Abstract: Maytansinol is a valuable precursor for the preparation of maytansine derivatives (known as maytansinoids). Inspired by the intriguing structure of the macrocycle and the success in targeted cancer therapy of the derivatives, we explored the maytansinol acylation reaction. As a result, we were able to obtain a series of derivatives with novel modifications of the maytansine scaffold. We characterized these molecules by docking studies, by a comprehensive biochemical evaluation, and by determination of their crystal structures in complex with tubulin. The results shed further light on the intriguing chemical behavior of maytansinoids and confirm the relevance of this peculiar scaffold in the scenario of tubulin binders.

Introduction

Maytansine (1a) is an ansamacrolide isolated from Maytenus ovatus; it is a highly potent antimitotic agent that exerts an antiproliferative effect by inhibiting microtubule assembly upon binding to tubulin.\(^{[1-3]}\) Despite a promising in vitro profile, clinical trials with maytansine in cancer patients failed because of poor efficacy and unacceptable systemic toxicity.\(^{[4,5]}\) Although the narrow therapeutic window precluded further clinical development of the parent compound, maytansine, its derivatives were successfully applied clinically as antibody-drug conjugates (ADCs) and thus continue to excite interest.\(^{[6-10]}\) This is mainly due their high cytotoxicity, much higher than that of vincristine and vinblastine.\(^{[11]}\)

Maytansinol (1b) was first obtained by Kupchan et al. both by isolation from Putterlickia verrucose and chemical removal of the acyl group from the hydroxy group at the C3 position.\(^{[11]}\) It showed weaker inhibitory activity on tubulin polymerization than maytansine, thus implying that the ester moiety at the C3 position of ansamitocins, maytansine, and maytansinoids plays an important role for biological activity and cell permeability.\(^{[12,13]}\) In fact, it has just recently been found that the carbonyl oxygen atom of the ester moiety forms a strong intramolecular interaction with the hydroxy group at position 9, fixing the bioactive conformation.\(^{[14]}\) Maytansinol has been regarded as a valuable precursor because acylation allows the preparation of both natural and new semisynthetic maytansinoids, differing in the ester side-chain substituents (Scheme 1).\(^{[15,16]}\) The acylation reaction of maytansinol is a crucial step in the preparation of maytansinoid ADCs or

Scheme 1. Structure of maytansine (1a), maytansinol (1b), and the generic acylation reaction of maytansinol.
nanoparticles, constituting an uprising class of targeted cancer therapeutics.[26–29] A few attempts to conjugate maytansinoids to peptides by this reaction have also been made very recently.[22,23] Furthermore, considering that the maytansine binding site is one of the most recently identified and least explored on tubulin, acylation of maytansinol may serve for the preparation of useful molecular probes to better understand the structure-activity relationships of maytansinoids or to identify new maytansine-site ligands.[24,25]

The potent biological activity of maytansinoids, the variety of possible applications, and above all the intriguing macrocyclic structure decorated by several sensitive functional groups, motivated us to study the formation of maytansinol derivatives induced by different reaction conditions with particular attention to the acylation reaction.

Pursuing our interest in the chemistry and biological activity of tubulin binders,[26–29] we report here a set of new maytansinoids, obtained as a result of maytansinol acylation reaction. Apart from the formation of esters at C3 position, maytansinol has been discovered to undergo a range of other structural transformations not previously reported. To evaluate the potential of our novel maytansinoids, we submitted the obtained compounds to a) computational studies, b) experiments for evaluation of the effect on tubulin polymerization dynamics, c) evaluation of the cytotoxicity and d) structure determination by X-Ray crystallography. The obtained results are crucial for both the design and the synthesis of new effective maytansinoids.

