the resulting MAAP **10**, CLB is linked through a biodegradable ester while PRED is linked through a biodegradable aliphatic carbamate.



(a) Pyridine, 90°C, 1h; (b) DMAP (6eq.) DCM, 0°C.

Scheme 1. Chemical activation of CAMP, AZA and PRED by *p*-nitrophenyl chloroformate.



(a) Fmoc-Lys(Alloc)-OH, DIPEA/DMF, rt, 90 min and then addition of 0.5 ml MeOH for 30 min; (b) 20% piperidine/DMF; (c) CLB, PyBOP, DIPEA/DMF, rt, 90 min; (d) $Pd(PPh_3)_4$ (0.1eq), barbituric acid/DMF, 3 h; (e) for **4a: YG-42**, PyBOP, DIPEA, DMF, rt, 90 min, for **4b: 2,** DIPEA, rt, (90 min x 2), for **4c: 1**, DIPEA, rt, (90 min x 2), (f) TFA/DCM (3:97), 0°C-rt, 90 min.

Scheme 2. Synthesis of Lys-diheteronuclear platforms **4a**–**c**.

2.2. Chemo-stability of drug-linkers

For screening of degradable linker structures appropriate for construction of drug-loaded MAAPs, several drug-linker derivatives were initially synthesized (Fig. 3).

According to a previous report, modification of the 20-hydroxy group in the lactone ring moiety of CAMP contributes to enhancing its stability [23]. Therefore, CAMP derivative **15**, with a carbamate functionality substituting the aliphatic OH was prepared [24]. Additionally, AZA derivative **16** [25], which includes activated phenolic carbamate, was obtained, and the amidated DNA intercalator AHMA derivative **17** was obtained from AHMA [26] precursor and glutaric anhydride [27]. The chemo-stability studies of **15**– **17** were performed at three pH values (pH 2.0, 5.0 and 7.4). Aliquots taken at selected time intervals were analyzed by analytical HPLC and ES-MS. The results are shown in Fig. 4.

All three tested compounds showed high stability at pH 2 and pH 5 (>6 days). However, in tris buffer (pH 7.4) at 37 °C, compounds **15** and **16** exhibited pseudo first- order kinetics drug release profile of CAMP ($t_{1/2} = 76.3$ h) and AZA ($t_{1/2} = 9.4$ h) respectively. These results are consistent with the expected stability of the phenolic carbamate in AZA, which is known to degrade more rapidly than the aliphatic carbamate in CAMP. AHMA derivative **17** remained stable (>6 days).

The chemo-stability of **8** and **9** was then also tested (Fig. 4), showing the same trend observed for **15** and **16**. Thus, the aliphatic ester of 8 was more stable than the phenolic ester of 9 ($t_{1/2} = 92.4$ and 6.9 h, respectively). As expected, the amide bond with the second CLB compound in **8** was much more stable than the ester bond with the first CLB compound, as demonstrated by the identical kinetics of the formation of CLB (the product of the ester bond hydrolysis) and of Ser-CLB (containing the amide bond). The stability of the phenolic ester bond of **9** was pH dependent, being much more labile at pH 2 ($t_{1/2} = 2.8$ h). All experiments were monitored by HPLC against premade standards.

The results of these experiments indicate that carbamate and ester chemodegradable linkages are superior to amides. This served as a basis for preparing peptide–multidrug conjugates.

