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Cryptophycins: cytotoxic cyclodepsipeptides with potential for tumor targeting

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Cryptophycins are a class of 16-membered highly cytotoxic macrocyclic depsipeptides isolated from cyanobacteria. The biological activity is based on their ability to interact with tubulin. They interfere with microtubule dynamics and prevent microtubules from forming correct mitotic spindles, which causes cell-cycle arrest and apoptosis. Their strong antiproliferative activities with 100-fold to 1000-fold potency compared with those of paclitaxel and vinblastine have been observed. Cryptophycins are highly promising drug candidates, as their biological activity is not negatively affected by P-glycoprotein, a drug efflux system commonly found in multidrug-resistant cancer cell lines and solid tumors. Cryptophycin-52 had been investigated in phase II clinical trials but failed because of its high neurotoxicity. Recently, cryptophycin conjugates with peptides and antibodies have been developed for targeted delivery in tumor therapy. Copyright © 2017 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: natural product; depsipeptide; total synthesis; biological activity; SAR; antimitotic agent; drug conjugate; bioconjugation; warhead

Introduction

Cancer represents the second leading cause of death in most industrialized countries followed by cardiovascular diseases. In particular, organs like lungs, colon, mammary gland, and prostate are most often affected. Conventional tumor therapy includes surgical removal and radiotherapy in the case of locally restricted tumors as well as chemotherapy when metastases have spread. In comparison, immunotherapy and gene therapy are not as frequently applied. In addition, medication is often applied in palliative treatment to improve quality of life by alleviation of symptoms for tumor types without any chances for cure. Tumor therapy is one of the major challenges for modern medicine, because of the similarity between tumor and healthy cells because tumors develop from endogenous tissue and do not differ very much from a non-pathological cell on the molecular level. However, tumor cells show an accelerated proliferation rate compared with healthy tissue, and therefore, the enhanced cell division is often used in a therapeutic approach. In chemotherapy, cell multiplication can be restricted or entirely prevented by administration of neoplastic agents. In fact, antitumor chemotherapeutics often lack specificity and thus inevitably damage other proliferating cells, leading to the chemotherapy-associated side effects such as hair loss, weakening of the immune system, and gastrointestinal complaints. The selective delivery of antitumor agents to tumor tissue might provide a solution to overcome systemic toxicity and undesired side effects. In this context, the drug is linked directly or through a suitable linker system to a tumor-targeting moiety (homing device). The use of monoclonal antibodies [1–4], hyaluronic acid [5,6], small molecules [7] like folic acid [8–10], and peptides [11–14] as tumor-targeting groups (homing devices) has been reported. Remarkable progress in cancer therapy has been made in the last years using such tumor-targeting drug delivery systems [11,15].

Nature provides a broad range of structurally diverse compounds with interesting biological properties like taxanes, quinoline alkaloids, anthracyclines, and epothilones. Such secondary metabolites provide a pool of pharmacologically promising lead structures. In particular, inhibitors of mitosis are one of the most potent classes of anticancer agents available to date [16]. These compounds can be subdivided into microtubule stabilizers and tubulin polymerization inhibitors [17,18]. Tubulin is a global hetero-dimeric protein with α-subunit and β-subunit, which polymerize to form tube-shaped filaments of approximately 240-Å diameters, the microtubules [19]. Among other functions, microtubules are especially important for the formation of the mitotic spindle and are responsible for the distribution of the chromatids to daughter cells during cell division [20]. This requires high dynamics of the microtubule ensured by constant polymerization and depolymerization [21,22]. The polymerization process is guanosine triphosphate (GTP) dependent and, therefore, depends on the phosphorylation of a nucleotide bound to β-tubulin, which influences the conformation of the protein structure. All antimitotic agents have in common that the microtubule dynamics is disrupted. Therefore, aberrant mitotic spindle formation leads to mitotic arrest in a prometaphase/metaphase-like state and to apoptosis. In fact, spindle microtubules display 10-fold to 100-fold more dynamics than interphase microtubules, and thus many microtubule-targeting compounds act as potent chemotherapeutic drugs [23]. The majority of the antimitotic drugs bind to one of the three well-established binding sites on β-tubulin: the *vinca* domain, the colchicine site, and the taxane binding site [24]. The *vinca* alkaloids vincristine and vinblastine as well as colchicine are known to prevent tubulin polymerization and reduce, in high concentrations, the microtubule polymer mass. Upon binding to

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Journal of PeptideScience

the *vinca* domain (e.g. of *vinca* alkaloids, dolastatins, and hemiasterlins) or the colchicine binding site (e.g. 2-methoxyestradiol, methoxybenzenesulfonamide, and combretastatin), respectively, these agents prevent correct formation of the spindle apparatus by inhibiting tubulin polymerization. Another mode of action was found for paclitaxel [25], being the first representative of a compound class known to act as microtubule stabilizers. Other natural products have been identified to promote the polymerization of tubulin dimers into microtubules and to stabilize preformed microtubules at high concentrations. These agents (e.g. taxanes, epothilones, and discodermolide) bind to the so-called paclitaxel site of tubulin. At low but clinically relevant concentrations, both tubulin-stabilizing and tubulin-destabilizing drugs interfere with microtubule dynamics while the microtubule mass is not affected. In addition, apoptosis is being induced [26–29]. The cryptophycins, 16-membered cyclic depsipeptides, were isolated from cyanobacteria *Nostoc* sp. ATCC 53789 in 1990 by researchers from Merck and were identified as a new class of microtubule destabilizers (Figure 1) [30]. Treatment of carcinoma cells with cryptophycin-1 in picomolar concentration quickly leads to morphological changes of the cells and to apoptosis.

With regard to total synthesis, all naturally occurring cryptophycins can be divided into the four building blocks unit A–D being amino or hydroxycarboxylic acids, respectively. Unit A is an α,β-unsaturated δ-hydroxy carboxylic acid typical for cryptophycins. In cryptophycin-1, this building block contains a benzylic β-epoxide, while many cryptophycin derivatives isolated from *Nostoc* sp. contain a styrene moiety instead of the styrene oxide. Such desoxy-cryptophycins are assumed to be biosynthetic precursors of the corresponding epoxides [31], but their cytotoxic activities are significantly reduced. In the case of cryptophycin-1, a D-tyrosine derivative that is *O*-methylated and chlorinated in 3′-position serves as unit B. Several analogs also contain a second chloro substituent in the other *ortho*-position relative to the methoxy group. The (*R*)-configuration of unit B is essential for high cytotoxicity. The relative configuration was proven by X-ray analysis of cryptophycin-3 [32]. In naturally occurring cryptophycins, unit C is either β-alanine or (*R*)-α-methyl-β-alanine, so that cryptophycins belong to the rare peptidic natural products containing a β^2-amino acid with a chiral center at C^{α}. Unit D is a α-hydroxy carboxylic acid, which in many cases is L-leucic acid. In addition, analogs bearing L-isoleucic acid, L-valic acid, or an α-*n*-propyl-substituted α-hydroxy acid subunit have been isolated.

Many cyanobacteria produce toxic secondary metabolites to protect themselves against other microorganisms [33,34]. The name cryptophycins was coined because of the high cytotoxicity of these compounds against yeast of the genus *Cryptococcus* [30,35,36]. Moore *et al.* isolated cryptophycin-1, the first representative, from the related *Nostoc* species GSV 224 and published a first proposal of the stereochemistry in 1994 [37]. The absolute configuration of a closely related analog of cryptophycin-1, namely, arenastatin A, was correctly assigned by Kobayashi *et al.* [38,39]. Arenastatin A was isolated from the marine sponge *Dysidea arenaria* and later renamed to cryptophycin-24. Today, over 28 naturally occurring cryptophycin derivatives have been isolated (Table 1) [32,40,41], and a large number of synthetic analogs have been reported in the context of structure–activity relationship (SAR) studies [42,43]. The ester moieties are susceptible towards cleavage, and hence linear derivatives of cryptophycins observed during the methanolic extraction from natural sources represent artifacts [32]. In biological screening assays, the high cytotoxicity and cytostatic activity of some representatives of this compound class were discovered with cryptophycin-1 being the most efficient one. In addition, cryptophycins display a remarkable cytotoxicity against multidrug-resistant (MDR) tumor cell lines [32,37], making this class of natural products interesting as potential chemotherapeutics [44]. With cryptophycin-52 (LY355703; Figure 1, Table 2), the most promising first-generation clinical candidate was identified by Eli Lilly [45]. This synthetic analog displays enhanced stability in aqueous solution compared with cryptophycin-1, while the high activity against MDR cells is maintained.

Biological Activity

The exact binding site of cryptophycins and the orientation of the drug inside the binding pocket are still unknown because no

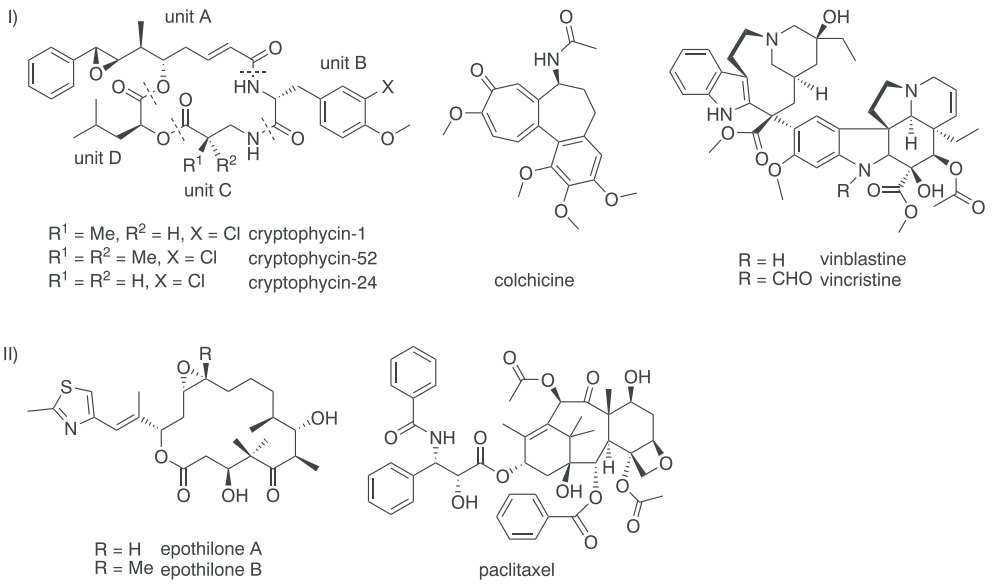
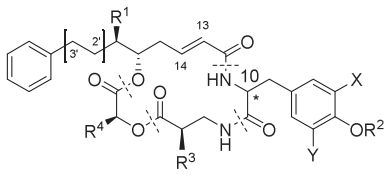
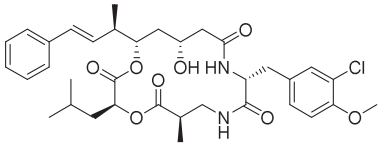


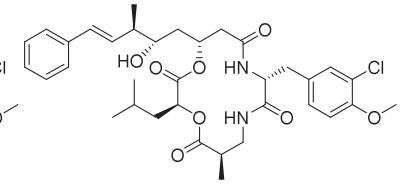
Figure 1. Naturally occurring antimitotic compounds classified according to their mode of action: (I) microtubule destabilizers (tubulin polymerization inhibitors) and (II) microtubule stabilizers (microtubule depolymerization inhibitors).