Results and Discussion

Chemical synthesis

Initially, maytansinol was subjected to alkylation by employing propargyl bromide as the alkylation agent. The reaction was performed in ACN/DMF using Cs₂CO₃, KI and TEBA as a phase-transfer catalyst. Interestingly, the 4-hydroxy 2-oxazinanone of maytansinol was cleaved providing the unknown unsaturated ketone 2. Repeating the same reaction without the addition of alkylation agent did not lead to the formation of any product, thereby suggesting that the elimination can occur only after the oxazinanone nitrogen alkylation that induces the formal release of carbon dioxide, water and propargyl amine. We then moved our attention to the acylation at C3 position. Due to the steric hindrance and the consequent poor reactivity of the maytansinol secondary alcohol, the first synthetic strategy was the use of acyl chlorides. Common aliphatic and aromatic acyl chlorides were studied in order to evaluate the influence of the side chain. In addition to the formation of the expected O-acylated product 4, other undescribed maytansinoids were formed (Figure 1; 3–7). The modifications included also the formation of the new double bond in C8-C9 position, as a consequence of the dehydration of the hydroxy group in the oxazinanone (3, 5–7), and the acylation of the oxazinanone nitrogen (6, 7). Attracted by the interesting structural transformations, we planned to monitor their formation depending on the conditions and acylating agents used.

The use of benzoyl chloride as acylating agent in presence of triethylamine and 4-pyrrolidinopyridine did not secure the attainment of only the desired ester, but led to a mixture of compounds 3a, 4a, 6a and 7a depending on the molar ratio and reaction time. Lowering the temperature (−20 °C) led only to the formation of 3a in very low yield, while the use of pyridine as solvent led to the formation of 4a in 25% yield.

Increasing the amount of benzoyl chloride shifted the composition of the reaction mixture exclusively in favor of the formation of 6a and 7a.

In contrast, the use of aliphatic acyl chloride, such as b and c (Figure 2), predominantly produced the derivative 4, showing a weak tendency to form the corresponding dehydrated compounds.

The common Steglich esterification procedure was applied using different coupling reagents. The use of a large excess of Dicyclohexylcarbodiimide (DCC)/4-dimethylaminopyridine; (DMAP) with a reaction time of 3–4 h gave positive results for obtaining the desired C3 acylated derivatives (4a–c). The selectivity was improved by adding an excess of ZnCl₂, and compound 4 was preferentially obtained, even if the reaction time increases. Stoichiometric amount of DCC with 4-(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) as acyl-transfer
agent instead of DMAP did not make the reaction selective, moreover the kinetics resulted very slow. The use of excess DCC affected the purity of the obtained products due to the difficult removal of the DCU by-product. The use of the EDC as coupling agent solved this problem and reduced the formation of the dehydrated products although it required a longer reaction time of 18–24 h.

In general, a longer reaction time increased the conversion of maytansinol, thereby favoring the formation of products 5 and 7. In summary, the use of different acylating agents (Figure 2), coupling reagents, reaction conditions, and reaction times showed a significant influence on the formation of diverse products, giving the possibility to shift the product formation preference (Table 1).

An HPLC method was refined in order to follow easily the conversion and to determine the composition percentage of the reaction mixture (Figure 3).

The spectroscopic characterization of the obtained derivatives required a detailed and sophisticated investigation. All compounds discussed were fully characterized using NMR data, and the complete \(^1\)H and \(^{13}\)C NMR assignments (Tables S1–S4 in the Supporting Information) have been determined based on 1D and 2D NMR spectra \(^1\)H and \(^{13}\)C NMR, COSY, HSQC, and HMBC). Diagnostic NMR peaks are listed in Table 2. The evaluation of the main differences between 1b and the O-acylated derivative 4, 5, 7 shows an evident shift of H-3 signal from 3.34 to about 5 ppm as a consequence of the successfully esterification at the OH-3. It is possible to observe the shift of the H-2 signals from 2.36 and 1.91 ppm to about 2.9 and 2.2 ppm. The corresponding signals of the compounds 3 and 6 did not undergo significant changes excluding an involvement of the hydroxy group. The presence of C8=C9 double bond in compounds 3 and 5–7 was confirmed by the merge of the H-8 signals in the range of 4.5–5.3 ppm. Furthermore, the characteristic H-7 signal was shifted at lower field and the multiplicity changes to a dd for 3 and 5, whereas a multiplet was observed for 6 and 7. Similarly, it is possible to note a new revealing of the H-10 over 4.15 ppm, whereas the olefinic protons signals H-11, H-12, and H-13 are all shifted slightly to higher fields. Finally, the disappearance of the NH signal indicates an acylation of oxazinanone as regard the compounds 6 and 7.

The intriguing structural novelty of the obtained compounds moved us to evaluate their biological activity in combination with their ability to interact with tubulin, with the aim to improve the knowledge on the structure-activity relationships of maytansinoids.