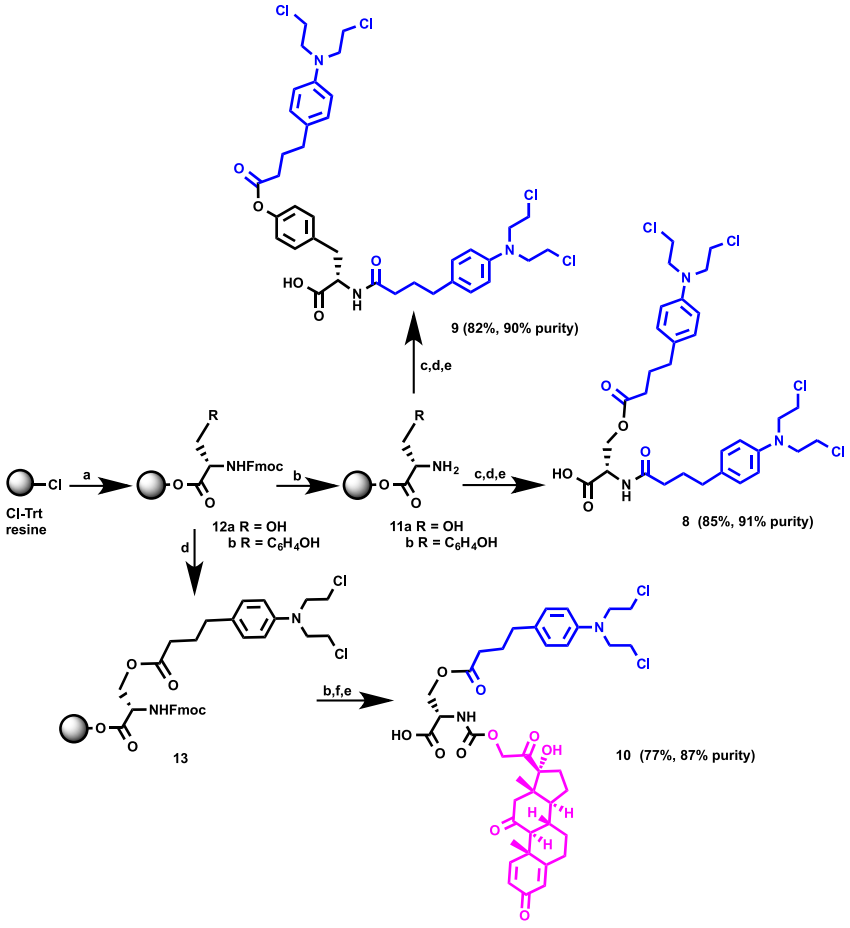
2.3. Bio-stability of bis-CLB MAAPs

We further characterized the bio-stability of simple L-Ser and L-Tyr G_1 MAAP **8** and **9** respectively in liver homogenates (LH) (see Supplementary Information), loaded with two equivalents of CLB, for measurement of CLB release rate *vs* linkage moiety. The results revealed an unstable phenolic ester linkage on L-Tyr platform **9**, ($t_{1/2} = 5.8$ min, (LH)), whereas the same drug linked to L-Ser platform **8** through an alkyl ester moiety exhibited prolonged stability ($t_{1/2} = 22.3$ min (LH), Fig. 5). In both experiments the mono-adduct, namely α-amidated AA-CLB, was observed and subsequently underwent decomposition. Interestingly, the half-life of the mono-adduct of L-Ser-CLB was longer than that exhibited by L-Tyr-CLB, most probably because of the higher stability of G_1 L-Ser MAAP. Notably, released CLB exhibited a significant accumulation profile, especially in L-Tyr G_1 MAAP, with slow CLB degradation rate.

These results indicate that drug – MAAP linkages can be based on ester, carbamate and amide functionalities, with esters being the most bio-cleavable moieties. This information allowed us to proceed to the biological tests of the compounds.

2.4. "Switch off/switch on" of drug cytotoxicity against cancer cells

CAMP is a potent inhibitor of DNA topoisomerase I (Topo I). By stabilizing the covalent binding of Topo I to DNA, it induces irreversible and lethal strand breaks of DNA during its replication [28]. Unfortunately, its unfavorable properties such as non-specific toxicity and negligible water solubility [29] have resulted in suspension of its clinical use in cancer therapy. To overcome these drawbacks, significant efforts have been made to develop various



Scheme 3. Synthesis of Ser and Tyr-dinuclear platforms **8**–**10**.

modifications such as CAMP prodrugs [30] and its combination with drug delivery systems [31], some of which have been assessed in clinical trials [32].

