Bachelor's project

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Epothilones as Anticancer Drugs

Bachelor's project in Chemistry

Supervisor: Eirik Johansson Solum

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Abstract

Cancer is a serious disease that is often hard to treat. New drugs that can limit the growth of the cancerous cells are therefore sought. A well known problem in the treatment of cancer is multi-drug resistant cells, but a group of molecules known as the epothilones can inhibit cell growth also for some resistant cell lines. The epothilones will inhibit uncontrolled growth by tubulin stabilizing. Since the discovery of the epothilones´ anticancer effect in the 1990s the epothilones have been modified, synthesised and tested to further increase this effect and emphasise the wanted properties. Researchers are making changes to these areas to increase the anticancer activity, decrease the toxicity, and improve the metabolic stability. When making modifications to the natural epothilones, some areas are more prone to alteration than others. Among these are the C3 hydroxy group, the C12-C13 epoxide group, C1 lactone and the C15 moiety. Another strategy to emphasise the wanted properties is to map the binding site of the epothilones. The mapping is done to increase the knowledge of what features that contribute to make a good bonding between the epothilones and tubulin. The current challenge is to make epothilone analogues that have even better anticancer effect than the epothilone drugs that are available today.

Contents

1 Introduction

Cancer is the second major cause of death to humans world wide, only exceeded by cardiovascular diseases[1]. In 2018 the most common cancer in women was breast cancer with a total of 2.09 million documented cases worldwide and 627 000 deaths[2]. Traditional cancer treatments include chemotherapy, radiation therapy, and surgery. Chemotherapy involves the use of cytotoxic drugs which will damage all cells with a high rate of cell division and cause apoptosis. Throughout human history natural compounds have played a vital role in medicine and the treatment of cancer is no exception[3]. In the search for new cytotoxic agents natural products have proven to be a leading source[4]. Among the chemotherapeutic agents used today more than 60% are derived from structures found in nature[4]. This includes the group of anticancer compounds discussed in this thesis, the epothilones.

The epothilones are a part of a group of microtubule-targeting drugs that earlier mainly consisted of taxanes. These drugs are important in the treatment of many types of cancer, including breast cancer[5]. Epothilones are of great interest in this field due to their antitumor activity, also against some taxane-resistant cell lines. In addition, the epothilones are easier to extract from bacteria and are more water soluble than taxanes, and therefore easier to deliver to the cancer cells[6,7]. Many derivatives of epothilone have been synthesised and tested, but challenges considering the epothilones´ microtubule-binding activity and pharmacokinetics have required a lot of effort. Despite this, the first epothilone-drug was approved by the US Food and Drug Administration (FDA) in 2007 and is now being used to treat breast cancer[8]. In addition, a compound of an unknown structure called utidelone (UTD1) is undergoing phase III clinical trials (last phase before possible FDA approval) for treatment of breast cancer[9].

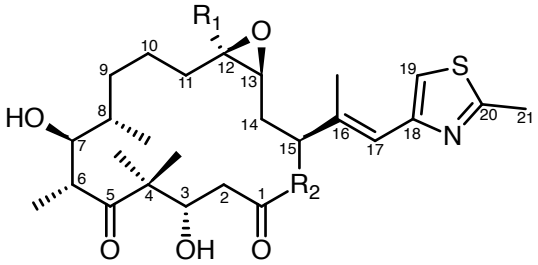
This thesis will study epothilones as anticancer drugs and elaborate on how to improve their anticancer activity based on knowledge about their binding mechanisms and binding site. There will also be given some information on the synthesis of this drug.

2 Theory

2.1 General

Epothilones are a class of natural compounds that have been shown to have antitumor activity[10]. The first epothilone was isolated and identified by Höfle *et al.* in 1996 from the *Sorangium Cellulosum* bacteria[11]. They observed that the epothilones had a narrow antifungural effect and a high cytotoxicity, and suggested that the compound could work as an antitumor agent. Since this discovery, there have been numerous scientists looking into the chemistry and function of epothilones. Different derivatives of epothilone have been classified in categories from A to F, where epothilone A (**1**) and B (**2**) are among the most researched. The only structural difference between these two derivatives is a methyl group located at C12 of **2**. Considering the difference in activity, it is observed that **2** has a 14-fold higher activity than **1** (see table 2.1)[7]. The lactam analogue of **2**, in which the C1 oxygen is replaced with a nitrogen, is called ixabepilone (**3**) and was the first member of the epothilone family to be approved for drug use[5]. Ixabepilone is known under the marketing name Ixempra and was approved by the (FDA) in October 2007 as an antitumor agent[5]. This discovery was motivated by the development of taxane-

resistant tumors in the mid-1990s and was a result of the synthesis and testing of over 350 synthetic derivatives of epothilone[3,12]. When comparing the IC_{50} values of **2** and **3** the activity of **3** is 0.90 times the activity of **2** (see table 2.1)[3]. The IC_{50}, the half-maximal inhibitory concentration, indicates how much of the drug is needed to inhibit a biological process by half and is therefore a measure of the drugs potency[13]. The main advantage of **3** is therefore not its activity, but its good metabolic stability[5]. This new epothilone joins a group of tubule-stabilising antitumour-agents that earlier consisted mainly of taxanes.



1, R_1 = H, R_2= O
2, R_1 = CH_3, R_2= O
3, R_1 = CH_3, R_2= NH

Figure 2.1: The structure of epothilone A (**1**), epothilone B (**2**) and ixabepilone (**3**). Adapted from Chen and Heal[14].