### Table 1. Reaction conditions used for the acylation reaction in dichloromethane (DCM) at room temperature.

| R   | Y   | Acyl eqiv. | C.A. | Base    | \(t\) [h] | Yield [%] | 1b | 3  | 4  | 5  | 6  | 7  |
|-----|-----|------------|------|---------|-----------|-----------|----|----|----|----|----|----|
| a\(a\) | Cl  | 0.5       |      | TEA, A  | 0.5       | 82        | 5  | –  | –  | –  | –  | –  |
| a\(a\) | Cl  | 1         |      | TEA, A  | 2         | 80        | 15 | 2  | –  | –  | –  | –  |
| a    | Cl  | 3         |      | TEA, A  | 6         | 75        | 11 | –  | –  | –  | –  | –  |
| a\(a\) | Cl  | 6         |      | TEA, A  | 4         | –         | –  | –  | –  | –  | –  | –  |
| a\(a\) | OH  | 3         | DCC  | DMAP   | 8         | 4         | 5  | 35 | 26 | –  | –  | –  |
| a\(a\) | OH  | 6         |      | DCC, DMAP | 48       | –         | –  | 15 | –  | 31 | –  | –  |
| a\(a\) | OH  | 3         | DCC  | DMAP, ZnCl\(_2\) | 48       | 29        | –  | 62 | 4  | 4  | –  | –  |
| a\(a\) | OH  | 1         | DCC  | DPTS   | 5d        | 55        | 4  | 18 | 5  | 4  | –  | –  |
| a    | OH  | 3         | EDCI | DMAP, TEA | 24       | 21        | 8  | 39 | 12 | –  | –  | –  |
| a    | OH  | 3         | EDCI | DMAP, TEA | 48       | –         | –  | 15 | 47 | –  | –  | –  |
| b    | Cl  | 2         | –    | TEA, A  | 6         | 51        | –  | 46 | –  | –  | –  | –  |
| c    | OH  | 3         | DCC  | DMAP   | 4         | –         | –  | 42 | 43 | –  | –  | –  |
| c\(a\) | Cl  | 8         | –    | TEA, A  | 7d        | 65        | 2  | 16 | 7  | –  | –  | –  |
| c\(a\) | OH  | 3         | DCC  | DMAP   | 3         | 7         | 9  | 37 | 30 | –  | –  | –  |
| c\(a\) | OH  | 3         | EDCI | DMAP, TEA | 18       | 17        | 4  | 36 | 15 | –  | –  | –  |

[a] Reaction performed at –20 °C; [b] Reaction performed in Py; [c] Percentage determination by HPLC; TEA = triethylamine; A = 4-pyrrolidinopyridine; Py = pyridine; EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

**Figure 3.** Representative HPLC chromatogram. ZORBAX SB C\(_8\) column (3.5 μm × 4.6 × 150 mm). Pressure: 85 bar; Flow rate: 1 mL/min. UV: 254 and 210 nm with DAD detection. Mobile phase: H\(_2\)O/ACN 1 min isocratic at 50% ACN, then gradient to 90% ACN over 10 min. Retention times: 1b, 3.10 min; 3, 4.08 min; 4a, 5.20 min; 5a, 6.90 min; 6a, 7.20 min; 7a, 9.40 min.
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Subsequently determined their binding affinities to tubulin and microtubules, we first probed their effect on microtubule assembly dynamics, and subsequently determined their binding affinities to tubulin dimers and their cell toxicity.

Table 2. Diagnostic $^1$H NMR spectroscopic data of maytansinoid compounds.