In light of these results, we asked whether the cytotoxic activity of CAMP could be controlled by chemical modification and appropriate delivery. Could this activity be switched off by converting the compound to a prodrug and then switched on again by conjugating the prodrug to a carrier, which would deliver it to the target cancer cell where it would undergo activation?

To test the "switch off" hypothesis, the growth of MBP and BCL1 hybridoma cancer cell lines was assessed following their culture for 48 h with free- and pro-drugs over a concentration range of 5–50 μM. MBP is a cell line expressing a surface antibody specific to a 13 amino acid fragment of the Myelin Basic Protein (MBP). BCL-1 is a cell line of similar lineage expressing a surface antibody to a different antigen. We previously used these cell lines to demonstrate the specificity of other peptide–drug conjugates for anticancer therapy [17]. More recently, we demonstrated that

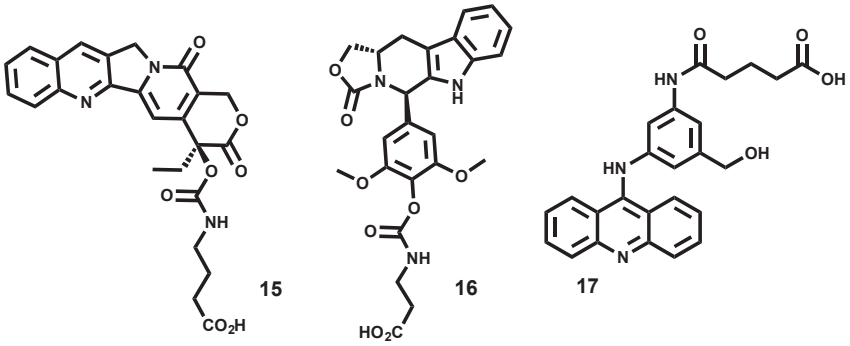


Fig. 3. CAMP, AZA and AHMA derivatives **15**–**17**, respectively, with various carboxylated linkers.