Table 1. Natural cryptophycins isolated by Moore *et al.* from *Nostoc* sp. [32,40,41]





cryptophycin-26



cryptophycin-30

compound	$C^{2'}$-$C^{3'}$	R^1	C^{13}=C^{14}	C^{10}	R^2	X	Y	R^3	R^4
cryptophycin-1	β-epoxide	CH_3	*trans*	(*R*)	CH_3	Cl	H	CH_3	*i*-Bu
cryptophycin-2	β-epoxide	CH_3	*trans*	(*R*)	CH_3	H	H	CH_3	*i*-Bu
cryptophycin-3	olefin	CH_3	*trans*	(*R*)	CH_3	Cl	H	CH_3	*i*-Bu
cryptophycin-4	olefin	CH_3	*trans*	(*R*)	CH_3	H	H	CH_3	*i*-Bu
cryptophycin-16	β-epoxide	CH_3	*trans*	(*R*)	H	Cl	H	CH_3	*i*-Bu
cryptophycin-17	olefin	CH_3	*trans*	(*R*)	H	Cl	H	CH_3	*i*-Bu
cryptophycin-18	olefin	CH_3	*trans*	(*R*)	CH_3	Cl	H	CH_3	*s*-Bu
cryptophycin-19	olefin	CH_3	*trans*	(*R*)	CH_3	Cl	H	CH_3	*i*-Pr
cryptophycin-21	β-epoxide	CH_3	*trans*	(*R*)	CH_3	Cl	H	H	*i*-Bu
cryptophycin-23	β-epoxide	CH_3	*trans*	(*R*)	H	Cl	H	CH_3	*i*-Bu
cryptophycin-24	β-epoxide	CH_3	*trans*	(*R*)	CH_3	H	H	H	*i*-Bu
cryptophycin-28	β-epoxide	H	*trans*	(*R*)	CH_3	Cl	H	CH_3	*i*-Bu
cryptophycin-29	olefin	CH_3	*trans*	(*R*)	CH_3	Cl	H	H	*i*-Bu
cryptophycin-31	β-epoxide	CH_3	*trans*	(*R*)	CH_3	Cl	Cl	CH_3	*i*-Bu
cryptophycin-38	α-epoxide	CH_3	*trans*	(*R*)	CH_3	Cl	H	CH_3	*i*-Bu
cryptophycin-40	olefin	H	*trans*	(*R*)	CH_3	Cl	H	CH_3	*i*-Bu
cryptophycin-43	olefin	CH_3	*trans*	(*R*)	H	H	H	CH_3	*i*-Bu
cryptophycin-45	olefin	CH_3	*trans*	(*R*)	H	Cl	Cl	CH_3	*i*-Bu
cryptophycin-46	olefin	CH_3	*trans*	(*S*)	CH_3	Cl	H	CH_3	*i*-Bu
cryptophycin-49	olefin	CH_3	*trans*	(*R*)	CH_3	Cl	H	CH_3	*n*-Pr
cryptophycin-50	β-epoxide	CH_3	*trans*	(*R*)	CH_3	Cl	H	CH_3	*n*-Pr
cryptophycin-54	β-epoxide	CH_3	*trans*	(*R*)	CH_3	Cl	H	CH_3	*s*-Bu
cryptophycin-175	olefin	CH_3	*trans*	(*R*)	CH_3	Cl	Cl	CH_3	*i*-Bu
cryptophycin-176	β-epoxide	CH_3	*trans*	(*R*)	H	Cl	H	H	*i*-Bu
cryptophycin-326	β-epoxide	CH_3	*trans*	(*R*)	CH_3	Cl	Cl	H	*i*-Bu
cryptophycin-327	β-epoxide	CH_3	*cis*	(*R*)	CH_3	Cl	H	CH_3	*i*-Bu

X-ray structure of a cryptophycin–tubulin complex has been published yet. Many inhibitors of mitosis interact with tubulin by binding to one of the three binding sites, namely, the *vinca* domain, the colchicine site, or the paclitaxel site. Cryptophycins do not interfere with binding of paclitaxel or colchicine to tubulin [45–47]. In contrast, cryptophycins non-competitively inhibit vinblastine binding to tubulin [44], and identical cleavage patterns are obtained upon tryptic and chymotryptic digestion of tubulin treated with cryptophycins and vinblastine, respectively [48]. These observations suggest that the cryptophycin binding site overlaps with the *vinca* domain. Additionally, the structurally diverse (depsi)peptides cryptophycin, dolastatin-10, hemiasterlin, and phomopsin A competitively inhibit their interaction with tubulin assuming a common binding site [49]. This prediction is supported by combined molecular dynamics simulation and molecular docking experiments [50] where the β-subunit of the tubulin heterodimer has been identified as the collective binding site [44], which is nowadays known as the 'peptide site' (Figure 2) [44,51]. As these peptides are of rather hydrophobic nature, their binding to tubulin reduces the exposed hydrophobic surfaces and, therefore, is entropy driven [50,52].

Cryptophycins bearing an epoxide moiety might either bind covalently to tubulin by nucleophilic attack of an amino acid side chain to the epoxide or by a non-covalent interaction. The latter

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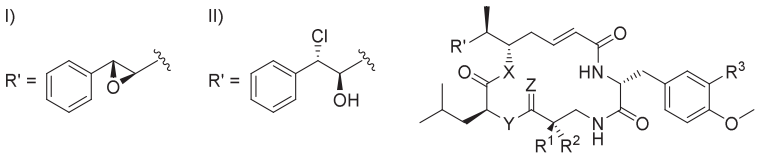
Table 2. Isosteric replacement of ester bonds and its impact on cytotoxicity of cryptophycin [98,101–103]



R'	R^1	R^2	R^3	X	Y	Z	IC_{50} [nM] (cell line)
I	Me	H	Cl	O	O	O	0.009 (KB) (C-1)
II	Me	H	Cl	O	O	O	0.019 (KB) (C-8)
I	Me	Me	Cl	O	O	O	0.022 (CCRF-CEM) (C-52)
II	Me	Me	Cl	O	O	O	0.027 (CCRF-CEM) (C-55)
I	Me	Me	Cl	O	O	H,H	1.1 (CCRF-CEM)
I	Me	Me	Cl	O	NH	O	0.014 (CCRF-CEM)
I	Me	H	Cl	O	NH	O	0.027 (KB)
II	Me	Me	Cl	O	O	H,H	2.7 (CCRF-CEM)
II	Me	Me	Cl	O	NH	O	0.016 (CCRF-CEM)
II	Me	H	Cl	O	NH	O	0.020 (KB)
I	H	H	H	O	O	O	0.005 (KB) (C-24)
I	H	H	H	O	CH_2	O	70 (KB)
I	H	H	H	O	O	H,H	40 (KB)
I	H	H	H	NH	O	O	4000 (KB)
I	H	H	H	O	NH	O	6 (KB)
I	H	H	H	NH	NH	O	6000 (KB)
I	H	H	H	O	S	O	0.9 (KB)

assumption is supported by the fact that cryptophycin-52 can be recovered from the corresponding tubulin complex after denaturation of the protein with heat or urea [52]. Moreover, the concentration of cryptophycin-1 remained the same when the drug was treated with tubulin followed by subsequent precipitation of tubulin with ethanol. Under these conditions, the solution would be depleted of cryptophycin-1, if it was bound covalently to tubulin [53]. Additionally, the epoxide function does not react with lysine, glutamic acid, dithiothreitol, and β-mercaptoethanol under physiological conditions because these compounds do not compromise the ability of cryptophycins to inhibit microtubule assembly [52,53].

A conformational change of free tubulin dimers occurs upon binding of cryptophycin [52,54], preventing the polymerization to microtubules. This even occurs when substoichiometric amounts of cryptophycin-1 are administered [46]. However, at high drug concentrations, the tubulin dimers form small ring-shaped oligomers. Cryptophycins can also associate with the surface of microtubule ends [55], inducing a conformational change in the protofilaments, which are cleaved from the microtubule and cyclize to tubulin oligomers. Ring structures consisting of eight or nine tubulin dimers were observed when microtubules were treated with cryptophycin-1 and cryptophycin-52, respectively [54,56]. Low cryptophycin concentrations lead to a strong suppression of

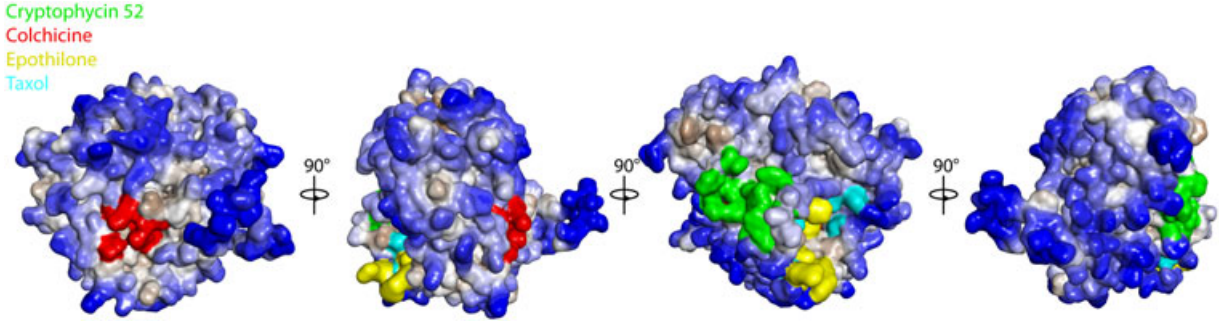


Figure 2. Representation of the different effector binding sites on β-tubulin (red, colchicine; cyan, taxol; yellow, epothilone; green, cryptophycin) [50].