2.2 Mechanism of antitumor properties

Epothilones are a part of a group of compounds with anti-neoplastic properties due to their ability to bind to α- and β-tubulin[15]. α- and β-tubulin are proteins that assemble to form tubular structures called microtubules (MTs). The α- and β-tubulin structures consist of strands of amino acids that are coiled together in secondary protein structures like β-sheets, α-helixes and loops[16]. The loops are mainly serving to bind the helixes to the sheets, but are shown to have an important role in the polymerization of MTs[17]. Variations in the loops can affect MT-flexibility and therefore alter the MTs function.

An average of thirteen tubulin-proteins interact to form circular structures (protofilaments) which again assembles to form the MTs[18]. MTs play a critical role in mitosis and are therefore an interesting target considering potential anticancer drugs[19]. During mitosis the MTs form a so called mitotic spindle, a bipolar structure designed to split the duplicated chromosomes of the mother cell into two identical sets before cleavage of the cell. The MTs will create a stable environment for the chromosomes to bind to the MTs, and then separate[18].

MTs are highly dynamic structures that undergo cycles of growth and shrinkage regulated by numerous proteins[19]. This property is called dynamic instability and is vital for the MTs ability to contribute in the splitting of chromosomes[18]. The correct splitting of chromosomes and a proper cleavage of the cell require the mitotic spindle to work rapidly, making this process extremely sensitive to microtubule-

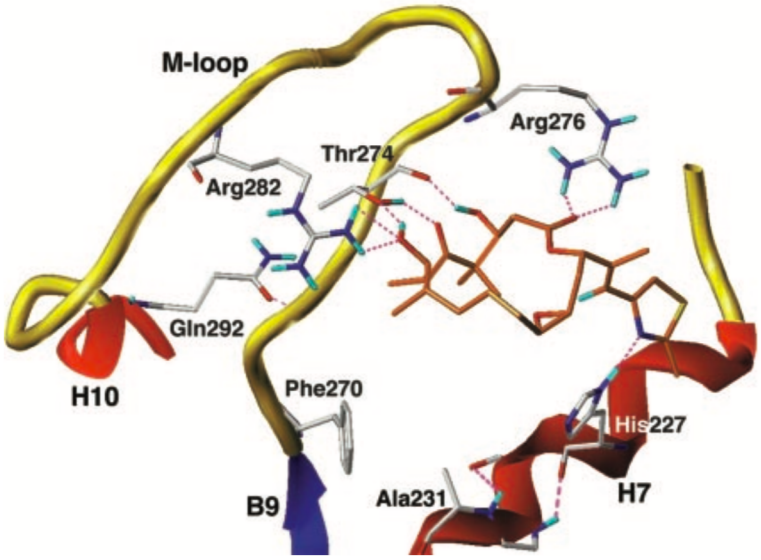


Figure 2.2: Structure of **1** binding to the M-loop and the H7 of tubuline. Hydrogen bonding of the oxygen atoms from C1 to C7 and of the thiazole nitrogen to thr274, arg282, arg276 and his227 is marked with dotted lines. From Nettles *et al.*[6]. Reprinted with permission from AAAS.

targeting drugs[15]. Jordan and Wilson have reported that drugs that can suppress the MTs dynamic properties can stop the mitosis and kill the cells[15]. Epothilones are doing this by binding to the interior surface of the MTs[15]. The presence of a single chromosome that is unable to bind to the spindle, and therefore unable to split, can be enough to prevent the cell from dividing and thus undergo apoptosis (programmed cell death)[15].

Much effort have been put in to mapping the epothilones specific binding mode to tubulin. It was, for a long time, assumed that the epothilones had a pharmacophore similar to that of taxol, but this turns out to be only partly true[6]. A pharmacophore describes the steric and electronic features that is acquired to achieve binding interactions with the target molecule and blocking its biological response[24]. Although the epothilones and taxols overlap in their binding site of tubulin, each one of the molecules exploits the binding pocket in a unique way. It is suggested that the epothilones bind to the following amino acids of the tubulin protein: thr274 (threonine), arg282 (arginine), arg276 and his227 (histidine)[6]. When bonding it is assumed that polar and hydrophobic groups of the epothilone will bind to the complementary site at the protein as shown in figure 2.2. This epothilone binding-model is a result of combining nuclear magnetic resonance (NMR), electron crystallography (EC) and molecular modeling and can give a great deal of information about the binding structure and binding-site of epothilones[6].

2.3 Synthesis of epothilones

Even though epothilones can be extracted from bacteria, the total synthesis of the compounds is of great importance due to their interesting biological properties as an antitumor agent. We can differentiate between three main strategies concerning the synthesis of epothilones[25]. All three routes follow the same strategy when it comes to the making of three buildingblocks which are then put together. The ap-