| Atom | 3$^{[a]}$ | 3$^{[b]}$ | 4$^{[a]}$ | 4$^{[b]}$ | 5$^{[a]}$ | 5$^{[b]}$ | 6$^{[a]}$ | 7$^{[a]}$ |
|------|---------|---------|---------|---------|---------|---------|---------|---------|
| 2    | 2.36, 2.13 | 2.32, 2.14 | 2.88–2.79, 2.26 | 2.91, 2.22 | 2.37, 2.12 | 2.99–2.87, 2.27 |
| 3    | 3.55 | 3.59 | 5.04–4.85$^{[c]}$ | 5.02 | 3.61 | 5.04 |
| 7    | 4.25 | 4.78 | 4.31–4.14 | 4.56 | 4.86 | 4.92–4.76$^{[d]}$ |
| 8    | 1.91, 1.41 | 5.02–4.96 | 1.59, 1.54–1.39 | 4.43 | 5.43–5.33 | 4.92–4.76$^{[d]}$ |
| 10   | 3.64 | 4.20–4.12 | 3.50 | 4.15 | 4.42 | 4.35 |
| 11   | 5.52 | 5.54 | 5.04–4.85$^{[c]}$ | 4.88 | 5.54 | 4.92–4.76$^{[d]}$ |
| 12   | 6.70 | 6.46 | 6.61 | 6.54 | 6.48 | 6.64 |
| 13   | 6.20 | 6.16 | 6.04 | 6.13 | 6.15 | 6.12 |
| OH-3 | 4.50 | – | – | – | – | – |
| OH-9 | 4.80 | – | 4.47 | – | – | – |
| NH   | 6.42 | 7.08 | 6.33 | 8.03 | – | – |

Chemical shifts (ppm) were determined with reference to TMS; Spectra determined at 400 MHz; [a] Solvent is [D$_6$]acetone; [b] Solvent is deuterated chloroform; [c], [d] Chemical shifts bearing the same symbol overlap.

Inhibition of tubulin assembly

Maytansine site targeting agents inhibit tubulin assembly by capping the plus end of β-tubulin subunits, thereby precluding further microtubule growth.$^{[25]}$ We therefore tested the selected compounds for their ability to inhibit tubulin assembly (Figure 4). Maytansine (1a), which completely abolishes tubulin polymerization, and maytansinol (1b), which has a noticeable effect at stoichiometric concentrations, were used as controls.

All the compounds assayed inhibited tubulin assembly into microtubules at stoichiometric ratios with tubulin, however, they showed different potencies. While compounds 4a–c, 5a, 5b, and 6a were strong inhibitors that completely abolished microtubule assembly, compounds 3, 5c, and 7a showed only a mild and compound 2a weak inhibition.

Binding affinities

In order to correlate the tubulin assembly inhibition with the binding affinities of the compounds for the maytansine site we determined using competition against Fc maytansine$^{[24]}$ (Table 4; Figure 5).

Surprisingly and unlike for other microtubule targeting agents, the potency did not correlate well with the determined binding affinities. The reason for this is that all the compounds assayed have at least micromolar affinity. Tubulin assembly

Table 3. Protein-ligand free energies of binding of the best docking geometries for each maytansinoid returned by AutoDock Vina calculations when docking to the structure of β-tubulin 4TV81.

| Maytansinoid | 3 | 4a-b | 4c | 5a-b | 5c | 6 | 7 |
|--------------|---|-----|----|------|----|---|---|
| $\Delta G^\circ$ [kcal/mol] | –7.5 | –7.4 | –8.2 | –6.7 | –6.8 | –7.5 | –7.4 |

Computational studies

We used docking to predict the spatial coordinates of the binding mode acquired by the synthesized maytansinoids within the maytansine structure, which is located in a shallow pocket on β-tubulin facing the inter-dimer interface.$^{[25]}$

To test the accuracy of the docking engine AutoDock Vina, the crystallographic structure of maytansine bound to β-tubulin was redocked in its site as a positive control measure. As a result, the geometry assigned by AutoDock Vina for maytansine overlapped with its crystallographic orientation. Therefore, since AutoDock Vina successfully reproduced the crystallographic findings, we could confirm the reliability of this docking software.

Subsequently, we successfully performed the docking of the derivatives 3, 4a–c and 5a–c to the maytansine binding site (Table 3). In all cases, the orientation of the maytansinol ring remained in the same spatial arrangement, acquiring a similar binding mode to the parent compound. The introduction of bulky substituents at position XO did not alter the predicted 3D arrangement of the core of the molecule. Thus, we assumed that binding of maytansinoids to β-tubulin is very tolerant to modifications of the X-hydroxy group and expected that the binding mode of the investigated molecules resembles the one of the parent compound.

