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„Impact of the grapevine-shoot extract vineatrol®30 and its constituents on human topoisomerases and DNA integrity“

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"Consistency is the last refuge of the unimaginative." Oscar Wilde

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1 List of Abbreviations

ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
BSA	Bovine serum albumine
CDK	Cyclin-dependent kinase
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA double strand repair
DDT	1,4-Dithiothreitol
DNA-PK	DNA-dependent protein kinase
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
p42/44 Erk 1/2	Mitogen-activated protein kinase with 42 or 44 kDa
Fapy Ade	4,6-diamino-5-formamidopyrimidine
FapyGua	2,6-diamino-4hydroxy-5-formamidopyrimidine
FCS	Fetal calf serum
Fpg	Formamido pyrimidine glycosylase
HRP	Horseradish peroxidase
HRR	Homologous recombination repair
Kb	Kilo bases
kDa	Kilodalton
kDNA	Kinetoplast DNA
MAPK	Mitogen-activated protein kinase
Mek1	Meiosis-specific serine/threonine protein
MIC	Minimal inhibition concentration
MMPs	Matrix metalloproteinases
Mre11	Nuclease
Nbs1	DNA damage repair protein, named after Nijmegen breakage syndrome
NFκB	Latent gene regulatory protein activated by various intracellular signaling pathways
NHEJ	Nonhomologous end joining
PI3K	Phosphatidylinositol-3-kinase
ROS	Reactive oxygen species
RT	Room temperature
Ser	Serine
TNF	Tumour necrosis factor

2 Introduction

Within the last decade there has been a growing interest in the study of the therapeutic potential of natural products. A variety of compounds have been isolated and tested in clinical trials. One of these compounds is resveratrol, which is the focus in over 4000 publications (stand October 2011, PubMed). Resveratrol, amongst others, has shown to exhibit beneficial effects in the prevention of different stages of carcinogenesis.

Much effort is observed concerning the improvement of the biological activity of resveratrol. One of these efforts is the development and commercialization of the grapevine shoot extract vineatrol®30. Vineatrol®30 contains not only a high amount of resveratrol but also resveratrol oligomers and –polymers. Since the extract was admitted as a dietary supplement, it is essential to understand the mechanisms underlying the possible anti-cancer potential of vineatrol®30 and its constituents.

In terms of cancer treatment, human topoisomerase II has become a central approach in the current research. These essential enzymes control the topology of DNA by creating transient breaks in the sugar-phosphate backbone of the double helix. The most effective topoisomerase II-targeting drugs induce enzyme-mediated DNA-damage by the generation of permanent instead of transient DNA double strand breaks. This effect is called topoisomerase-poisoning. A recent study showed that resveratrol inhibits human topoisomerase II and induces DNA double strand breaks (Leone *et al., 2010*). Therefore, the effect of resveratrol on topoisomerase II as well as its potential in the induction of DNA strand breaks must be confirmed.

In the current project, it must be determined whether the extract vineatrol®30 acts as an inhibitor of the catalytic activity of human topoisomerase II, or as a poison. Furthermore, the bioactive constituents of vineatrol®30 concerning the inhibition of human topoisomerase II must be identified. Furthermore, it must also be determined whether these substances are more effective than the reference substance resveratrol.

Tumours containing higher levels of topoisomerase II provide potential for targeted and improved cancer treatment. For an efficient cancer therapy it is important to understand the underlying enzyme-drug-interaction. If the compound is capable of poisoning human topoisomerase II, it could be a potential candidate for the application in cancer chemotherapy. In the case of a catalytic inhibition, the substance can overcome the DNA damaging effect of topoisomerase II poisons.

3 Theoretical background

3.1 Human topoisomerases

3.1.1 General features

DNA topoisomerases control and influence the topology of DNA within the cell. These essential enzymes maintain the double helical organization of DNA and the circular chromosome shape (Champoux, 2001).

Usually, DNA is negatively supercoiled. This underwound state facilitates strand separation as well as initiation, elongation of replication and transcription. During the processes of transcription and replication, massive alterations of DNA topology occur. Due to the activity of the replication fork, positive supercoiling (overwinding) is induced, which cannot be unwound by helicases (Figure 1). Consequently, positive supercoils result in a replication or transcription stop and generally lead to suppression of other cellular processes. Moreover, nuclear processes influence the genetic material by creating knots and tangles. This topological alteration will prevent DNA tracking systems from the separation of the strands. Tangles in chromosomes disturb the initial de-condensation in the cell division leading to cell death (McClendon and Osheroff, 2007).