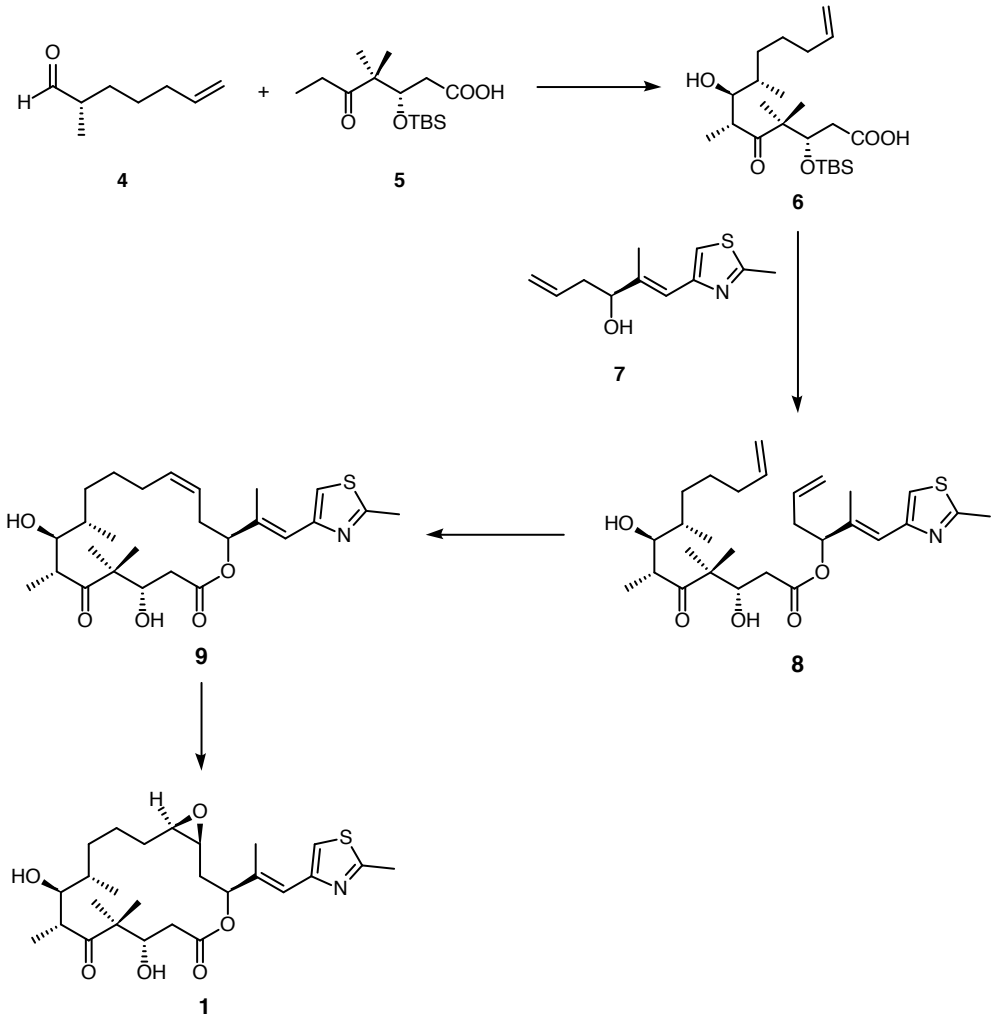
Table 2.1. Cytotoxicity of some modified analogues of **1** and **2**[20–23]. The values given here are relative and based on the cytotoxic values of **1** and **2**. The values are collected from different studies and are therefore just meant to be used to compare the activity of the modified structures relative to **1** and **2** and not relative to other modified structures. [a] From studies using leukemia cell lines. [b] From studies using overian cell lines. [c] Average IC_{50} values based on several cell lines.

Structure	1	2
1	1.0[b]	0.069[b]
2	14.5[b]	1.0[b]
3	-	0.90[c]
10	0.12[a]	0.016[a]
11	0.28[a]	0.037[a]
12	1.4[b]	0.059[b]
13	1.4[b]	-
14	~ 0[b]	~ 0[b]
15	40.0[b]	16.5[b]
16	-	2.0[b]
17	-	0.12[b]
18	-	0.035[b]

proaches were based on three different ring-closing reactions, namely a RCM (ring-closing metathesis) reaction at the C12-C13 making a dobble bond, ring-closing by esterification at C1-C15 or intramolecular aldol reaction at C2-C3. At last, all three ring-closing methods was followed by the removal of protective groups at the oxygens and epoxidation at C12-C13[25].

Among the first to conduct the total synthesis of an epothilone was Yang *et al.* who synthesised epothilone A in 1997[26]. The plan was to make three buildingblocks (**4**, **5** and **7**) and put them together to make the 16 membered ring, using the RCM strategy. Structure **4** and **5** are joined together in an aldol reaction and the prefered *6R,7S*-isomere (structure **6**) made the dominant product in a 2:1 ratio. Esterification of the mix with structure **7** gave the new compound **8** with a 70% yield of the prefered isomere. The two isomeres were then separated by chromatography and the pure *6R,7S*-isomere proceeded in a RCM reaction to produce **9** in a 50% yield. At last, structure **9** went through an epoxidation at the C12-C13 bond. Epoxidation at the C16-C17 bond was found as a biproduct. These two products were separated by chromatography and the final product (**1**) was found to be identical to the authentic natural **1** by the use of ^1H- and ^{13}C-NMR, mass spectrometry, TLC and HPLC[26].

In later times the esterification have shown to be the most efficient method for the closing of the ring[25]. This is due to the poor selectivity in the aldol reaction and the unsatisfactory *E:Z* ratios achieved using RCM. A study by Panek and Zhu gives an example of the use of esterification at C1-C15[27]. This ring-closing has a total yield of 62%, slightly higher than the synthesis by Yang *et al.*[26,27].



Scheme 2.1. Synthesis of **1** from the three buildingblocks **4**, **5** and **7**. Adapted from Yang *et al.*[26].

Another imperfection in the synthesis conducted by Yang *et al.* was the poor stereoselectivity in the aldol reaction joining **4** and **5**[25,26]. This have been shown to be one of the most critical steps in epothilone synthesis because of the great importance in achiving the *6R,7S*-stereochemistry. The selectivity will vary depending on both the aldehyde (**4**) and the ketone (**5**). The reactants used in the synthesis by Yang *et al.* generally gives low selecvitity (2:1) while other synthesis gives significantly higher ratios. In later times there have been done many modifications to the natural epothilones with the goal of increasing the medicinal effect as an anticancer agent. Some are successful, others not.