Table 4. Binding affinities of maytansinoid compounds.

| Compound | $K_\text{d}$ [M$^{-1}$] | $K_\text{d}$ [nM] |
|----------|----------------|----------------|
| 1a       | $9.0 \pm 1.3 \times 10^{-7}$ | 14 ± 2 |
| 1b       | $1.3 \times 0.06 \times 10^{-7}$ | 780 ± 40 |
| 2        | $1.42 \pm 0.08 \times 10^{-7}$ | 720 ± 50 |
| 3        | $1.20 \pm 0.03 \times 10^{-7}$ | 830 ± 20 |
| 4a       | $2.0 \pm 0.6 \times 10^{-7}$ | 51 ± 3 |
| 4b       | $9 \pm 2.1 \times 10^{-7}$ | 11 ± 1 |
| 4c       | $5.4 \pm 0.5 \times 10^{-7}$ | 20 ± 2 |
| 5a       | $9.2 \pm 0.3 \times 10^{-7}$ | 1090 ± 40 |
| 5b       | $1.17 \pm 0.04 \times 10^{-7}$ | 860 ± 30 |
| 5c       | $5.6 \pm 0.4 \times 10^{-7}$ | 1800 ± 120 |
| 6a       | $6 \pm 2 \times 10^{-7}$ | 3000 ± 100 |
| 7a       | $5.0 \pm 0.5 \times 10^{-7}$ | 2000 ± 200 |

Biological evaluation

To evaluate the activity of the representative compounds 2, 3, 4a–c, 5a–c, 6a and 7a on tubulin and microtubules, we first probed their effect on microtubule assembly dynamics, and subsequently determined their binding affinities to tubulin dimers and their cell toxicity.

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inhibition is dependent on two factors. First, the binding affinity of the compound for the site has to be significant at the concentrations of the inhibition assay. As the concentrations of both tubulin and drugs employed in the assay were nearly one order of magnitude higher than the weakest dissociation constant measured for \( \text{6 a} \) (3 mM), we expected that all the ligands employed were bound to the protein. Therefore, the binding affinity should not influence the in vitro assembly inhibition activity in the way we observed.

According to the mechanism of action proposed by Prota et al., ligand binding to the maytansine site should have a strong influence on MT-assembly. Maytansine site ligands bind at a shallow pocket at the top of the tubulin β-subunit where the interaction with a longitudinal aligned tubulin dimer in the protofilament takes place, thereby inhibiting the addition of tubulin subunits at the plus ends of growing microtubules.

From the ligands studied, \( \text{4 b} \) (11 nM) and \( \text{4 c} \) (20 nM) showed the highest affinities with dissociation constants close to the one of maytansine (14 nM), thus indicating that both small and bigger substituents can easily replace the \( \text{N-} \text{acetyl-N-methyl-L-alanine} \). On the other hand, compound \( \text{4 a} \) with a phenolic ester at position C3 was a slightly weaker binder (51 nM). The series of compounds lacking the hydroxy group at
position C9 showed binding affinities in the 1 μM range (2 720 nM, 3 830 nM, 5a 1090 nM, 5b 860 nM), thereby suggesting that the C9-hydroxy group might serve as a critical anchoring point to allow establishing the interaction with the site. Finally, the remaining three compounds also lacking the hydroxy group at position C9 with modifications either at the C3, the oxazinanone nitrogen or both, displayed affinities in the sub-mM range: 5c (1800 nM), 6a (3000 nM) and 7a (2000 nM).

In summary, two modifications resulted in a strong impact on the binding affinity, namely the lack of esterification of the C9-hydroxy group. Moreover, changes in the acid esterification on the binding affinity, namely the lack of esterification of the corresponding ligands maytansine, maytansinol and the vehicle (Figure 6).

Cytotoxicity

To correlate the potency of binding with the toxicity, and to investigate the potential of the compounds to overcome membrane pumps mediated multidrug resistance, we determined the cytotoxicity of the compounds both in A549 (small cell lung carcinoma) and in the isogenic pair A2780/A2780AD (pGp overexpressing) cell lines (Table 5).

Cytotoxicity requires effective binding of a ligand to tubulin at concentrations, which are about one order of magnitude lower than the dissociation constants of the corresponding ligand. The cytotoxicities determined in this study correlated well with the binding affinities: compounds with high affinity C9 double-bond on the binding pose, we also tested the analogues C8 – C9 double-bond on the binding pose, we also tested the analogues C8 – C9 double-bond on.