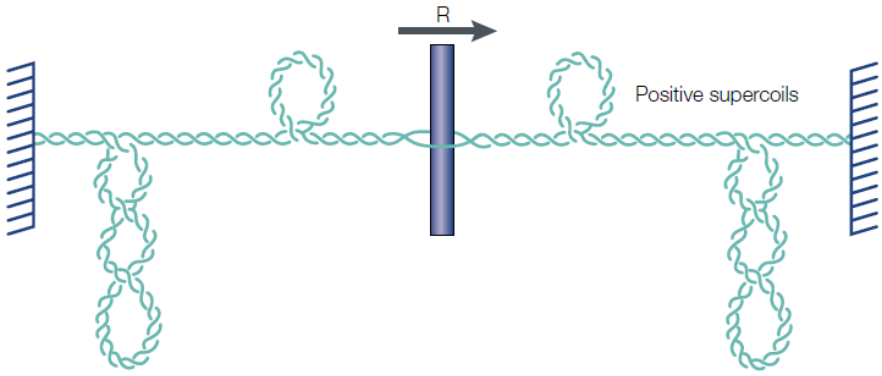


Figure 1: Generation of oppositely supercoiled domains by transcription. The transcription machinery (R) is shown as a rod. It includes RNA polymerase and assistant proteins as well as nascent RNA with associated proteins (Wang, 2002).

Consequently, in processes where the strands have to be separated such as replication, transcription or recombination, topological alterations have to occur (Wang, 1996).

Topoisomerases are responsible for unwinding of tangles or knots in double stranded DNA (McClendon and Osheroff, 2007). Before cytokinesis can proceed, catenated DNA resulting from replication has to be unlinked by topoisomerases (Pommier *et al.,* 2010). These enzymes are also involved in recombination, separation, condensation as well as de-condensation of chromosomes (McClendon and Osheroff, 2007).

Taken together, topoisomerases solve the structure-related problems and optimize the function of DNA by the fine-tuning of the topological state (Wang, 1996). For that purpose, topoisomerases are able to create transient breaks in the sugar-phosphate backbone of the double helix (McClendon and Osheroff, 2007).

3.1.2 Classification

Topoisomerases are classified according to their cleavage mechanism. If they induce cleavage of one DNA strand, they are defined as type I enzymes. If they induce DNA double strand breaks, they are defined as type II topoisomerases (Sng *et al.*, 1999).

Type I topoisomerases pass one strand of DNA through a break in the opposite strand or perform a controlled rotation about the break. These enzymes eliminate superhelical twists and decrease torsional tension in double stranded DNA. When tracking systems lead to topological alterations, this class of topoisomerases plays an important role maintaining genomic integrity (McClendon and Osheroff, 2007) (Figure 2a).

Type II topoisomerases catalyse the ATP-dependent transfer of a DNA duplex strand through a double-stranded gap (Champoux, 2001) (Figure 2b).