2.4 Ixabephilone

The natural epothilones have shown to have a high antitumor activity *in vitro*, but their activity *in vivo* is modest due to poor pharmacokinetics and poor metabolic stability[5]. One of the structural sites that can cause the low metabolic stability is the C1 lactone-structure. At this site esterase cleavage is possible. By

exposing **2** to mouse plasma, research have shown that this leads to a loss of cytotoxic activity and gives a structure with a mass 18 atomic units higher than the parent drug[3]. This change in mass is equivalent to the addition of one oxygen and two hydrogen atoms, and suggests that an opening of the ring has happened. This gives an acid and a hydroxy group[3]. By substituting the ester group the molecule will be allowed to stay in the body for a longer period of time without undergoing esterase cleavage. This will be a great advantage even though it does not affect the activity of the drug directly. This discovery leads to more efforts being put into the synthesis of the lactam analogues of **1** and **2**. It was shown that a semi-synthetic approach, with the use of **1** or **2** as the starting point, gave sufficient results[3]. This was done by a palladium-catalyzed ring opening reaction to form a π-allylpalladium complex. This complex can be trapped by azide (N_3^-) and reduction of the azide accompanied by cyclization completes the synthesis. The lactam analogue of epothilone is the compound which now is known as ixabephilone (**3**)[3]. The concept of exchanging one part of a molecule in order to improve some properties while maintaining others is a widely utilised concept called biososteric replacement[28]. This method is often used in the final, optimizing phase in the discovery of new drugs.

3 Discussion

Many different modifications of the natural epothilones have been made as attempts to improve the tubulin-binding affinity, the metabolic stability, or to decrease the toxicity. Although there are numerous different ideas on how to improve the epothilones´ antitumor activity, there are some areas of interest that reveal themselves more prominently than others. Among these are the C3 hydroxy group, the C12-C13 epoxide region, and the C15 moiety.

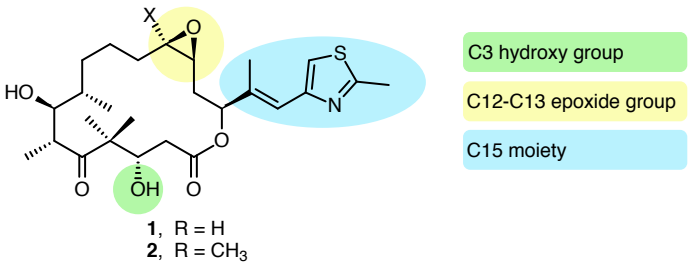


Figure 3.1: Structure **1** and **2** showing the areas where modifications have been made to increase the structures´ antitumor activity. Adapted from Chen and Heal[14].

3.1 The C3 hydroxy group

Concerning the C3 hydroxy group, it has been shown that this location might have an important role in the tubuline-binding mechanism of the epothilones[29]. Studies by Carlomagno *et al.* were using NMR to look at the conformation of tubuline-bound **1** in comparison to the free conformation[29]. This study investigated changes in the C4-C8 region of the molecule which affects the position of the two protons

at C2 and the hydroxy group at C3. The C3 hydroxy group which, in the free conformation, points to the inside of the macrocycle, moves towards the exterior of the macrocycle in the tubuline-bound conformation[29]. This implies that the C3 hydroxy group is significant in the tubulin binding of **1** because of the hydroxy groups ability to form hydrogen bonds to tubulin. On the contrary the α,β-unsaturated analogue of **1** is almost as active as **1** itself, which suggests the opposite. Carlomagno *et al.* suggests that this is due to the double bonds electron donating properties which can mimic the electron donation from the hydroxy group[29]. The same study also insinuates that neither the C5-C8 region nor the C10-C15 region exhibit any significant role in the tubulin binding. This correlates with the fact that these regions are seldom mentioned in literature concerning the alteration of epothilones to increase their antitumor activity.

More specifically the hydroxy group at C3 will bind to threonine (thr274) of tubulin[6]. This applies only to the original *S*-configuration of C3 while the *R*-confirmation is 86 times less active when it comes to tubulin stabilizing[6]. Threonine (thr274), together with arginine (arg282 and arg276) will also corporately engage in the binding of the C3, C5 and C7 oxygen atoms. This will likely happen through hydrogen bonds to the hydroxy group and amino groups of threonine and arginine, respectively. The information above is derived from research by Nettles *et al.* which is not only based on one analysing technique, but three; NMR, EC and molecular modeling[6]. Thus, there is no definitive truth concerning the C3 hydroxy group. Several studies choose to remove this group, while some do not. There is, however, an agreement concerning the conformation of this group due to the fact that the R-conformation eliminates approximately all antitumor activity.

3.2 The C12-C13 epoxide group

The C12-C13 epoxide group has shown to be of great interest, mainly because of the toxicity it causes. Even in dosages lower than those giving cytotoxic effect, **2** is highly toxic[4,20]. Toxicity is reduced by the removal of the epoxide-group. In a study by Chou *et al.* a dose of 0.6 mg/kg of **2** lead to the death of all six mice used, whilst the mice given a dose of 25 mg/kg of **11** all survived and did not show any sign of being poisoned[20]. The epoxide can be substituted with a double bond between C12 and C13. These compounds are named epothilone C (**10**) and epothilone D (**11**), containing a proton or a methyl-group at C12 respectively.