In order to validate the above computational analysis and to complement the biological assays, we sought to determine the crystal structures of the respective tubulin–maytansinoid complexes. To this end, we tested the compounds 3, 4a–c. Moreover, to evaluate the impact of the C8–C9 double-bond on the binding pose, we also tested the analogues 5a–c. Crystals of the TβR-TTL protein complex, containing two αβ-tubulin dimers, the stathmin-protein RB3 and the tubulin tyrosine ligase TTL were grown as described by Prota et al. The above listed compounds were soaked into the crystals over 6 h, which allowed us to solve the TβR-TTL-maytansinoid structures at high resolution, ranging between 2.25 and 2.7 Å (Table 6). Coordinates and structure factors for all tubulin-maytansinoid complexes were deposited at the Protein Data Bank (www.rcsb.org), the respective accession numbers are indicated in Table 6. All of the tested ligands were bound to the maytansine site of the β-tubulin chain in the TβR-TTL complex.
The overall T\(_2\)R-TTL-maytansinoid structures superimposed very well with the protein structure obtained in the absence of a ligand (PDB ID 4I55; Table 6), thus suggesting that the binding of the maytansinoids has no effect on the overall conformation of tubulin.

The binding pose of all the maytansinoids within the maytansine site closely resembles the one described for the parent compound\(^{[25]}\) and all the main interactions are conserved. Briefly, all compounds form hydrogen-bonds between the C1-O and the main chain nitrogen atom of Val181 and between the C24-O and the side chains of Lys105 and Asn102. Additionally, 4\textsubscript{a-c} establish a hydrogen bond to the main chain carbonyl group of Gly100 via their C9-OH group. In Figure 7, we show the binding pose of the best resolved maytansinoid 4\textsubscript{a}.

**Figure 6.** Effects of maytansinoids on cells in interphase and mitosis. The effect of these compounds on the microtubule network and mitotic spindle was characterized in A549 tumor cells by fluorescence microscopy. Cells were treated for 24 h with the different compounds analyzed: A)–C') control (DMSO 0.5 %), D)–E') 1\textsubscript{a} 5 nM, F)–G') 100 nM, and H)–I') 4\textsubscript{a} 5 nM. Cells were immunostained for A)–I') α-tubulin or A')–I') DNA, and A'')–I'') the images obtained were merged (tubulin in green and DNA in magenta). A)–A') Interphase cell treated with DMSO; notice the regular microtubule network evenly distributed in the cytoplasm. B)–B') Control metaphase cell with a normally distributed bipolar mitotic spindle in which all chromosomes are positioned in the metaphase plate. C)–C') Control late-anaphase cell in which sister chromatids are observed segregating to the daughter poles through a bipolar anaphase spindle; note that no anaphases are later observed in treated cells. D)–D'', F)–F'', H)–H'') Interphase cells displaying a range of less-dense microtubule networks and reduction of the microtubule mass compared with A–A'' (multinucleated heteroploid cells -D', H'- or single-nucleated cells -F'- may appear in any of the treatments with the three compounds). E)–E'', G)–G'', I)–I'') Blocked mitotic cells with condensed chromosomes showing less microtubular mass (when compared with B–B'') organized in star- or comet-shaped pseudopoles (one cell shown in E–E'', three in G–G'' and two in I–I''). All images are shown at the same scale; scale bar: 10 μm.
and compare it to 5a, as well as the maytansine orientation. The detailed binding poses of the other maytansinoids are provided in Figures S23 and S24.

In the studied maytansinoids, all the modifications introduced at the C3 position point towards the solvent and do not perturb the close environment of the maytansine site. For the larger C3 substituents, such as the phenyl ring, we observed a slight reorientation of the C3 carbonyl group, which increases the distance to the Asn101 carbonyl group, thereby weakening this interaction. However, our biological assays showed that this minor change in coordination of the ligand does not have an impact on the efficacy and the binding constants. For the three C3-modified maytansinoids 4a-c the determined values are close to the ones of maytansine (Tables 4 and 5). Thus, we can conclude that even attachment of larger groups at this position has no apparent impact on the binding pose, affinity or efficacy.

The structural analysis of the maytansinoids 3, 5a–c further reveals that the elimination of the C9-hydroxy group does not affect the binding mode of the ligands. As shown in Figure 7D, the heterocycle is anchored by two hydrogen bonds established between the C24-O and the side-chains of Asn102 and Lys105, which highlights that the interaction is not affected by the introduction of the double bond.