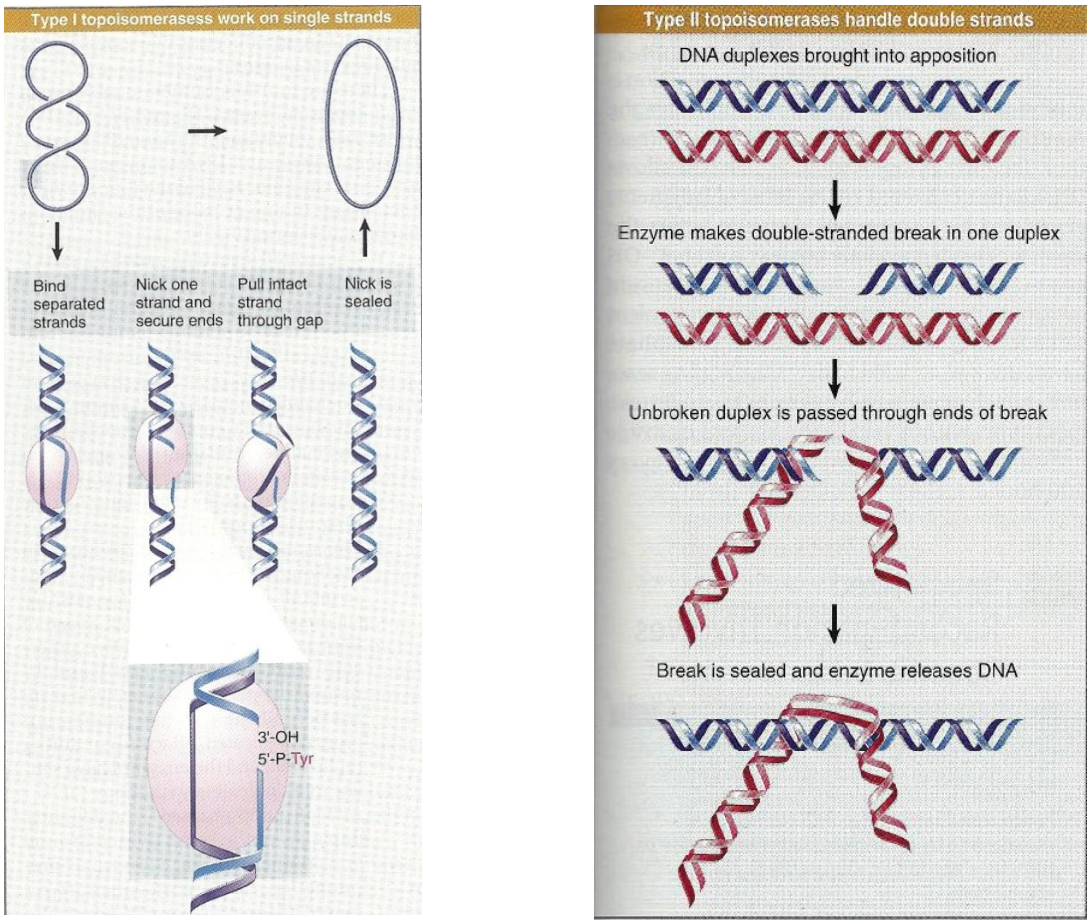


Figure 2: a) Type I topoisomerase recognize partially unwound segments of DNA and pass one strand through a break made in the other b) Type II topoisomerases can pass a duplex DNA through a DNA double strand break in another duplex (Lewin, 2008).

Type A subfamily members generate a protein link to 5´ phosphate and type B topoisomerases bind to 3´ phosphate on DNA (Sng *et al.*, 1999). Selected subfamilies of topoisomerases are shown in table 1.

Subfamily	Representative members
IA	Mammalian DNA topoisomerase IIIα und IIIβ
IB	Eukaryotic DNA topoisomerase I
IIA	Bacterial gyrase
	Yeast DNA topoisomerase II
	Drosophila DNA topoisomerase II
	Mammalian DNA topoisomerase IIα und IIβ
IIB	*Sulfolobus shibatae* DNA topoisomerase VI

Table 1: Subfamilies of DNA topoisomerases. Mammalian topoisomerase IIα and IIβ are highlighted as these enzymes were used in the experiments described later in chapter 5 (modified according to Wang, 2002).

3.1.3 Topoisomerase II

3.1.3.1 Catalytic cycle of topoisomerase II

The mechanism catalyzed by the homodimeric protein topoisomerase II is based on a DNA double-strand passage reaction. After binding two distinct duplexes of DNA, it cleaves one of the segments, the G (gate) segment, and passage the second segment, the T (transported) segment, through the nucleic acid gate. The enzyme ligates the DNA strand break and passes the trans-located segment through a gate in the protein (Figure 6). With closing the protein gate, a new catalytic cycle can be started (Fortune *et al.*, 1999). The energy for the translocation of one DNA double strand through the other is provided by ATP (Osheroff *et al.*, 1983). In total, topoisomerase II requires two molecules of ATP (Lindsley and Wang, 1991). In detail, the catalytic cycle of topoisomerase II can be divided into six steps summarized in figure 4:

Step 1: DNA binding

The initial interaction of DNA and topoisomerase II occurs without the presence of any cofactors. However, the association rate is stimulated by divalent cations (Osheroff, 1987). Although no characteristic features of DNA recognition sequences are recognizable, the enzyme prefers binding to bent DNA (Bechert *et al.*, 1994). The rate of topoisomerase II binding and catalytic activity depends on the topology of the DNA substrate as the enzyme prefers binding to negatively or positively supercoiled over relaxed molecules (Osheroff, 1986). The reason for the differentiation of the enzyme between the topological states of DNA lies in the recognition of DNA crossovers or nodes. Intramolecular crossovers are found in supercoiled DNA substrates, whereas intermolecular crossovers are observed in catenated molecules (Zechiedrich and Osheroff, 1990; Roca *et al.*, 1993).