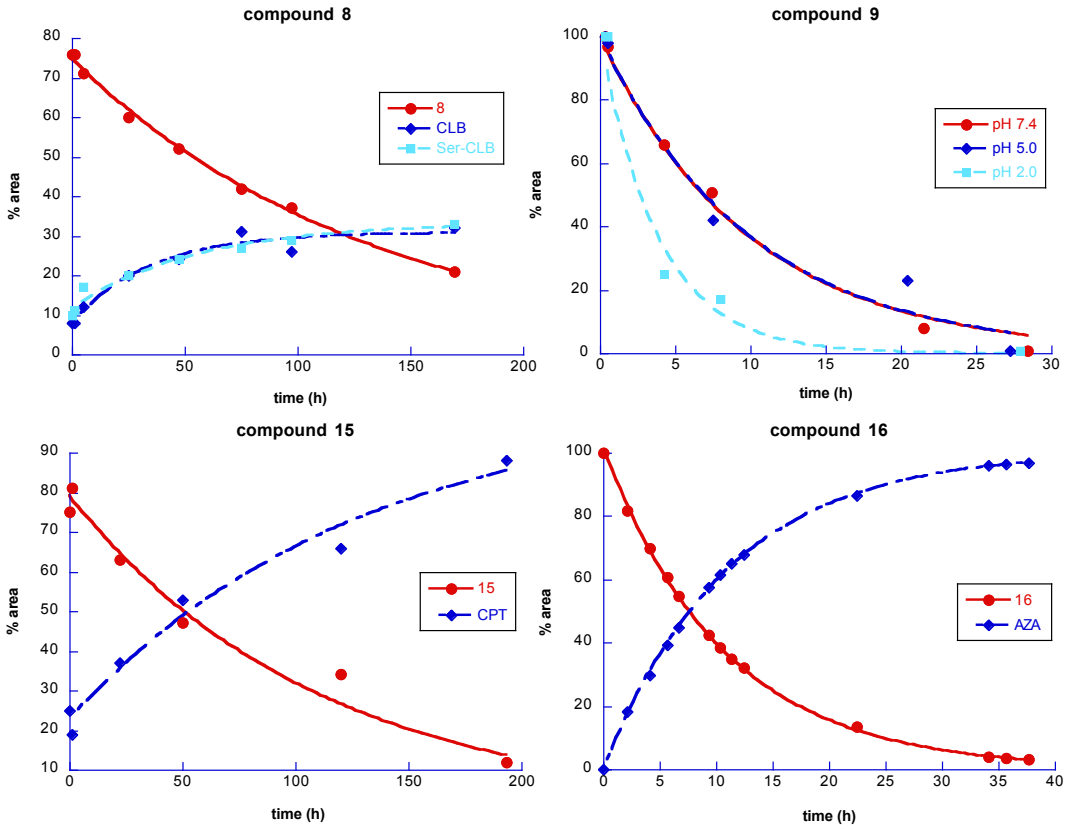


Fig. 4. Chemo-stability of drug-linker of compounds **8**, **9**, **15** and **16**. Stock solutions were made of 2–5 mg of the measured compound dissolved in 500 μl DMSO. 100 μl of stock solution were diluted to 2.5 ml with PBS buffer pH 7.4. The samples were incubated at 37 °C. Aliquots were taken at various time points along the incubation, filtered and analyzed by HPLC. Chemo-stability of **9** is also shown at pH 5.0 and 2.0. The points correspond to the experimental data, and the lines represent fitting to first order kinetics.

multifunctional Lys platforms bearing several CLB molecules covalently bound to a single copy of the MBP peptide fragment induced enhanced growth inhibition in target cells compared to PDCs carrying a single CLB molecule [8].

Fig. 6A shows that the only cytotoxic compound was free CAMP. As free CLB alone was not effective, the results for the mixture of free CAMP and free CLB represent the effect of CAMP. The effect of the drug was non-specific as it equally inhibited the growth of both MBP and BCL-1 cells. Significantly, the CAMP + CLB MAAP prodrug **4c** was not active against either cell line. During the 48 h of incubation, there was probably some degradation of the prodrug and release of free drug, but this seems to be of consequence to the cells only at higher concentrations (50 μM). These results support the first hypothesis, namely that CAMP activity can be "switched off" by transforming it into a prodrug as a MAAP conjugate.

In contrast, Fig. 6B demonstrates how CAMP activity can be "switched on" again, following specific delivery to target cells. The linkage of CLB and CAMP to the Lys MAAP (**4c**) and subsequent conjugation to the MBP carrier to produce MBP-Lys(CLB)CAMP **14** (Scheme 4) is described in the Supplementary Information.