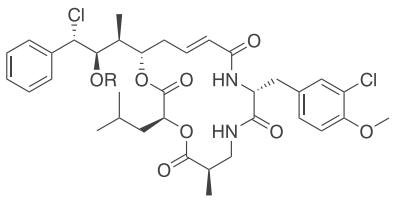
the microtubule dynamics [53,55]. The disruption of the microtubule dynamics leads to apoptosis (programmed cell death) that occurs very rapidly without a prolonged mitotic arrest [57,58]. Additionally, the characteristic morphological changes are induced more quickly with cryptophycin than those of other antiproliferative compounds like paclitaxel, cisplatin, and etoposide [32]. In contrast, cryptophycin disrupts mitotic spindles at concentrations where no loss of interphase microtubules is observed [57,59]. As a result of the disturbance of microtubule dynamics, the apoptosis effector enzyme caspase 3 and the phosphorylation of Bcl2 are activated. Bcl2 is a regulatory protein functioning as an apoptosis suppressor. Bcl2-phosphorylation induced by cryptophycin-52 or its chlorohydrin, cryptophycin-55, is three orders of magnitude higher than that of paclitaxel [60,61].

Cryptophycins show an extraordinarily high biological activity both *in vitro* and *in vivo*, exceeding that of paclitaxel and vinblastine by the factor of 100 to 1000. The IC_{50} values of cryptophycin-1 against various tumor cell lines are in the low picomolar range, e.g. 4.58 pM against KB cells and 7.63 pM against the LoVo cell line [37]. In addition, cryptophycins exhibit a very high cytotoxicity against MDR tumor cell lines. In many cases, expression of P-glycoprotein (permeability glycoprotein, P-gp), the gene product of the MDR1-gene, is responsible for the development of resistance to anticancer agents. This adenosine triphosphate-dependent drug efflux pump has low substrate selectivity and removes a broad range of structurally diverse xenobiotics out of the cells, keeping the intracellular drug concentration at a low level [62]. Polar or amphiphilic compounds are good substrates for P-gp [63], while cryptophycins are unpolar and, therefore, rather poor substrates. In this context, the cytotoxicity of cryptophycins against MDR cell lines exceeds that of vinblastine, paclitaxel, and colchicines.

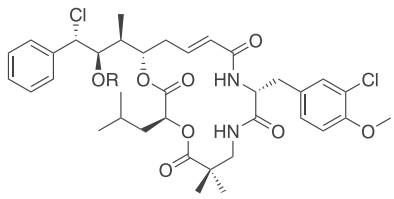
In general, cryptophycins have a higher affinity towards tubulin than other antimitotic compounds [52] and form very stable drug–tubulin complexes [55]. The stability of such complexes results in low concentrations of free cryptophycins inside the cells, and thus, the drug is not a substrate of P-gp. This might also be a reason for the high activity of cryptophycins against MDR cell lines. Cryptophycins are also very rapidly up taken by cells and accumulated inside the cells. In this context, HeLa cells were treated with ^{3}H-labeled cryptophycin-52 at a constant concentration of 11 pM. Within 20 h, the intracellular drug concentration was increased to 8 nM, which is 730 times higher than the environmental concentration [55]. Their biological activity is maintained after treatment, and cell growth is inhibited for a longer time after the end of administration than in the case of paclitaxel and vinblastine, presumably owing to the tight cryptophycin–tubulin complex [59,64]. A prolonged delay of tumor growth was observed for a combination of cryptophycins with other antitumor agents relying on various modes of action, e.g. paclitaxel (tubulin-interacting agent), doxorubicin (topoisomerase II inhibitor), fluorouracil (antimetabolite), or platinum complexes (DNA-damaging agents). The combination therapy was especially effective when cryptophycin was applied together with a substance of a different mode of action. In this case, the overall efficiency exceeded the sum of the individual effects [65].

Hence, cryptophycin-52 is a very active antitumor agent with superior cytotoxicity compared with many of the conventional drugs used in oncology. Therefore, cryptophycin-52 was considered as a potential chemotherapeutic agent, and it was chosen as the first-generation cryptophycin to undergo clinical evaluation [66]. Phase I clinical trials indicated that toxicity was rather linked with the cumulative dose than with schedule dependence [66,67]. As a result of these studies, cryptophycin-52 was applied as second-line therapy in phase II clinical studies on treatment of metastatic colorectal cancer and non-small cell lung cancer where patients had been previously treated with platinum-based therapy [68]. Unfortunately, a lack of efficacy *in vivo* combined with toxicity effects such as arthralgia, constipation, myalgia, and neuropathy was observed, leading to a discontinuation of the clinical trials. In particular, the high neurotoxicity is a major side effect of all currently used antimitotic drugs because microtubules are essential for cells of the nervous system. Neurons are the most abundant source of tubulin inside the body [23]. However, partial response was reported for some patients with platinum-resistant advanced ovarian cancer, but in many cases, the observed toxicities prevented the administration of effectively high doses of cryptophycin-52.

In addition, cryptophycin-55, the chlorohydrin correlate of cryptophycin-52, and the glycinate of this compound (cryptophycin-55gly), were found to be highly active in preclinical trials (Figure 3). Cryptophycin-55 exhibits an improved pharmacokinetic and rapid distribution into various tissues after intravenous application into mice. Even a pronounced tumor selectivity was observed (relative exposure; tumor 80.8%, plasma 3.9%, kidney 3.4%, liver 1.1%, and intestine 2.8%) [69]. Cryptophycin-8, the chlorohydrin of cryptophycin-1, showed high antitumor activity *in vivo* against a number of MDR tumors, and moreover, it was found to be less toxic [70]. In conclusion, the chlorohydrins are distinguished by promising pharmacokinetics and a 100-fold to 1000-fold increased antitumor activity compared with the epoxide parent compounds [71]. It is noteworthy that the chlorohydrins were found to be converted to the corresponding epoxides under physiological conditions and in animal studies [71]. This conversion was also observed in formulation (containing 5% ethanol and 5% cremophor) under different storage conditions, preventing the formulation of stable solutions. Enhanced stability as well as improved water solubility of the chlorohydrin compounds was obtained after esterification with glycine. In particular, cryptophycin-249 (the glycinate of cryptophycin-8) shows a high antitumor activity against an MDR mammary tumor



R = H cryptophycin-8
R = $COCH_2NH_2 \cdot HCl$ cryptophycin-249



R = H cryptophycin-55
R = $COCH_2NH_2 \cdot HCl$ cryptophycin-55gly

Figure 3. Cryptophycin chlorohydrins and related chlorohydrin glycinates.

Journal of PeptideScience

model and has been, therefore, considered as a promising second-generation clinical candidate [71]. As the glycinate analogs as well as the chlorohydrins can be converted to the related epoxide-containing cryptophycins, these derivatives can be considered as prodrugs of the epoxide parent compound.

Cryptophycin Analogs and SAR Studies

Besides the naturally occurring cryptophycins, numerous synthetic derivatives have been synthesized for SAR studies to improve tumor selectivity and water solubility. In this context, the influence of virtually any structural motif on the bioactivity of this compound class has been investigated with emphasis on modifications at units A and C.

Unit A

The methyl substituent of unit A together with (*S*)-configuration at the adjacent stereogenic center is crucial for a high biological activity irrespective of the substitution pattern of this region [32,72,73]. The most striking feature of unit A in cryptophycins is the epoxide moiety, and several analogs with modifications in the C2′–C3′ position have been synthesized to clarify its role. Meanwhile it is accepted that the epoxide is not opened to form a covalent bond with tubulin; however, this structural motif is of significant importance for high biological activity. In this regard, both the presence of the epoxide and the (*R*,*R*)-configuration are essential. The replacement of the epoxide function by a thiirane leads to a 200-fold reduced cytotoxicity, while replacement by an aziridine or a *trans*-styrene provides compounds with approximately 70-fold lower cytotoxicity (Figure 4) [74]. An even stronger decrease of the bioactivity was observed after inversion of the stereochemistry of one or both stereogenic centers: the (*S*,*R*)-configured *cis*-epoxide [41] is 8000 times less active and the (*R*,*S*)-isomer is 72,500 times less active. In the case of the (*S*,*S*)-configured α-epoxide [72,75], a considerable loss of cytotoxicity by the factor of 6000 was obtained (Figure 4). The hydrolysis of the epoxide moiety to the corresponding diol [32] leads to a 500-fold reduced cytotoxicity *in vitro*. However, the corresponding halohydrins obtained after acidolysis of the epoxide function with HX (X = Cl, Br, I) retain the *in vitro* activity of the parent compound [37,76]. Compared with the epoxide-containing cryptophycins, the analogous chlorohydrins, e.g. cryptophycin-55, exhibit a 100-fold to 1000-fold increased bioactivity *in vivo*. Additionally, they are of outstanding interest because of their wider therapeutic window [71]. Their stability in aqueous solutions is decreasing with increasing atomic number of the halogen owing to the higher reactivity, and over time, the epoxides are regenerated in an intramolecular S_N-reaction [76]. In particular, cryptophycin-55 is converted to the corresponding epoxide with a turnover of 9% within 48 h [71].

Stable derivatives of cryptophycin-55 are obtained upon esterification of the secondary alcohol with glycine (*vide supra*). It is noteworthy that they display increased *in vivo* cytotoxicity [71]. As mentioned earlier, the halohydrins and their glycinate derivatives can be considered as cryptophycin prodrugs because they are converted to the biologically active epoxides under physiological conditions. A library of chlorohydrin esters was synthesized to evaluate the cytotoxicity *in vitro* and activity *in vivo* based on the substitution pattern of the carboxylic acid [77,78]. While cryptophycin-55gly showed the highest cytotoxicity *in vitro*, cryptophycin-249, the chlorohydrin glycinate derived from cryptophycin-1, had the best activity *in vivo* against a variety of MDR tumor cell lines (Figure 3) [71].

Truncated cryptophycins have also been studied based on cryptophycin-24 (arenastatin A), as a parent compound [79]. These modifications led to a dramatically reduced cytotoxicity (Figure 5). The best analog, which contains a *para*-nitrophenyl substituent, showed a strong inhibition of α-tubulin and β-tubulin owing to the binding to β-tubulin in the same manner than other cryptophycins.