Although the removal of the epoxide group gives a decreased toxicity, the cytotoxicity also decreases. In table 2.1 the cytotoxic activities of **10** and **11** is given relative to the activity of **1** and **2**. E.g. structure **11** shows an activity only 0.037 times the activity of **2**. Considering the removal of the epoxide group to be highly necessary, this reduction in activity can not be avoided. Therefore, instead of just removing the C12-C13 epoxide, some researchers have tried replacing the epoxide group with a cyclopropyl[21]. These researchers found that the *cis*-12,13-cyclopropyl epothilone (**12**) gives an increased activity compared to that of **10** and **11** (see table 2.1). The *trans*-isomer of the same molecule did not show the same increased activity[21].

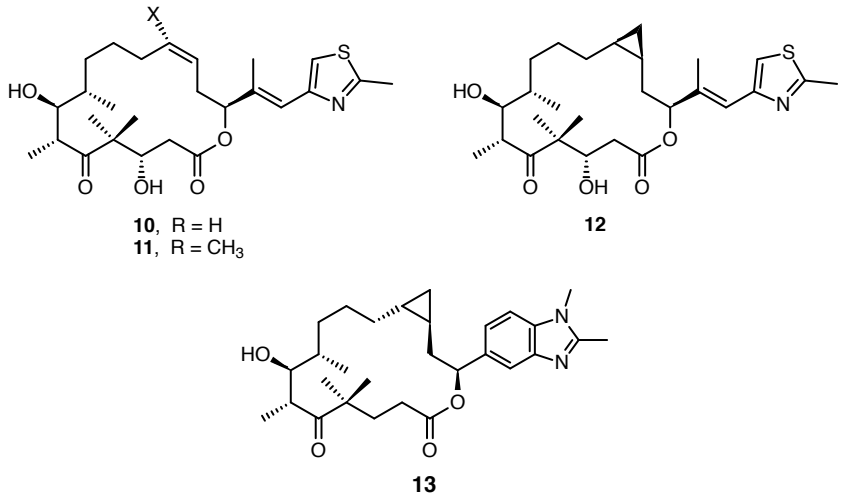


Figure 3.2: Structure of epothilone analogues showing modifications in the C12-C13 region. Adapted from Chou *et al.*, Nicolau *et al.* and Kuzniewski *et al.* [20,21,30]

In a study by Nettles *et al.* it was observed that the C12-C13 epoxide ring is oriented beneath the macrocyclic ring[6]. The epoxide and the C12 side group are then placed in an underlying hydrophobic site of the tubulin protein and the C12 side group will therefore not interrupt the epothilone´s antitumor activity[6]. This means that the extension of the C12 methyl side group of **2** will not eliminate activity, but neither increase the activity. Importantly, even though the extension of the C12-methyl does not increase the activity, the C12-methyl itself increases the activity significantly compared to the structure lacking this group (**1**)[7]. The difference between **1** and **2** is defined by this methyl-group, and **2** shows a 14.5-fold higher activity than **1** (see table 2.1)[7]. It is important to concider this aspect when evaluating the activity of different epothilone analogues. Analogues showing a good activity compared to **1** can not necessarily compete with **2**.

In a study by Kuzniewski *et al.* an epothilone analogue, deviating even more from the **1**, was synthesised and tested[30]. They synthesised a molecule differing from **1** by lacking the C3 hydroxy-group and the C16 methyl-group. In addition, they replaced the *cis*-epoxide ring at C12-C13 with a *trans*-cyclopropane moiety (see structure **13**)[30]. Compared to **1** this new structure shows a slightly increased activity (see table 2.1)[30]. Based on studies regarding the loss of the C3 hydroxy group and the C12-C13 epoxide discussed earlier, these changes should lead to the loss of activity[20,29]. This means that the increased activity **13** exhibits must be caused by the substitution at the C15-moiety. This is supported by the increased polymerization as a result of the substitution in the C15 moiety shown in structure **20**[23]. In this study the substitution of the thiazole ring of **2** with the aromatic, polycyclic group of **20** gives a 9% increased tubulin polymerization.

Similar to the researchers mentioned above, Kuzniewski *et al.* chose to investigate the *trans*-isomer of the C12-C13-cyclopropyl analogue[21,30]. They found that the C10-C11-dehydro analouge of **13** was virtually inactive compared to **13** itself and, suggested that this is due to the restriction of the torison angle

about this bond to 180° [30]. Data from X-ray crystallography shows that both the C9-C10 and the C10-C11 bonds of **13** are antiplanar when bound to tubulin. This implies that the ability to adjust the torison angle of these bonds are crucial for the bonding of 12,13-*trans*-epothilones to tubulin. The restriction of these angles to 180° by introducing a double bond will therefore harm the compounds antitumor activity. For the 12,13-*cis* analogues of **13**, it is not the case. The X-ray crystallography results from these molecules shown planar confirmations of the C9-C10 and C10-C11 bonds when bound to tubulin. This leaves the conclusion that the *trans*-analogues depends on the adjustment of the C9-C11 region to be able to bind to tubulin while the *cis*-analogues does not need to make the same adjustments [30].

3.3 The C15 moiety

The C15 moiety, with its thiazole ring, are also pointed out as an important site when it comes to the antitumor properties of epothilones [6,22,23,29,31]. By investigating the changes in conformation that occurs when epothilones bind to tubulin with X-ray crystallography, Carlomagno *et al.* have characterized the nitrogen atom of the thiazole ring as important [29]. They suggest that the change in the conformation that occurs when the epothilone binds to the tubulin makes the nitrogen atom more accessible for potential hydrogen-bonding. The same study points out the C16-C17 double bond as significant in determining the orientation, and thereby the binding of the thiazole ring to tubulin [29]. When evaluating these results it should be taken into account that this study uses non-polymerized tubulin *in vitro*, which differs from the polymerized tubulin found *in vivo* [29].