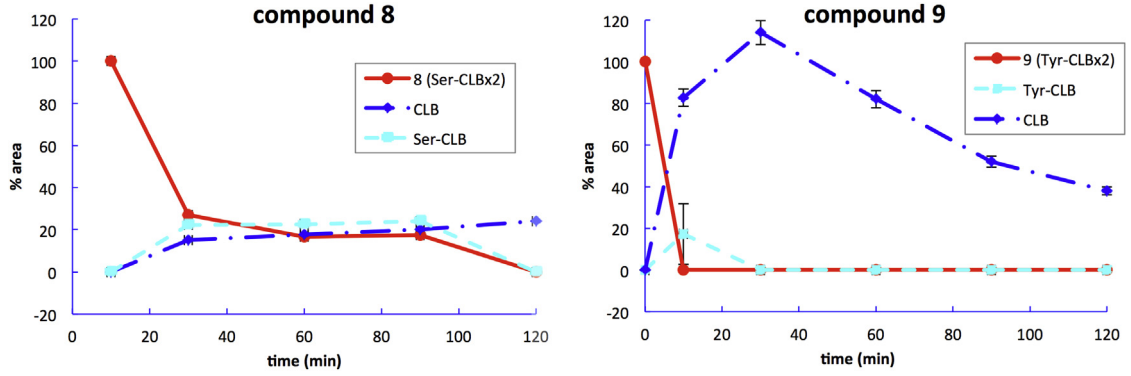


Fig. 5. Bio-stability of G_1 MAAPs L-Ser **8** (left) and L-Tyr **9** (right). General procedure: to prepare the stock solution, 5 mg of **8** or **9** were dissolved in 500 μl DMSO. 50 μl of stock solution were diluted with 2 ml of fresh mouse liver homogenate. The mixture was incubated at 37 °C. Aliquots of the mixture were collected at 10, 30, 60, 90 and 120 min and immediately mixed with 2.5 volumes of ethanol. To remove the precipitate the samples were centrifuged at 14,000 for 15 min. Supernatants were collected carefully, filtered and analyzed by HPLC. Each graph point represents the mean and SD of at least three measurements.

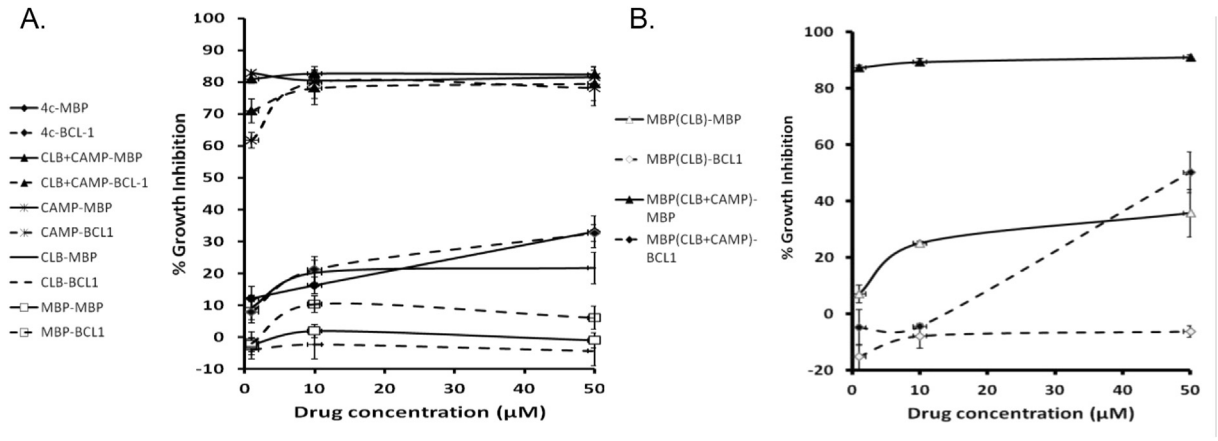


Fig. 6. "Switch off/switch on" regulation of CLB + CAMP "cocktail" cytotoxicity by conjugation to a cell targeting MBP peptide. **A**: Free or platform-linked drugs. **B**: MBP-conjugated drugs. The effect of free-, platform-linked and peptide-conjugated drugs on cancer cells growth was studied. MBP or BCL-1 hybridoma cells were cultured for 48 h either alone or together with different compounds in increasing concentrations (5–50 μM). At the end of the culture period, cell growth was estimated by XTT assay. After 4 h incubation of the cells with the XTT reagent, optical density (OD) of the reduced XTT product was measured at 480 and 680 nm. Percentage of growth inhibition by a test compound was calculated by comparison of the treated culture versus a control culture (free of any compound). The result shown for each concentration point represents the mean ± standard error calculated from 3 different experiments. In each experiment the compounds were tested in triplicates.