Modifications of the phenyl ring of unit A in cryptophycin-52 were another focus of SAR studies (Figure 6). A methyl substituent in *meta*-position or a *meta*-methyl substituent and a *para*-methyl substituent have only a limited influence on cytotoxicity, which is marginally decreased by a factor of 7 or 2, respectively. In contrast, a methyl group in both *ortho*-position and *para*-position results in 20,000-fold reduced activity, and the corresponding analog is almost inactive. Additionally, the exchange of the phenyl ring against a methyl substituent or a thiophene ring leads to decreased bioactivities [74]. The *para*-hydroxy functionalized cryptophycin-51 derivative exhibits a significantly reduced bioactivity, which, however, most likely is due to the missing epoxide moiety [77,80]. The low water solubility of cryptophycin-52 is one of its major drawbacks, and therefore, more polar cryptophycin-52 unit A analogs have been investigated. The introduction of a *para*-hydroxymethyl group results in a 5-fold to 10-fold increased activity against the human leukemia cell lines CCRF-CEM [80] and HL-60 [77], but at the same time, the activity against the MDR subclone HL-60/Vinc is decreased by a factor of 27. Three *para*-alkoxymethyl analogs have been synthesized, aiming to improve bioactivity and

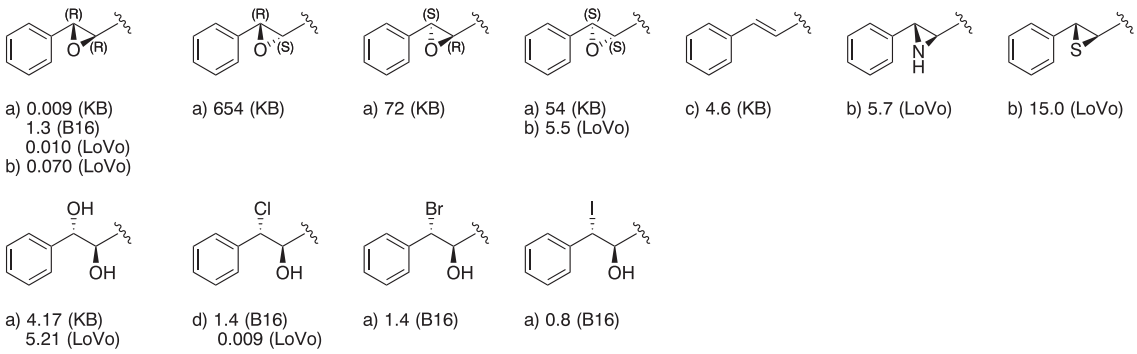


Figure 4. Structures and cytotoxicities of cryptophycins with modified epoxide moieties (IC_{50} values in nM). Parent compounds: (a) cryptophycin-1, (b) cryptophycin-52, (c) cryptophycin-51, (d) cryptophycin-55. Cell lines: KB, human cervical carcinoma; B16V, murine melanoma; LoVo, human colon carcinoma.

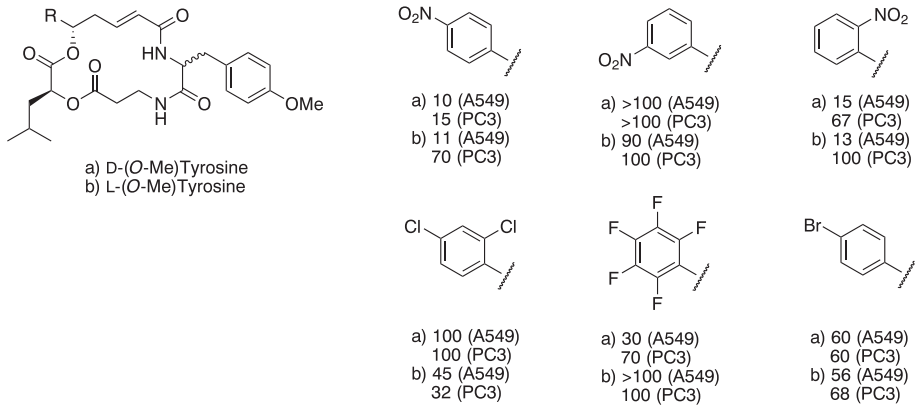


Figure 5. Structures and cytotoxicities of cryptophycin with truncated epoxide chain (IC_{50} values in μM). Cell lines: A549, lung cancer; PC-3, cervical cancer.

water solubility, while the high activity of cryptophycin-52 against MDR cells is retained [81]. These derivatives were evaluated using the non-MDR human cervical carcinoma cell line KB-3-1 and its MDR subclone KB-V1. Best results were obtained for the *para*-methoxymethyl analog, which is more cytotoxic than the parent structure. However, it is also a slightly better substrate for P-gp in the KB-V1 cells, which leads to a fivefold decreased activity against this MDR cell line. The cytotoxicity of the *para*-isopropoxymethyl analog is comparable with that of cryptophycin-52, while the activity against the MDR cells was reduced by a factor of 3.5.

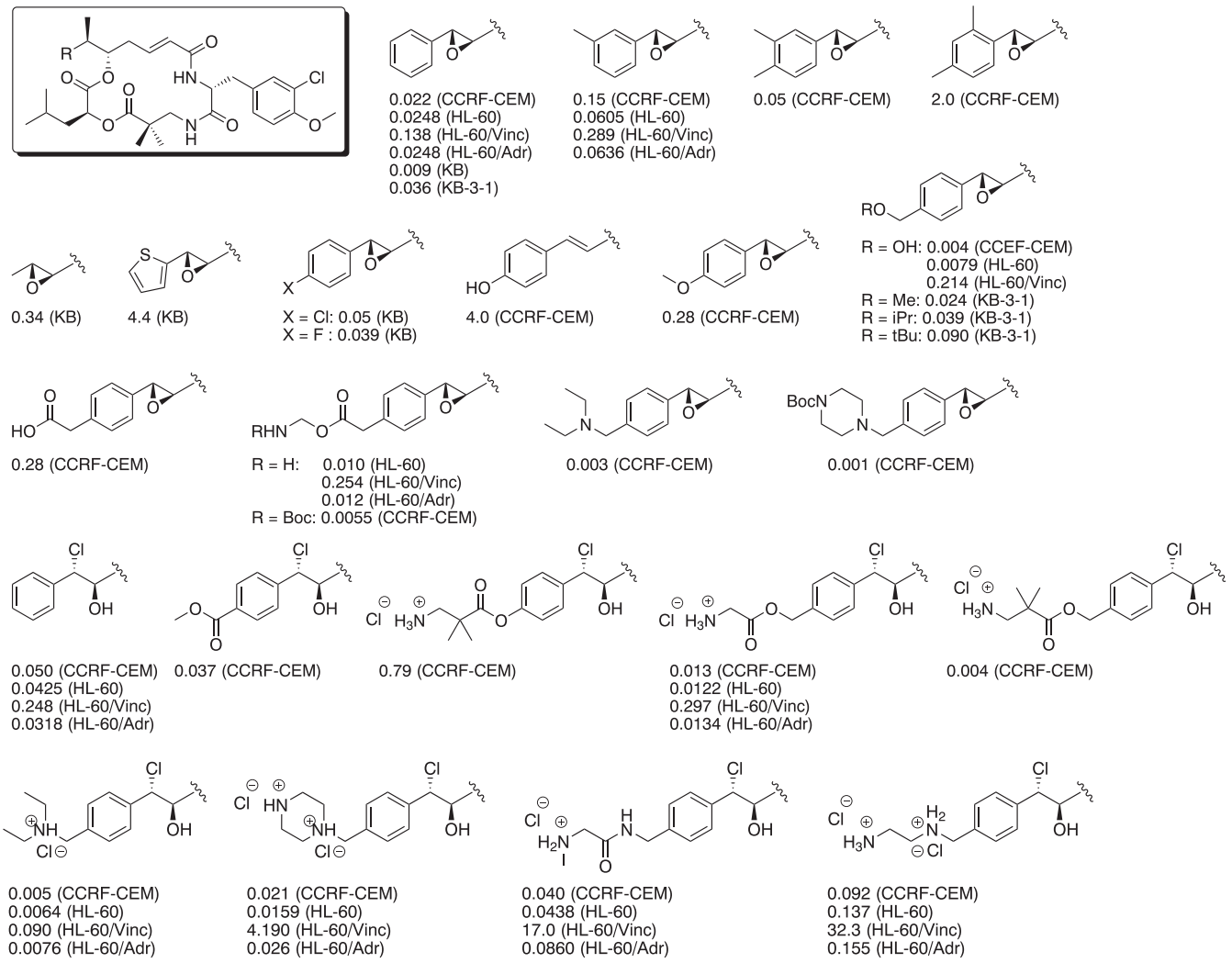


Figure 6. Cytotoxicities of unit A-modified cryptophycin-52 analogs (IC_{50} values in nM). Cell lines: CCRF-CEM, human T-cell leukemia; HL-60, human acute myelocytic leukemia; HL-60/Vinc, P-gp-expressing vincristine-resistant subclone of HL-60; HL-60/Adr, MRP1-expressing adriamycine-resistant subclone of HL-60; KB-3-1, human cervical carcinoma.

Journal of PeptideScience

The *para-tert*-butoxymethyl analog exhibits slightly decreased bioactivities against both cell lines presumably owing to the steric bulk of the alkoxy substituent. The resistance factor of an MDR cell line for a specific drug is defined as the ratio of IC_{50} values for the MDR cell line and the non-resistant cell line. Hence, it is a measure for reduced cytotoxicity by resistance mechanisms.

Amino derivatives are very active *in vitro*, but *in vivo* experiments in mouse model show high systemic toxicity and, therefore, a narrow therapeutic window [80]. Polar substituents increase water solubility and lead to an enhanced biological activity of the corresponding cryptophycin derivatives on the one hand [77,80], but on the other hand, such polar analogs display reduced cytotoxicity against MDR tumor cell lines compared with the parent structure cryptophycin-52 [77]. The transmembrane ABC transporter P-gp is reported to prefer hydrophobic or slightly cationic substrates [82,83]. In this context, cell lines expressing P-gp, e.g. HL-60/Vinc cells, show a very strong resistance against the polar modified cryptophycins, while HL-60/Adr cells that express the MRP1-transporter are barely resistant against these analogs. Consequently, polar substituents at the phenyl ring are assumed to render cryptophycins into better substrates of the P-gp efflux pump [77]. Amino-functionalized cryptophycins exhibit particularly high resistance factors.