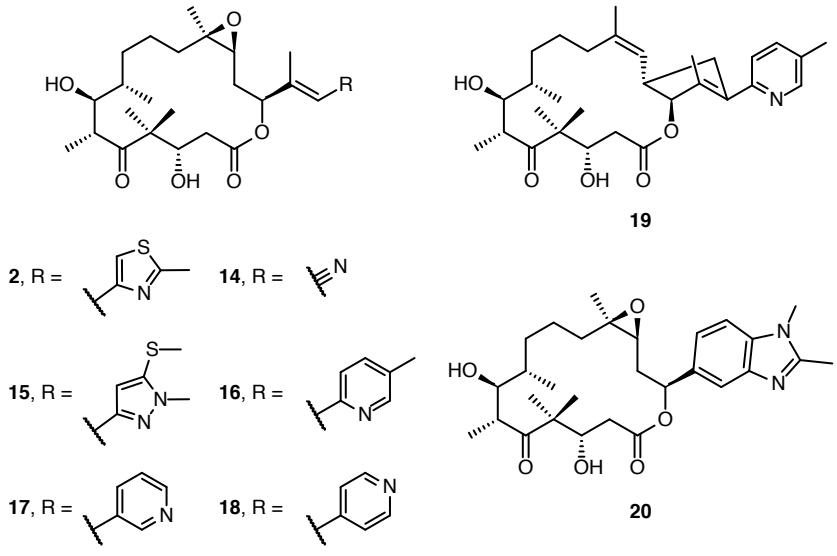


Figure 3.3: Structure of epothilone analogues showing modifications in the C15 moiety. Adapted from Nicolaou *et al.* and Alhamadsheh *et al.* [22,23,31]

In a study by Alhamadsheh *et al.*, a different approach was used [31]. Under the assumption that the role of the nitrogen atom is to bind to tubulin through hydrogen bonds, they replaced the thiazole ring with a methylpyridine ring containing the nitrogen in an *ortho* position (**16**). This gave a twofold increased

activity (see table 2.1). This was also confirmed by another research group which conducted similar studies where they let the nitrogen atom "walk" around the ring[23]. As can be seen from table 2.1 the *ortho* analogue showed a significantly higher activity than the *meta* (**17**) and *para* (**18**) analogues. Alhamadsheh *et al.* believed that making the nitrogen atom as free from steric interactions from other groups as possible would enhance the molecules activity[31]. They therefore used a new approach where they introduce a methylene bridge between C14 and C17 of **11** (see structure **19**). This was meant to rigidify the movement of the side chain and especially the C16 methyl group, while still permitting movement of the pyridine ring. This new compound turned out to have the enhanced antitumor activity that the researchers wanted, with approximately a threefold increase compared to **11**[31]. Surprisingly this increase did not apply to the breast cancer cell lines, but to cell lines originating from leukemia[31]. In the same study rigidifying of other parts of the molecules was also tested, but with no positive results. The same kind of rigidifying was also tested by Nicolaou *et al.*[23]. They replaced the C15-C16 double bond with an aromatic ring making a polycyclic ring at the C15 moiety (see structure **20**). This structure shows a 99% degree of polymerization compared to 90% for **2**, which equals a considerable improvement. Considering studies looking at tubulin polymerization, it is important to remember that the ability to induce tubulin polymerization and the cytotoxic activity are not equivalent. There are more factors concerning the antitumor activity of a compound that its ability to polymerize tubulin, e.g. uptake into cells[23].

By conducting Structure-Activity Relationship (SAR) studies different epothilone derivatives with different substitutes for the ring-containing side chain at C15 have been tested. In the study by Nicolaou *et al.* 21 side chain substituents were tested and their cytotoxity was compared to that of taxol, **1**, and **2**[22]. This study does not conclude with any simple relation between cytotoxic activity and conditions as polycyclic- vs monocyclic-substituents, substituents containing halogens or size of the substituent. The researchers found that the non-cyclic nitrile substituent (**14**) did not have any cytotoxic effect. They also showed that structure **15** had extremely high potency against most kinds of cell lines compared to both **1** and **2**[22]. The potency of **15** equals a 40-fold increase compared to **1** and a 16.5-fold increase compared to **2** (see table 2.1). This suggests that the modifications done to structure **15** increases the compounds ability to bind to tubulin[22].

The specific binding site of the C15 moiety, and especially the thiazole group, is a topic of discussion. By using NMR, EC and molecular modelig, it is shown that the nitrogen atom in the *ortho* position at the thiazole binds to the histidine (his227) of the tubulin protein[6]. This is dependent on the stereochemistry at C15 being the same as in **1**[23]. With the wrong stereochemistry the C15 moiety will not be able to dock in the right binding site of tubulin. In a study by Ginnakakou *et al.* it was suggested that the Phe270 (phenylalanine) was also located in the area of the binding site of the C15 moiety[7]. This assumption originated from observations regarding the mutation of this amino acid. They observed that a mutation of this amino acid to valine had a negative threefold effect on the activity of **2**. This change in activity can be caused by the decrease of space in the C15 moiety binding cavity when phenylalanine was substituted in favor of valine. Valine has a higher molecular weight than phenylalanine and they are both non-polar. This assumption was confirmed by examining the activity of the even bulkier pyridine analogue of **2**. This analogue showed a 10-fold change in activity when exposed to the mutated

cell lines[7]. Based on this and the other studies concerning the C15 moiety, there is an agreement that this site is important for the tubulin binding-properties of epothilones. There is also a common understanding that the rigidifying of the area around the nitrogen atom of the thiazole group gives positive outcomes considering anticancer activity. The rigidifying will make the nitrogen atom more accessible for hydrogen-bonding to the amino acids of tubulin without having to use energy to adjust during the binding process.