The results clearly support the second hypothesis, that CAMP activity can be "switched on" again by conjugating the prodrug to a carrier peptide that delivers the peptide–drug conjugate (PDC) to the target cells. The MBP-CAMP/CLB PDC was extremely specific, being non-toxic to BCL-1 at 5 and 10 μM. The results shown for the MBP-CLB PDC are consistent with our previous report in which we compared the efficacy of Lys platforms bearing 1, 2 or 4 CLB molecules covalently bound to the MBP peptide [8].

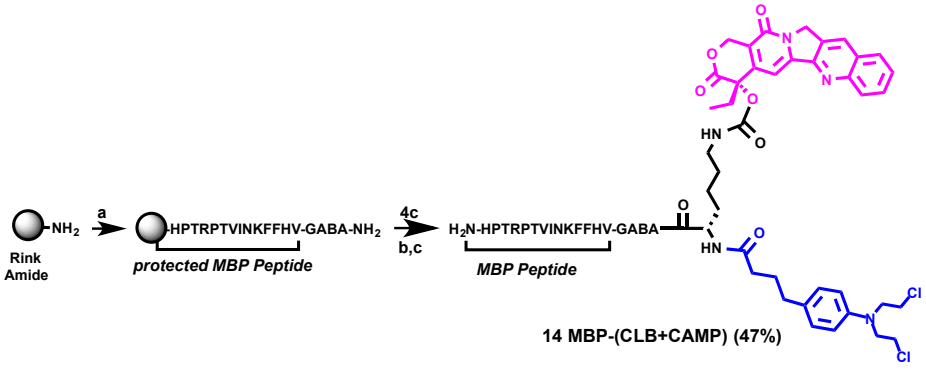
3. Conclusions

The versatility of the synthetic approach presented here suggest that utilization of other L and D amino acids like Thr, Asp, Glu and Cys may also be successful for construction of MAAPs. Bearing functional side chains suitable for drug linkage, these amino acids can form remarkable architectures with highly versatile and tunable drug linkage/release capabilities. Moreover, other semi-orthogonal and orthogonal combinations of protecting groups are applicable in such methodology, for instance: Dde/Fmoc, o-Nosyl/Fmoc, Teoc/Fmoc, Acm/Fmoc. More acid sensitive resins can also be employed leading, after cleavage, to various non-carboxylic anchor points such as amines or thiols for conjugation properties. Importantly, the developed facile SPOS is suitable for combinatorial synthesis of loaded MAAPs, significantly accelerating the discovery of favorable parameters matching essential for defining desired release profiles of the drugs from the platforms.

Our results showed high variability in the bio-stability of the drugs linked through various moieties and therefore strongly support our hypothesis that exposure to cleavage enzymes will lead to differing kinetics of drug release from the loaded MAAP.

We have demonstrated that linkage of a cytotoxic drug cocktail consisting of DNA alkylator CLB and Topo I inhibitor CAMP to the Lys platform and subsequent conjugation to the MBP carrier gains "switch off/switch on" capabilities, specifically activating the cocktail in the target MBP hybridoma cells. In this study, a cocktail of free CLB and CAMP was non-specifically cytotoxic to hybridoma cell lines. Platform Lys(CLB)CAMP was not cytotoxic to any cell line (switch off), while MBP peptide – Lys(CBL)CAMP conjugate showed remarkable specific cytotoxicity (90% growth inhibition/1 μM) on MBP hybridoma (switch on).

Based on these results we are now constructing and assessing more sophisticated MAAPs bearing diverse chemotherapeutic "cocktails" in different linkage variations. These are being conjugated to peptide carriers of clinical significance for preclinical cancer therapy assessment.



(a) Fmoc Chemistry SPPS; (b) 4c, PyBOP, DIPEA/DMF, rt, 90 min; (c) TFA/H_2O/TIPS/DCM (3:2:2:93), 0°C-rt, 90 min.

Scheme 4. Solid phase synthesis of MBP-(CLB + CAMP) **14** on Rink Amide resin.

Acknowledgment

The authors thank Mrs. Cherna Moskowitz for generous stipends to Y.G., B.R. and E.R.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.07.073.

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