Unit B

The native 3-chloro-*O*-methyl-D-tyrosine building block of cryptophycin-1 and cryptophycin-52 is indispensable for high biological activity. Modifications at unit B with small polar substituents are tolerated to a certain extent but in general lead to less active analogs [32,41,75]. Improved cytotoxicity by variation of the native unit B fragment could not yet be achieved. In this regard, the exchange of the 3-chloro-4-methoxyphenyl residue by a phenyl ring results in an eightfold decreased bioactivity (Figure 7). Homologation with an additional methylene group leads to a 3000 times less active analog compared with the phenyl modified compound. The chloro substituent is also important for cryptophycin-1 analogs, as its omission leads to eightfold decreased bioactivity, while a more significant loss is observed when an additional chloro substituent is introduced in *ortho*-position to the *para*-methoxy group [75,84]. The cytotoxicity of cryptophycin-1 is also decreased significantly when both the *O*-methyl group and the chloro substituent are omitted. A less significant decrease is observed for an additional chloro substituent, which is in *ortho*-position to the hydroxyl group. Similar results are obtained in case of a phenyl ring substituted with a *para*-amino group. Such an analog shows a 460-fold decreased cytotoxicity, while the corresponding derivative with *para*-amino group and an adjacent chloro substituent gave only a 26-fold loss of activity. Interestingly, high biological activity is observed for the chloro substituted *para*-dimethylamino analog, for which the cytotoxicity is reduced by only a factor of 2.5 compared with that of cryptophycin-52. The replacement of the aryl group against structurally aberrant aryl or alkyl residues, for example, a cyclohexyl ring, results in a substantial loss of activity; an even bigger loss is observed in the case of the α-naphthyl and β-naphthyl substituted cryptophycins. Inversion of the stereochemistry at C^{α} results in an almost inactive cryptophycin-24 analog [72].

Two cryptophycin analogs with an elongated unit B fragment have been reported (Figure 8) [85]. The Arndt–Eistert reaction

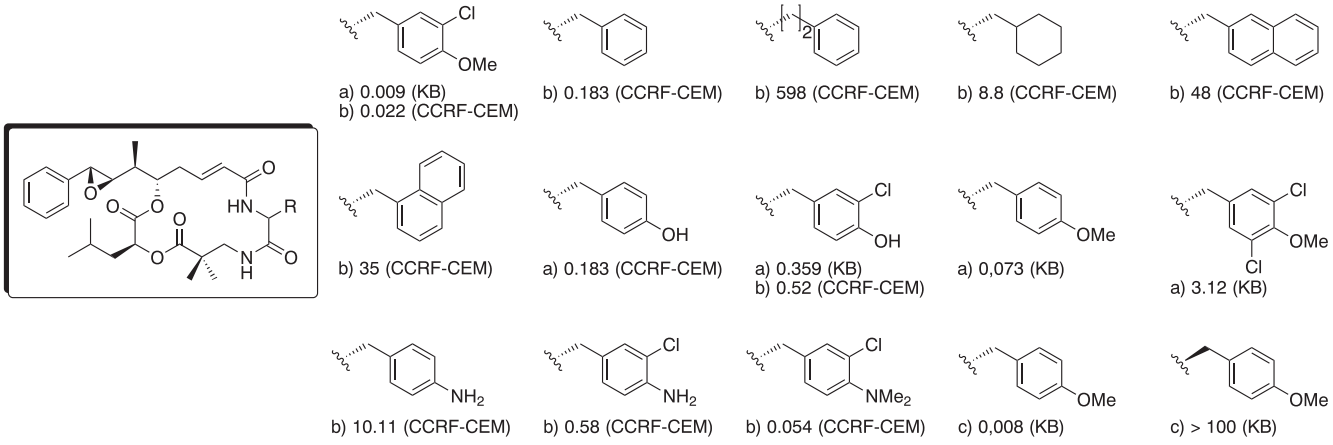


Figure 7. Structure–activity relationship studies of unit B building block (IC_{50} values in nM). Parent compounds: (a) cryptophycin-1, (b) cryptophycin-52, and (c) cryptophycin-24. Cell lines: CCRF-CEM, human T-cell leukemia; KB, human cervical carcinoma.

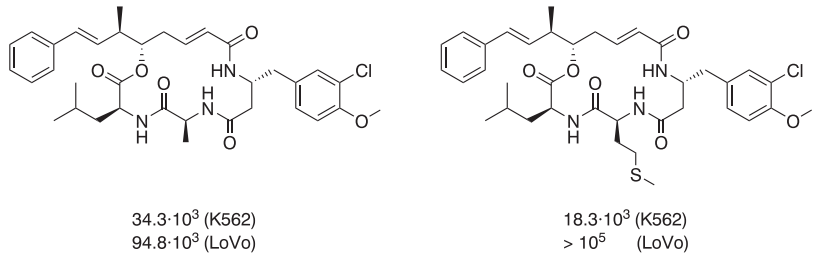


Figure 8. Cryptophycin analogs with an elongated unit B building block (IC_{50} values in nM) [85]. Cell lines: K562, multidrug-resistant human leukemia; LoVo, human colon carcinoma.

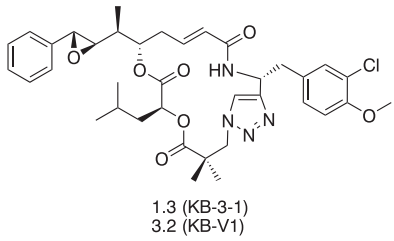


Figure 9. The cryptophycin-52 triazole analog can be considered as a mimic of the *trans*-amide parent structure (IC_{50} values in nM) [92].

served as the key step in the synthesis of the corresponding homologated unit B building block, a β-amino acid. At the same time unit C, natively a β-amino acid was replaced by the α-amino acids D-alanine or D-methionine, respectively. This keeps the size of the macrocycle constant but virtually shifts the amide group. Simultaneously, the ester bond between units C and D was replaced by a more stable amide bond. Moreover, both analogs lack the β-epoxide, which might explain the low cytotoxicities observed against 15 cell lines. In each case, the IC_{50} value exceeded 3 μM, adding further proof that modifications at unit B result in a significantly reduced bioactivity.

Conformational analysis of cryptophycin derivatives revealed a *trans*-amide bond between units B and C both in solution and in solid state, while a *cis*-amide bond prevails between units A and B [32]. Although size and dipole moment of 1,4-disubstituted triazole rings are larger in comparison with *trans*-amide bonds [86], these triazole rings have been suggested to act as *trans*-amide mimetics because they exhibit almost identical physicochemical properties [87]. Furthermore, the triazole ring is metabolically inert because it cannot be hydrolytically cleaved, oxidized, or reduced under physiological conditions [88]. The bioisosterism of 1,4-disubstituted 1*H*-1,2,3-triazoles and *trans*-amide bonds have been proven in many cases [89–91], and therefore, the peptide bond between units B and C was replaced by a 1,4-disubstituted triazole ring to corroborate this bioequivalence (Figure 9) [92]. For the synthesis of such a cryptophycin-52 triazole analog, an alkyne-functionalized unit B building block was coupled via Cu^{I}-catalyzed azide–alkyne cycloaddition to an appropriate azide-modified unit C fragment. Indeed, the high biological activity of cryptophycin-52 was largely maintained for the corresponding triazole analog: The cytotoxicity of the triazole analog (IC_{50} = 3.2 nM) against the P-gp-expressing MDR cells KB-V1 decreased by fivefold only compared with cryptophycin-52 (IC_{50} = 0.7 nM) [92]. This preservation of cytotoxicity for the triazole analog is remarkable because the 16-membered macrocycle of cryptophycin-52 is extended to a 17-membered ring.

Unit C

Natural cryptophycins contain either (*R*)-β-aminoisobutyric acid (e.g. cryptophycin-1) or the α-unsubstituted building block β-alanine (e.g. cryptophycin-24). Upon extraction of cryptophycins from their natural sources, Moore and co-workers observed acyclic products by methanolysis, giving the first hint that the ester bond between units C and D might be sensitive towards hydrolysis [32]. In this context, cryptophycin-24 is hydrolysis sensitive under physiological conditions owing to the absence of an α-substituent and, hence, shows a half-life period of only 10 min in mouse serum [93]. Additionally, the *in vivo* activity is marginal. With increasing steric shielding of this ester bond by additional adjacent substituent, the stability of the corresponding cryptophycins increases and, therefore, cryptophycin-1 is more stable *in vivo* and exhibits a higher *in vivo* activity than cryptophycin-24.

In order to enhance the stability of cryptophycins by increasing the steric demand of the unit C–D ester bond, different α,α-dialkylated β-amino acids ($\beta^{2,2}$-amino acids) have been incorporated as unit C building blocks into the cryptophycin macrocyle (Figure 10) [94]. The exceptionally potent analog

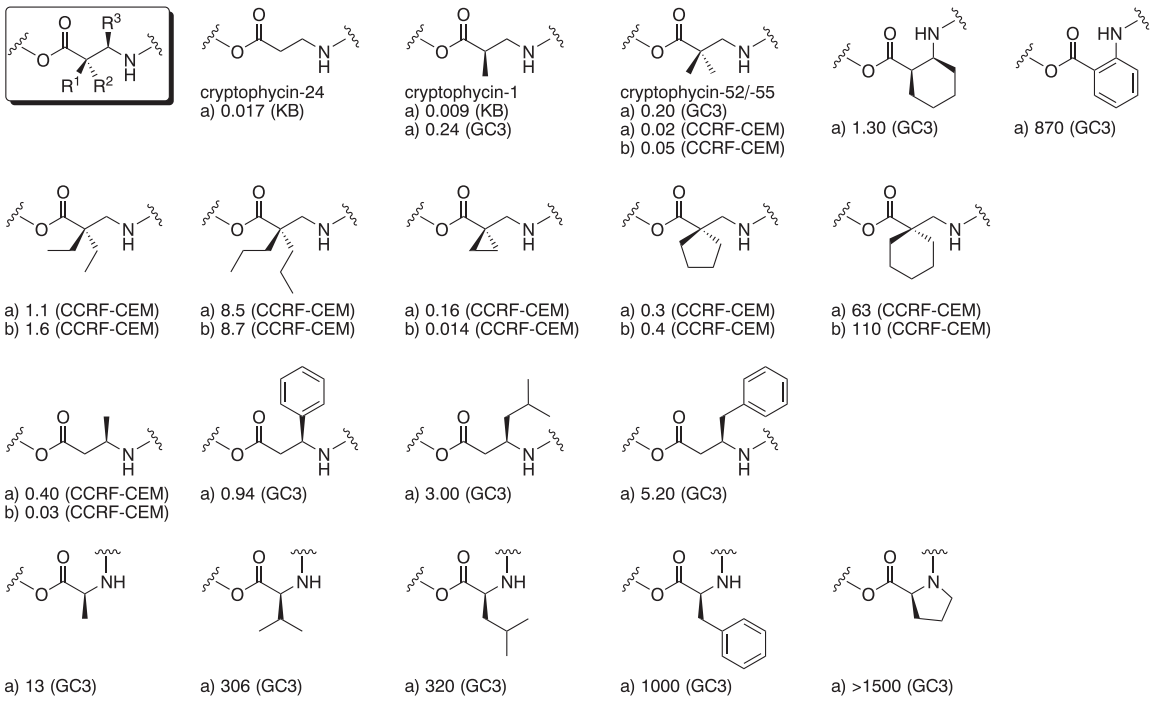


Figure 10. Structure–activity relationship studies of unit C building block (IC_{50} values in nM). Parent compounds: (a) epoxide and (b) chlorohydrin. Cell lines: CCRF-CEM, human T-cell leukemia; GC3, human colon carcinoma; KB, human cervical carcinoma [32,94,95].