Step 2: pre-strand passage DNA cleavage/re-ligation

The presence of a divalent cation leads to an equilibrium between cleavage and re-ligation of the G segment before strand passaging occurs (Osheroff, 1987). DNA double strand breaks are generated because topoisomerase II nicks each of the opposite strands of the double helix leading to two 5`-overhangs with the lengths of 4 bp (Sander and Hsieh, 1983). The cleavage reaction implies the interaction between a specific tyrosine residue of each enzyme monomer and the simultaneously generated 5´end of the broken double strand, resulting in a transient phosphodiester bond (Pommier *et al.,* 2010). The DNA cleavage intermediate is shown in figure 4 in brackets.

Mechanistically, the cleavage reaction functions as a trans-esterification induced by the active tyrosine of the enzyme which performs a nucleophilic attack to the phosphate end of the simultaneously generated DNA break. Consequently, a transient covalent complex between the protein and DNA is generated. The reaction is reversible by the re-ligation of the DNA backbone bond and the release of the tyrosine residue (Wang, 2002) (Figure 3).

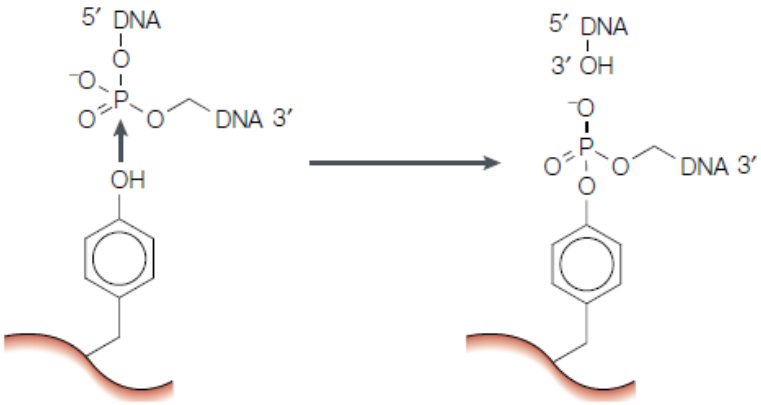


Figure 3: Catalysis of transient breakage of DNA by DNA topoisomerases (Wang, 2002).

Step 3: strand passage

With the binding of ATP as cofactor the clamp conformation of the enzyme is converted from an open to a closed form. This conformational change triggers DNA double strand passage (Pommier *et al.,* 2010). The energy for the strand passage reaction is taken from the triphosphate portion of the high energy cofactor (Osheroff *et al.*, 1983).

Step 4: post-strand passage DNA cleavage/re-ligation

As before the translocation, the DNA cleavage of the G segment is compensated by the re-ligation. Compared to the complex that is generated before ATP binding, the enzyme-DNA complex shows a 2-4-fold higher stability with the energy of the cofactor (Robinson and Osheroff, 1991).

Step 5: ATP hydrolysis

By performing ATP hydrolysis, the protein clamp of the enzyme is opened in order to release the G segment. Topoisomerase II does not essentially require DNA binding for ATP hydrolysis (Osheroff, 1986).

Step 6: enzyme recycling

Topoisomerase II can start a new round of catalysis, either on the same DNA substrate remaining associated or on another DNA double strand dissociating from the former substrate (Osheroff *et al.*, 1983).