3.4 Analyzing methods

There are many different methods to determine the binding pose and binding position of epothilones, including molecular modeling, NMR-studies, photoaffinity labeling (PAL) and EC. Although these methods show some similar results there are considerable differences. For instance, the C3 hydroxy group which shows importance in the tubulin binding in studies using molecular dynamic (MD) simulations, but no affinity to tubulin in PAL-studies[32,33].

PAL is a method used to covalently bind chemical tags to the target protein. In this case, the proteins of tubulin[34]. The epothilones can be used as a transporter to transport the tags in to the tubulin structure. The chemical tags, being photoactivatable, can then be activated and will bind to the nearest amino acid[34]. By using this method Ranade *et al.* conducted the first specific labeling of the β-tubulin amino acids that binds the epothilone[32]. They identified the amino acid sequences TARGSQQY and TSRGSQQY. This study points out the C7 hydroxy group, the C1 lactone carbonyl-oxygen and the nitrogen atom of the thiazole ring to be important sites of binding . From the amino acid sequence identified in this study two amino acids match those identified by Nettles *et al.* (thr and arg)[6]. This will strengthen the hypothesis suggesting that these amino acids are located in the epothilone binding cite, especially considering that the Nettles *et al.* study was utilising both NMR, EC and molecular modeling in their research.

Another method for determining the binding mode of epothilone to tubulin is by MD simulations. In an attempt to rationalize the activity trends of epothilone analogues on a molecular level Jimènes *et al.* conducted MD simulations on 20 epothilone analogues[33]. The analogues included changes in the epoxide at C12-C13, the stereochemistry of C12, the stereochemistry of C15 and the modification of the heterocyclic moiety. The intermolecular interactions between the epothilone and tubulin found in this study were located at the C1 carbonyl group, the C3 hydroxy group, the C7 hydroxy group and the nitrogen atom of the thiazole ring. These are pointed out as regions that are capable of making hydrogen bonds to polar amino acids. They also suggest that the C4, C6, C8 and C22 methyl groups make hydrophobic interactions with apolar amino acids. However, the amino acids pointed out as the binding locations in this study only partly match the peptide sequence in the PAL experiment mentioned above[32,33]. This study points out thr, gln (glutamine) and Asp (aspartic acid) as amino acids located in the binding cavity. Of these gln is also mentioned in the PAL-study and thr is mentioned in both the PAL study and the study by Nettles *et al.*[6,34]. The research conducted by Jimènes *et al.* also suggests that there is a correlation between the flexibility of an epothilone analogue and its ability to bind to tubulin[33]. This property can be related to the ability to adjust to the correct conformation of the tubulin-binding site with minimal

energy required.

In a study by Giannakakou *et al.* another approach is used[7]. They wanted to find a common pharmacophore shared by taxanes and epothilones because they belived it could provide a tool to develop new improved tubulin stabilizing drugs. By using SAR-studies and molecular modeling they identified some of the amino acids in the epothilone binding site. Like many other anticancer drugs, the epothilones do not effect all cell lines. Some cell lines are epothilone-resistant due to mutations in the β-tubulin primary structure[7]. The known mutations involve alteration of the thr274 to isoleucine (ile) and the arg282 to gln. At closer examination it becomes clear that the substitution of arg to ile equals the loss of a polar hydroxy group in exchange for a non-polar hydrocarbonic chain. This will clearly affect the epothilones´ binding at this site, considering that this bonding is based on the hydrogen bonds between the C7 hydroxy group of the epothilone and the hydroxy group of the thr.

When it comes to the arg282 to gln mutation the effect on the binding site is not as clear. It is pointed out that the arg282 is located on the M-loop of the β-tubulin. With M-loops being the part of the β-tubulin responsible for the joining of several β-tubulins to form MTs, a mutation at this site will be likely to affect the tubulin polymerization[17]. Cell lines with this mutation have a decreased tubulin polymerization rate, but whether this is because of the defect in the M-loop or a change in the epothilones´ ability to bind to tubulin is unclear. In non-resistant cells, 150 nano molar (nM) of **1** resulted in polymerization of 98% of the tubulin while 5000 nM of **1** was unable to cause substantial polymerization in the resistant cell lines[7].

It can be challenging to evaluate the results considering the vast range of information from different studies utilising different methods. Considering the binding site of the epothilones many different amino acids are mentioned in different studies, but only some are overlapping. Among the amino acids identified with more than one method are gln, which is identified with both PAL and MD simulations[33,34]. Arg is identified in both PAL studies, the studies by Giannakakou *et al.* performing SAR studies with mutated cell lines, and in the study by Nettles *et al.* using EC, NMR and molecular modeling[6,7,34]. Finally, thr is mentioned in the same three studies as arg in addition to the study by Jimènes *et al.*[33]. The amino acids mentioned here can be said, with some certainty, to be located in the epothilone binding cavity while the amino acids that are only mentioned once are more uncertain. It is also important to keep in mind that the results are dependent on what cell lines and what tubulin is used.