Journal of PeptideScience

cryptophycin-52 bears an α,α-dimethyl substituent. The related chlorohydrin, cryptophycin-55, also exhibits a high *in vitro* cytotoxicity, while the corresponding α,α-diethyl compounds display 30-fold to 50-fold less activity. The biological activity is further decreased by a factor of 175 to 400 in case of the α,α-di-*n*-propyl analogs. One might assume that the interaction of the higher substituted analogs with tubulin is hindered by steric effects. Additionally, the water solubility of these analogs is reduced with increasing lipophilicity leading to lower efficiency. The same tendency is observed for the corresponding cyclopropyl-substituted, cyclopentyl-substituted, and cyclohexyl-substituted spiro compounds. In particular, the cyclopropyl analog of chlorohydrin cryptophycin-55 shows an activity comparable with that of its parent. Considerably lower *in vitro* cytotoxicities are observed for the higher substituted spiro compounds [94]. While the *in vitro* activity of cryptophycin-1 could be improved with the analogs cryptophycin-52 and cryptophycin-55, the same is not generally true for the *in vivo* activity [77].

Shifting the methyl substituent of unit C of cryptophycin-1 from the α-position to the β-position seems to influence the *in vitro* activity for the cryptophycin-1 analog with an epoxide in unit A, but not for the related chlorohydrin. However, with an increasing steric demand of the alkyl or aryl residues at the β-carbon atom, the *in vitro* cytotoxicity is more and more decreased [95]. In addition, the *in vivo* activity of the α-unsubstituted analogs in mouse serum was low as well because of the hydrolytic instability of the sterically non-shielded unit C–D ester bond. Introduction of the α,β-disubstituted unit C building block *cis*-2-amino-cyclohexanoic acid results in a sevenfold decreased cytotoxicity, while the conformationally restricted 2-aminobenzoic acid analog is virtually inactive [95].

Replacement of the unit C β^2-amino acid by various α-amino acids leads to a drastic loss of activity [95]. The incorporation of L-alanine results in a 30-fold to 50-fold less active analog, while the cytotoxicity decreases dramatically in the case of the higher substituted L-amino acids valine, leucine, phenylalanine, and proline. The corresponding cryptophycins are almost inactive presumably because of the constricted 15-membered macrocycle, which is not able to adopt the required conformation for an efficient interaction with tubulin.

Recently, the synthesis and biological evaluation of cryptophycin-1 analogs modified by polar unit C building blocks were reported (Figure 11) [96]. Additionally, the influence of the stereochemistry at the α-position of this fragment was investigated. Many of these analogs prove to be highly active against KB-3-1 cells with IC_{50} values in the picomolar range. However, the IC_{50} values against the MDR subclone KB-V1 are only in the nanomolar range compared with cryptophycin-52 (IC_{50} = 0.7 nM). The amphiphilic nature of these cryptophycins presumably makes them better substrates for the P-gp efflux pump.

In summary, there is a certain tolerance concerning the introduction of moderately bulky, non-polar alkyl substituents into the native unit C building block. Particularly, the steric hindrance of the labile unit C–D ester bond by two geminal methyl groups in the α-position is favorable. However, the bioactivity decreases with increasing alkyl residues. There is no clear-cut tendency regarding the stereochemistry. The β-amino acid framework is substantial for the active 16-membered macrolide structure and to provide the necessary conformational flexibility. The polar unit C functionalized cryptophycins show high cytotoxicities against KB-3-1, but they are less active against MDR tumor cells.

Unit D

Unit D modifications have not yet been investigated extensively, although it has been observed that some variation in that region is tolerated. With this regard, even cryptophycins isolated from natural sources contain one of four different α-hydroxy acids as the unit D fragment. The most potent cryptophycin derivatives bear an isobutyl group, but the alternative *sec*-butyl, isopropyl, and *n*-propyl residues lead to only slightly decreased cytotoxicities (Figure 12) [32]. Interestingly, the bulky non-natural neopentyl group proved to be equally as effective *in vivo* as the isobutyl residue [65]. Furthermore, unit D is the only building block of the cryptophycin backbone in which inversion of the stereochemistry does not have an extremely deleterious effect, although naturally

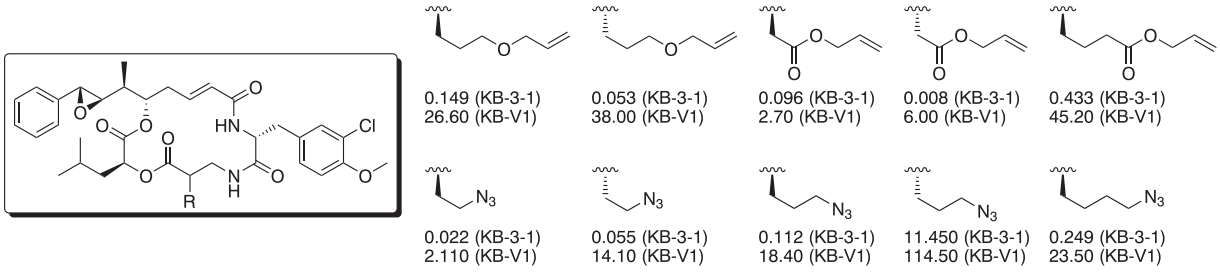


Figure 11. Polar functionalized unit C analogs of cryptophycin-1 and the corresponding cytotoxicities (IC_{50} values in nM) [96]. Cell lines: KB-3-1, human cervical carcinoma; KB-V1, P-gp-expressing multidrug-resistant subclone of KB-3-1.

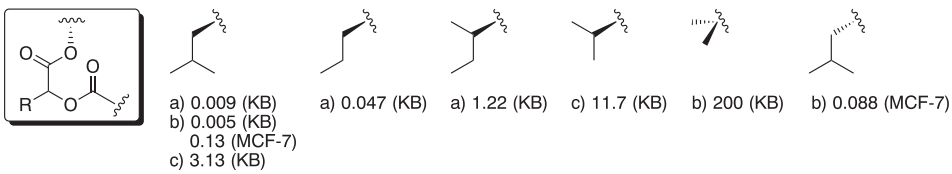


Figure 12. Structure–activity relationship studies of unit D building block (IC_{50} values in nM) [32,65,97,98]. Parent compounds: (a) cryptophycin-1, (b) cryptophycin-24, and (c) cryptophycin-3. Cell lines: KB, human cervical carcinoma; MCF-7, human breast adenocarcinoma.

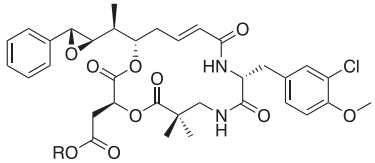
occurring cryptophycins exclusively possess L-configured α-hydroxy acids [97]. A cryptophycin-24 analog with (*R*)-configuration at unit D was almost as potent against MCF-7 cells as the (*S*)-configured natural parent. By contrast, a significant reduction of bioactivity by the factor of 40,000 was found when an achiral α,α-disubstituted unit D fragment was incorporated into cryptophycin-24 [98]. It can be excluded that the low activity results from a labile ester bond between units C and D because this analog was stable in mouse serum, but rather it can be concluded that the alkyl side chain of unit D is involved in the interaction of cryptophycin with tubulin.

More recently, the synthesis of two cryptophycins with functional groups in unit D was reported (Figure 13) [99]. The allyl ester protected analog can be considered as the precursor for the free acid derivative, and the deprotection step using $Pd(PPh_3)_4$ catalyzed allyl transfer to morpholine provided the cryptophycin analog with a free carboxy group in good yield. Both unit D cryptophycin analogs containing an allyl ester or a free carboxylic acid show high activities against the KB-3-1 cells with IC_{50} values of 14.5 or 88.6 pM, respectively. In the case of the P-gp-expressing MDR cell line, the allyl ester modified analog (IC_{50} = 0.66 nM) remains highly potent, while the cytotoxicity of the free acid cryptophycin (IC_{50} = 372 nM) is reduced by a factor greater than 1000. This analog might be a better substrate for P-gp owing to the increased amphiphilicity [100].

Isosteric Replacement of Ester Bonds within the Cryptophycin Backbone

In order to enhance the stability of cryptophycins towards hydrolysis, labile ester bonds were substituted by more stable functionalities (Table 1). The ester bond between units C and D can be replaced by an amide bond without compromising cytotoxicity, because the corresponding derivatives of cryptophycin-1, cryptophycin-8, cryptophycin-52, and cryptophycin-55 are almost as potent as the parent compounds [101]. However, an ether bond at the same position is tolerated to a minor extent, and such cryptophycin analogs exhibit 50-fold to 100-fold reduced cytotoxicities presumably owing to the missing hydrogen bond accepting carbonyl group.

Replacement of the ester bond between units C and D by a more stable amide bond in case of cryptophycin-24 results in a 1,200-fold decreased bioactivity [98,102]. A thioester bond at the same position does not affect cytotoxicity as much, and the corresponding cryptophycin-24 analog is 180-fold less active than the parent structure [98], although the thioester is even more hydrolysis sensitive in mouse serum than the analogous ester. In contrast, the exchange of the same ester bond by a 4-deoxy methylene ketone leads to a hydrolytically stable compound, but the bioactivity drops by a factor of 14,000. When the ester bond between units A and D is replaced by an amide bond, a 800,000-fold less active analog is observed. The labile C–D ester bond is still present in this compound, presumably causing the low activity, and additionally, this isoster is poorly water soluble [102]. The replacement of both ester bonds by amides leads to a cyclopeptide analog, which is completely inactive [102]. This compound is extreme hydrolytically stable but almost insoluble in polar solvents like water, dimethylsulfoxide, or alcohols, which might be the reason for the missing activity. It is noteworthy that such cryptophycin-24 amide that contains polar unit A building blocks are both significantly more water soluble and more potent *in vitro* as well as *in vivo* [103].



I) R = $CH_2CH{=}CH_2$ 0.015 (KB-3-1) 0.662 (KB-V1)
II) R = H 0.089 (KB-3-1) 372.2 (KB-V1)

Figure 13. Unit D-modified cryptophycin-52 analogs with functional groups in the side chain for bioconjugation and prodrug design [99]. Cell lines: KB-3-1, human cervical carcinoma; KB-V1, P-gp-expressing multidrug-resistant subclone of KB-3-1.