However, the loss of one hydrogen bond between the C9-OH group and the main chain carbonyl of Gly100 cannot fully account for the observed lower affinities and efficacies of these maytansinoids compared to their hydroxylated analogues, suggesting a contribution by other factors such as compound solubility or decreased stability (Figure 7B).

In order to validate the docking results, we superimposed the crystal structures obtained for each maytansinoid and the

| Compound | PDB ID | Resolution [Å] | RMSD overall/chain D (over No. of Cα atoms) |
|----------|--------|----------------|---------------------------------------------|
| 3        | S5B8   | 2.30           | 0.25 (1974)/0.163 (380)                      |
| 4a       | S5B9   | 2.50           | 0.259 (1958)/0.189 (384)                     |
| 4b       | S5BA   | 2.25           | 0.216 (1937)/0.163 (375)                     |
| 4c       | S5BB   | 2.25           | 0.232 (1962)/0.153 (369)                     |
| 5a       | S5BC   | 2.32           | 0.258 (1946)/0.173 (369)                     |
| 5b       | S5BD   | 2.25           | 0.274 (2004)/0.154 (362)                     |
| 5c       | S5BE   | 2.75           | 0.266 (1966)/0.197 (394)                     |

Figure 7. X-ray analysis of the T2R-TTL-maytansinoid complexes. A) Overall view of the interaction between the maytansinoids (blue) and tubulin (gray) in relation to the bound nucleotides (purple). The maytansine binding site is located on the β-tubulin surface in close proximity to the bound GDP molecule. The tubulin molecule is in ribbon representation (gray), and the interacting maytansinoid and nucleotides are in surface and stick representations, respectively. B) Close-up of the interaction between the maytansinoid 4a (PDB ID: S5B9, blue) and tubulin (gray). The interacting residues and ligand are represented as sticks. Oxygen atoms are in red, nitrogens are blue, and the chlorine atom is in green. Hydrogen bonds are displayed as black dashed lines. C) Superposition of the T2R-TTL-maytansine structure (PDB ID: 4TV8, maytansine in gray) and the 4a-T2R-TTL structure (4a in blue). The dashed lines indicate the hydrogen-bond interactions established by the maytansine molecule. 4a adopts the same binding pose as its parent compound, except for the interaction of the acyl group with Asn101, which is less pronounced and thus not displayed in the 4a structure, all interactions are conserved. D) The superposition of the T2R-TTL-4a (4a in blue) and the T2R-TTL-5a (5a in orange) structures shows that the elimination of the C8-OH group in 5a has no major effect on the coordination of the ligand. Although the heterocycle moiety is flattened by the double bond in 5a, the position of the ring is anchored at its position by coordination to Asn102 and Lys105.
best corresponding conformer resulting from the docking studies.

In Figure 8 we show the superposition for the 4a models; the results for the other maytansinoids can be found in the Supporting Information. For all molecules the docking results agreed well with the models determined by X-ray crystallography.

These results confirm the reliability of the performed computational docking studies and provides confidence for further usage of the described protocol to investigate novel maytansine-site binders.

Conclusion

In summary, we have synthesized a series of maytansinoids containing novel scaffold modifications through extensive investigation of the maytansinol acylation reaction. We were able to shift the reaction towards the specific product formation by screening different reaction conditions. The subtle changes between these structures were carefully analyzed and fully characterized by using NMR spectroscopy. Encouraged by the positive results of our docking studies, we submitted a selection containing novel scaffold modifications through extensive approaches. This provides us with a solid foundation for future design, synthesis and analysis of next-generation maytansinoids.

Acknowledgements

We thank Ganadería Fernando Díaz for calf brains. We thank the staff of the Swiss Light Source responsible for beamline X06DA for their support during X-ray data collection. This work was supported by the H2020-MSCA-ITN-2019 (860070 TUBINTRAIN), Ministerio de Ciencia e Innovación PID2019-104545RB-I00, PIE 201920E111 and PIE 202020E301 from CSIC, Fundacion Tatiana Pérez de Guzmán el Bueno. Open Access Funding provided by $INSTITUTION within the CRUI-CARE Agreement.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: maytansinol · maytansine binding site · tubulin · microtubules · tubulin binders

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