4 Conclusion

Epothilones have shown to be a group of molecules with great potential for treatment of cancer. Compared to other cytotoxic drugs, like taxol, the epothilones demonstrates an increase in both effect and number of cell lines they can affect. By targeting the MTs, the epothilones are able to slow down the uncontrolled cell division of the cancer cells and cause apoptosis. By using different methods, researchers have tried to identify the binding mechanism and the binding site of epothilone, but this have given various results with only some similarities. Much of the disagreements may be a consequence of the use of different analytic methods such as NMR, molecular modeling, PAL, SAR and EC. On the positive

side researchers are making some progress in this field using the information they can derive from the methods they use. The approval of ixabepilone is a proof of this progress.

Among the researchers one point of agreement is which locations of the epothilone that are the most active and which of the amino acids of tubulin they bind to. It can be said, with high certainty, that the C1 lactone, the C3- and C7 hydroxy-groups, the C12-C13 epoxide and the thiazole ring are important locations when it comes to the tubulin binding activity. It is also agreed that hydrogen-bonds are an important when it comes to binding of the epothilones to tubulin. Based on the literature used in this thesis there are some characteristic features that are pointed out as properties for making the epothilone highly active. This includes hydroxy-groups at C3 and C7 that have the right conformation and are available for hydrogen-bonding, the rigidifying of the C15 moiety to prevent the nitrogen atom of the thiazole ring from being sterically hindered, as well as substituting the C12-C13 epoxide with a cyclopropyl to decrease the toxicity. The substitution of the C1 oxygen with a nitrogen atom is also important for the function of the epothilone as a drug because it helps increase the molecules metabolic stability. These are all modifications that will benefit the epothilones´ abilities as anticancer drugs.

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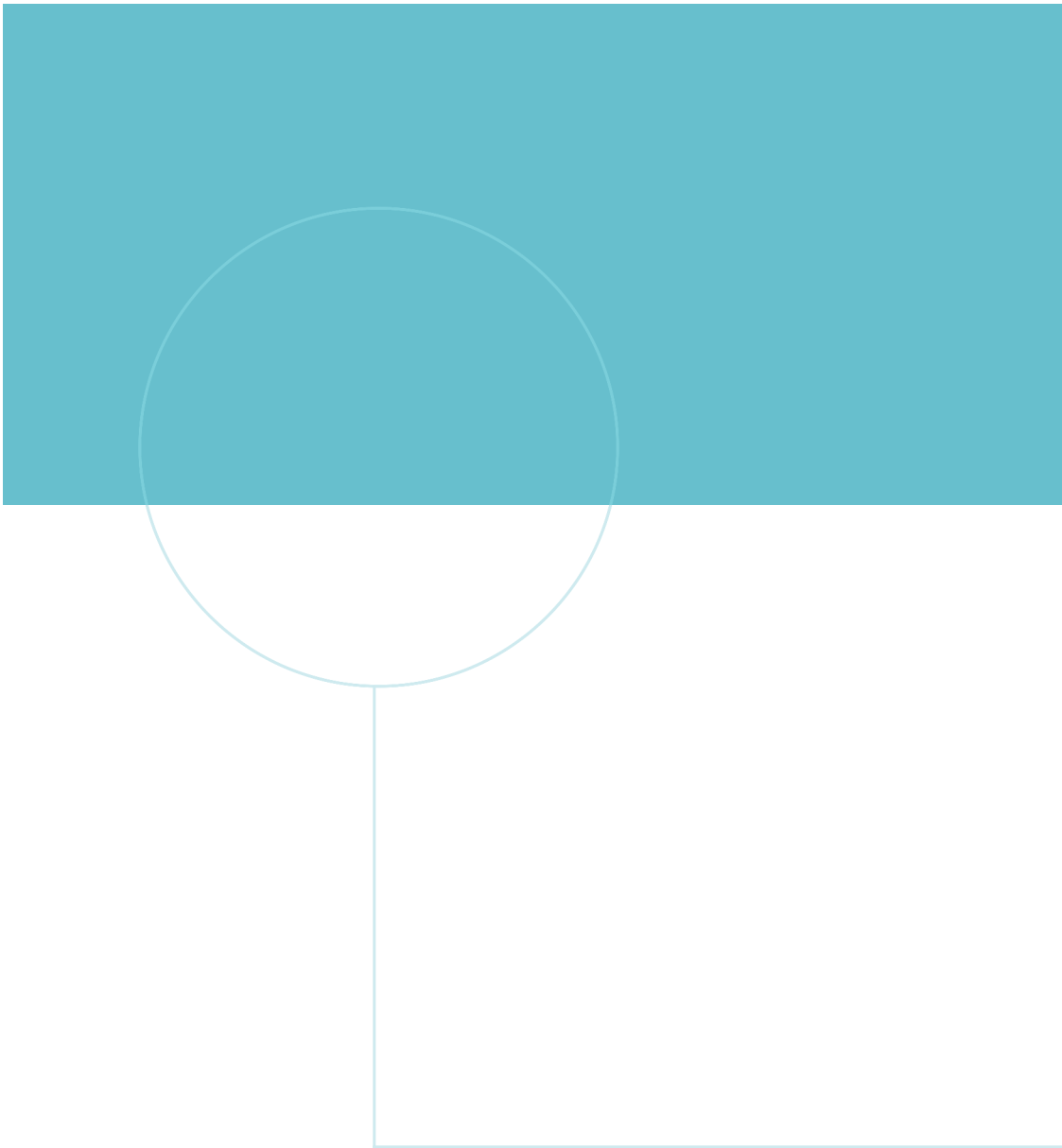

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