Fluorinated Cryptophycins

Elementary fluorine is very rare in nature owing to its high redox potential. Consequently, and because of the low abundance of free fluoride ions in the environment compared with chloride ions, there are only very few naturally occurring fluorinated compounds known [104]. One of the first synthetic fluorinated pharmaceuticals is the antineoplastic agent 5-fluorouracil, which was reported for the first time in 1957 and brought to market by Hoffmann-La Roche in 1962. Since then, fluorinated drugs have become more and more important over the last decades, and presently, more than 20% of all pharmaceuticals contain at least one fluorine atom [105–107]. Fluorination is supposed to improve bioavailability and receptor selectivity [108]. Hydrogen can be exchanged by fluorine without significant changes of the molecular size or shape because of the similar van der Waals radius [109]. The high electronegativity of fluorine substituents substantially affects the electronic properties of the parent compound.

Moore *et al.* reported the total synthesis of analogs of cryptophycin-1 and cryptophycin-52 fluorinated in unit A (Figure 14) [110]. Derivative **B** bearing a fluorine substituent in *para*-position proved to be remarkably active with an IC_{50} value of 39 pM against the human cell line KB-3-1. Hence, its *in vitro* activity is well comparable with that of cryptophycin-1 (IC_{50} = 29 pM) [111]. Additionally, the chlorohydrin of compound **E** (IC_{50} = 33 pM, CCRF-CEM), an analog based on cryptophycin-52 (IC_{50} = 22 pM, CCRF-CEM), was patented by Eli Lilly as a very promising clinical candidate [112]. The same research group produced the unit B fluorinated cryptophycins **F** and **G** by fermentation (Figure 14). For this purpose, strains of *Nostoc* sp. were cultivated in media enriched with the appropriate phenylalanine derivatives, which served as precursors for the unit B building blocks. However, the biological activity of these analogs against KB-tumor cells has not been explicitly described.

Cryptophycin-52 analogs with more than one fluorine substituent have recently been published (Figure 15) [113]. The CF_3-functionalized compound is about fivefold less active against the tumor cell line KB-3-1 in comparison with cryptophycin-52, while the pentafluorophenylalanine analog shows a significant loss of cytotoxicity, most likely owing to the absence of the methoxy group. Substantially lower activity was noted for both derivatives against the MDR subclone KB-V1. The amphiphilicity presumably leads to increased clearance by the P-gp efflux pump. Despite reduced activity *in vitro*, it would be very interesting to evaluate *in vivo* cytotoxicity of these analogs and to further investigate the influence of fluorine substituents.

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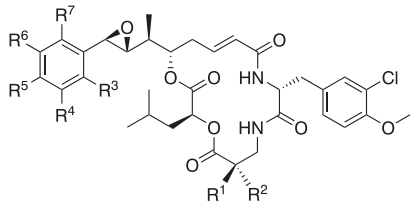
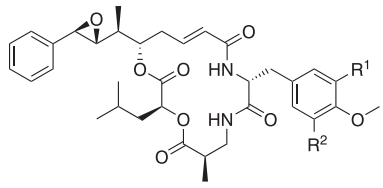
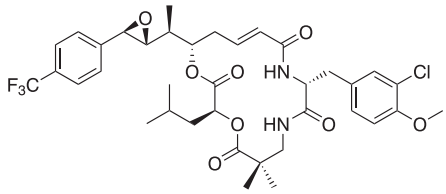


compound	R^1	R^2	R^3	R^4	R^5	R^6	R^7
A	Me	H	F	H	H	H	H
B	Me	H	H	H	F	H	H
C	Me	H	H	F	H	F	H
D	Me	Me	H	F	H	H	H
E	Me	Me	H	H	F	H	H

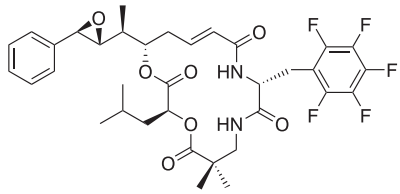


F: R^1 = F; R^2 = H
G: R^1 = Cl; R^2 = F

Figure 14. Fluorinated analogs of cryptophycin-1 and cryptophycin-52 [110–112].



0.066 (KB-3-1)
10.11 (KB-V1)



2.97 (KB-3-1)
98.36 (KB-V1)

Figure 15. Highly fluorinated cryptophycin-52 analogs and the accompanying cytotoxicities (IC_{50} values in nM) [113]. Cell lines: KB-3-1, human cervical carcinoma; KB-V1, P-gp-expressing multidrug-resistant subclone of KB-3-1.

Cryptothilone 1 – A Cryptophycin–Epothilone Hybrid

Cryptophycins and epothilones are both very potent antimitotic natural products that inhibit cell proliferation by interfering with tubulin polymerization. However, they are of largely different origin. While cryptophycins have been isolated from cyanobacteria [30,37,59], the latter originate from the myxobacteria *Sorangium cellulosum* [114]. It is noteworthy that they bind to different sites of the microtubule and differ in their effect on tubulin polymerization. As described earlier, the cryptophycin family acts as microtubule destabilizers binding to the ends of microtubules and, hence, leads to degradation of the tubulin polymers. In contrast, epothilones are microtubule stabilizers known to bind to an interior region of the microtubule adjacent to the taxol binding site. Epothilones reduce the dissociation rate of tubulin dimers upon binding to the β-subunit of tubulin while acting as a stapling device and stabilizing the polymeric structures.

Comparing the structural features of natural cryptophycin-4 and *trans*-epothilone C, a synthetic analog with similar tubulin polymerization properties compared with the natural 12,13-*cis* isomer, striking similarities along with one significant difference become obvious (Figure 16) [115]. Both compounds have in common (i) a 16-membered ring, (ii) an aryl substituent joined to a conjugated double bond (that is epoxidized in some cryptophycin analogs), (iii) a methyl substituent at or in close proximity to the conjugated double bond, (iv) an (*S*)-configured stereogenic carbon with an oxygen substituent to involved in lactonization, and (v) an alkene (which is epoxidized in epothilones A and B) segregated from the acyloxy carbon by one methylene moiety. Besides these similarities, the cryptophycin segment comprising the two amide bonds is rather rigid, albeit the C8–C11 region of epothilones is quite flexible. A hybrid structure comprising the similarities was synthesized to evaluate the different tubulin binding modes typical for each compound class. Cryptothilone 1 encloses the upper half of cryptophycin-4 and the lower part common to most epothilones (Figure 16) [115]. However, this type of hybrid molecule turned out to be inactive with respect to interfering with tubulin polymerization or depolymerization at concentrations up to 40 μM. Hence, it is assumed that this compound has affinity neither for the cryptophycin nor for the epothilone/taxol binding site.

Cryptophycin Conjugates

Practically all cytotoxic agents that may be used in tumor therapy do not display any selectivity between healthy tissue and tumor tissue, as the cytotoxic agent targets cellular structures like microtubules or DNA. Consequently, many cytotoxic agents display a very narrow therapeutic window and, hence, cannot be applied in tumor therapy. Conjugates of cytotoxic agents (also called 'payload') and a homing device that would direct the conjugate selectively to tumor tissue have been designed to overcome this problem. As homing devices, monoclonal antibodies, peptides, and small molecules have been proven amenable. In particular, antibody–drug conjugates (ADCs) have been emerging as a novel therapeutic concept [2–4]. The homing devices selectively address receptors that are overexpressed on the tumor cell surface. Upon binding of the conjugates to the target receptor, these complexes

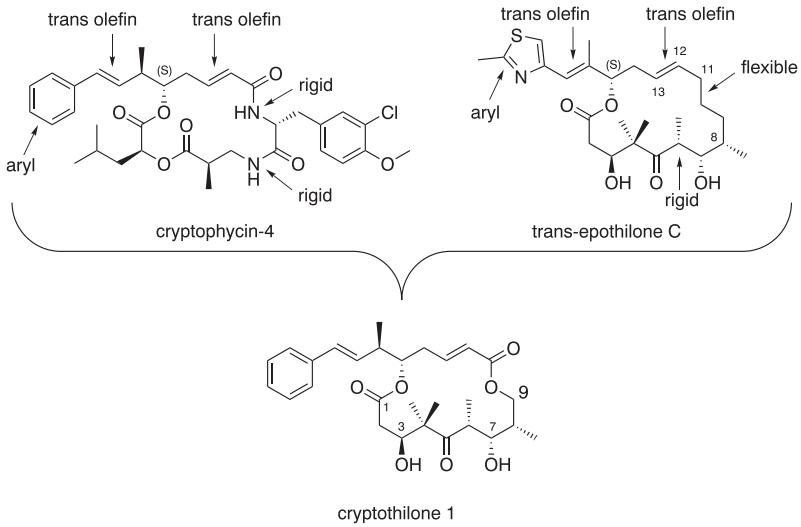


Figure 16. Structural homology of cryptophycin-4 and *trans*-epothilone C as well as the hybrid molecule cryptothilone 1.

are internalized by the cell, e.g. by receptor-mediated endocytosis. Ideally the conjugates do not display significant cytotoxicity, but the toxic payload is being liberated in the lysosomes after endocytosis. This strategy enables the application of highly toxic compounds that would otherwise also damage healthy cells.

Having failed in clinical phase II studies, cryptophycin-52 still remains a promising cytotoxic agent, provided targeted delivery to tumor cells can be achieved. A conjugate involving folic acid as the targeting device together with a polar linker, a disulfide moiety for intracellular release, and a carbonate based on cryptophycin-55 has been claimed by *Endocyte* (Figure 17) [116]. The folate-based conjugates address cancer cells as they are known to overexpress folate receptors. The IC_{50} value against KB cells was in the low nanomolar range and cytotoxicity could be competed against by the addition of free folate.

The aromatic ring of unit A in cryptophycin-52 can be subjected to modification in *para*-position without significantly compromising biological activity. These modifications have been employed by Sanofi-Aventis as attachment points for antibody conjugation [117,118]. Either cleavable or non-cleavable linkers were used to connect the cytotoxic payload and the antibody hu2H11, a monoclonal antibody addressing the EphA2 receptor (Figure 18). Some conjugates display sub-nanomolar cytotoxicity against the breast gland cancer cell-line MDA-MB-231, which can be competed against by free antibody.

Genentech has also used the *para*-position of the aromatic ring of unit A as a handle for the conjugation to an antibody [119]. Specifically, the payload was conjugated to the cysteine mutant A118C that targets HER2 and CD22 receptors (Figure 19). The conjugates displays sub-nanomolar cytotoxicity against cell lines

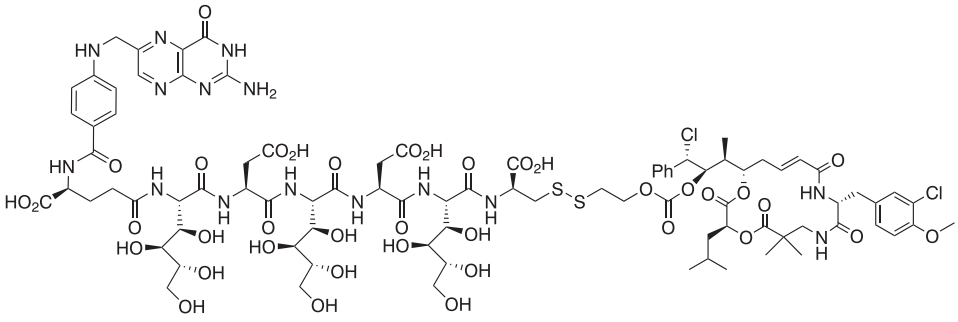


Figure 17. Cryptophycin conjugate with folic acid as the homing device [116].

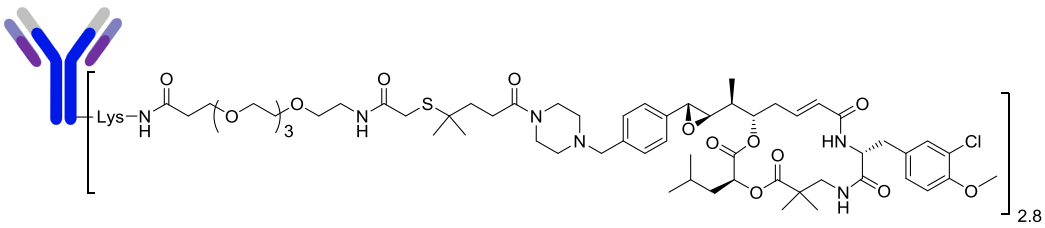


Figure 18. Antibody–drug conjugates with a cryptophycin-52 derivative modified in unit A connected across a cleavable *tert*-butylthio linker to the monoclonal antibody hu2H11 [117,118].

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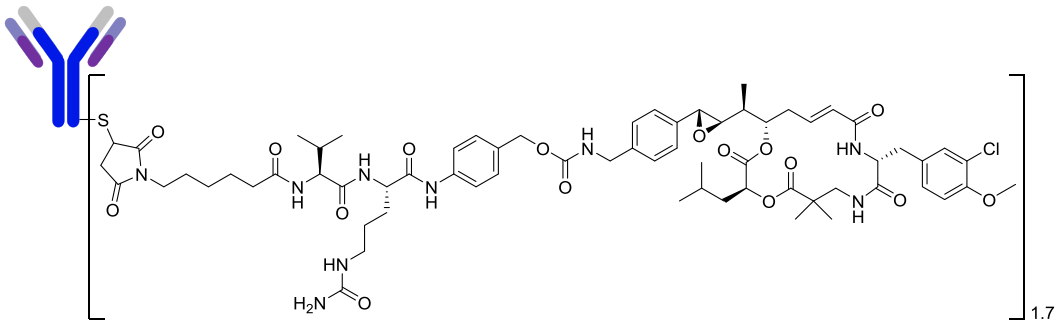

Figure 19. Antibody–drug conjugates with a cryptophycin-52 derivative modified in the unit A that targets HER2 and CD22 receptors [119].

overexpressing HER22 or CD22 receptors, while they are inactive with cell lines that do not overexpress these receptors.

More recently, the use of cryptophycin-55 glycinate as a prodrug has been acknowledged in a patent [120]. The cryptophycin-55 glycinate has been conjugated to the antibody trastuzumab, which targets HER2 receptors, through a cleavable linker (Figure 20). The conjugate displayed nanomolar activity against SK-BR3 cells and inactivity in other cell lines that were not expressing the HER2 factor.

Peptides might represent a viable alternative to protein-based homing devices. Peptides can be obtained by chemical synthesis and are, therefore, easier to purify than recombinant proteins. For targeting purposes, RGD peptides have been envisaged. A conjugate comprising a cyclic RGD peptide connected across a modified carboxyfluorescein residue to a unit C modified cryptophycin (Figure 21) has been proven to bind to the integrin $\alpha_V\beta_3$ that is highly expressed on some tumor cells [96]. The complex undergoes internalization by integrin-mediated endocytosis. Confocal microscopy studies with this conjugate and WM-115 human epithelial cancer cells proved the lysosomal localization of the fluorescent conjugates. This result shows that integrin $\alpha_V\beta_3$ is a suitable target structure for tumor targeting.

The prodrug cryptophycin-55 glycinate has also been used to prepare a conjugate with octreotide peptide (Figure 22) [120].

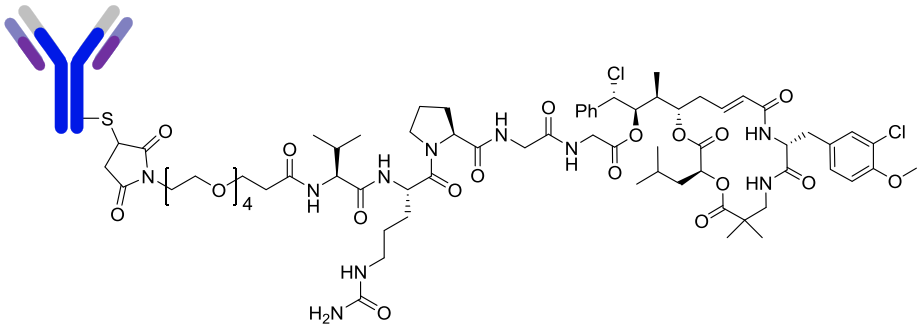

Figure 20. Antibody–drug conjugates with a cryptophycin-55 glycinate as a prodrug that targets HER2 receptors [120].

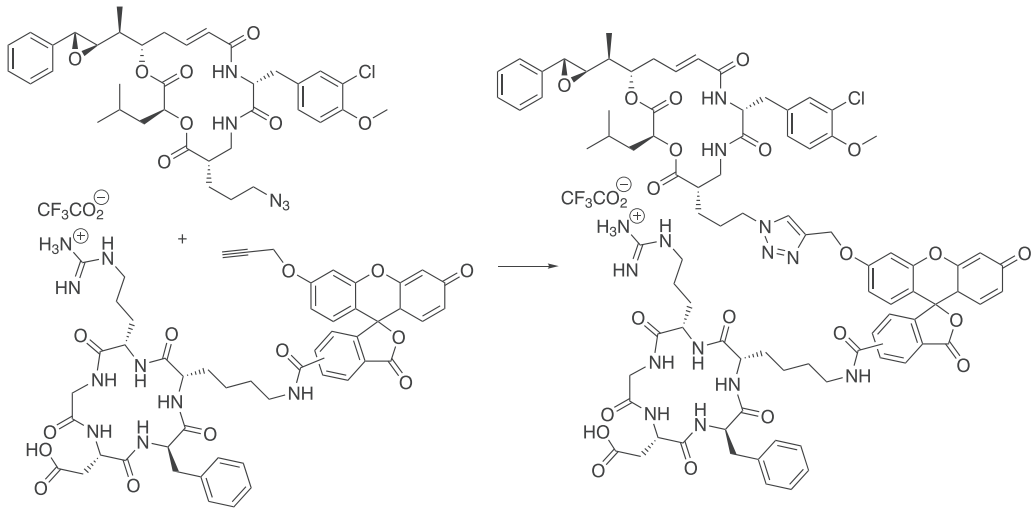

Figure 21. Conjugate of a cryptophycin modified in unit C with a fluorescently labeled RGD peptide [96].

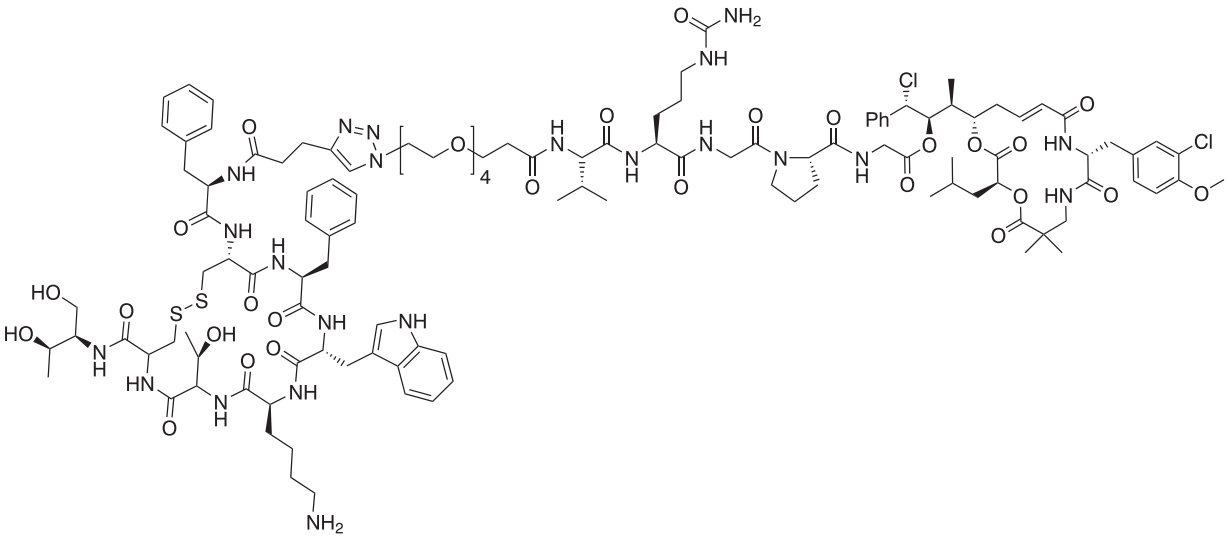

Figure 22. Conjugate of cryptophycin-55 glycinate with an octreotide peptide [120].

Octreotide targets somatostatin receptor subtypes that are highly expressed in neuroendocrine tumors. Cytotoxic assays of the conjugate show promising selectivity for cell lines overexpressing somatostatin receptors.

Conclusions

Many cryptophycin derivatives have been isolated from natural sources or have been obtained by total synthesis in order to provide extensive SAR data. The eminent biological activity of some cryptophycin analogs especially against MDR tumors renders them promising candidates for treatment of such tumors. Although cryptophycin-52 failed in clinical phase II studies, there is still an interesting perspective for the application of cryptophycin derivatives as bioconjugates in tumor-targeting approaches.

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