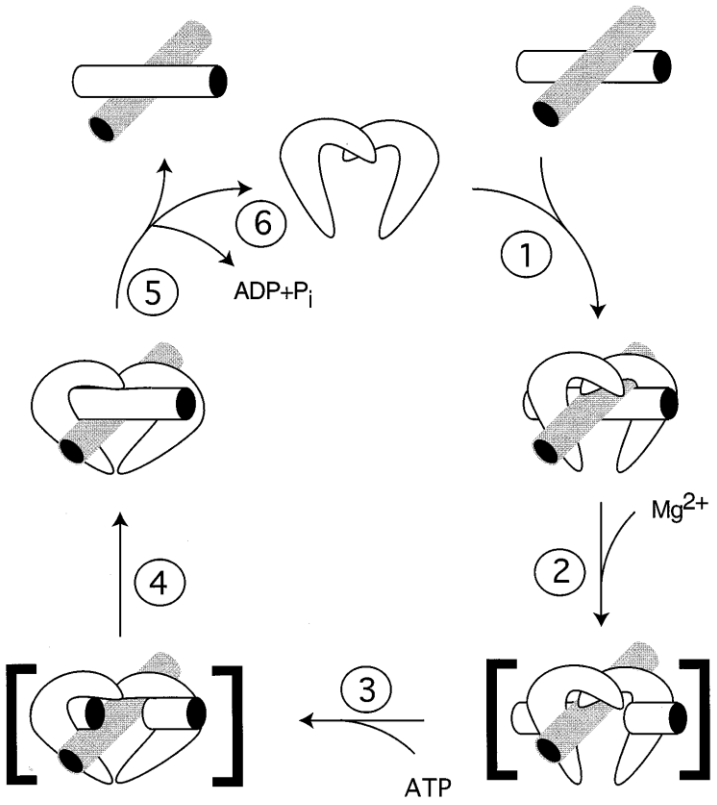


Figure 4: The catalytic cycle of topoisomerase II. The individual steps of the catalytic cycle are shown. (1) DNA binding of the enzyme; (2) pre-strand passage cleavage/re-ligation; (3) DNA strand passage; (4) post-strand passage cleavage/re-ligation (5) ATP hydrolysis (6) enzyme recycling. Brackets indicate that the enzyme-DNA-complex is a short-lived intermediate (Burden and Osheroff, 1998).

As mentioned before, there are specific sites of enzyme-mediated-DNA cleavage within its binding/recognition sites (Spitzner and Muller, 1988). These recognition sites are conserved (Muller *et al.*, 1988). Therefore, any topoisomerase II can be used to obtain a reproducible characteristic pattern of cleavage sites on a DNA molecule (Burden and Osheroff, 1998).

The formation of covalent DNA/topoisomerase II-intermediates is a critical step in the cell. Therefore, the concentration of these transient enzyme/DNA-complexes is tightly regulated. (Wilstermann and Osheroff, 2003). The topoisomerase II-associated DNA double strand breaks are transient under normal cellular conditions, as intermediates during the catalytic cycle of the enzyme (Osheroff *et al.*, 1991). The DNA double strand breaks occur in low steady-state-levels which are tolerated by the cell (Kohn, 1996). If the activity of topoisomerase is reduced**,** the chromosomes could not be separated resulting in impaired mitosis and cell death (Wilstermann and Osheroff, 2003). If the level or the lifetime of the transient enzyme/DNA-intermediates is increased, DNA aberrations will occur (Corbett and Osheroff, 1993).

The transient enzyme/DNA-complex can lead to permanent DNA double strand breaks if polymerases or helicases pass the interaction site and disrupt the complex (Wilstermann and Osheroff, 2003). Consequently, these double strand breaks are no longer surrounded by the two monomers of topoisomerase II and repair pathways are activated.

With positive supercoiled DNA, the level of covalent topoisomerase II/DNA-intermediates is reduced 2-to 4-fold. Therefore, the probability of a permanent strand breaks decreases as a collision with a tracking system is less likely to occur (McClendon and Osheroff, 2007). With this substrate, topoisomerase II becomes a safer tool in DNA processes on the one hand, but the lethal effect of topoisomerase II-targeting-anti-cancer drugs is reduced on the other hand (McClendon and Osheroff, 2007).

3.1.3.2 Isoforms and structure

In vertebrates two isoforms of topoisomerases II are expressed, IIα and IIβ. Although the isoenzymes differ in their molecular mass (170 and 180 kDa, respectively) and are encoded by different genes, they show high amino acid conformity. However, topoisomerases IIα and IIβ are expressed differently and are involved in distinct processes (Velez-Cruz and Osheroff, 2004).

Topoisomerase IIα plays an important role during cell proliferation and is essential for active cell growth. Topoisomerase IIα levels depend on the proliferative state of the cell and show the highest rate in G2/M phase. As this isoform is associated with the chromosomes and located at replication forks during mitosis (Woessner *et al.*, 1991), it is mostly involved in DNA replication and chromosome segregation (Wang, 2002). Furthermore, it was shown that the enzyme relaxes positive DNA supercoils 10-fold faster in comparison to negative supercoils (Corbett *et al.*, 2004).

In contrast to topoisomerase IIα, the IIβ isoform is not involved in cell proliferation as it dissociates from the chromosomes during mitosis. Therefore, topoisomerase IIβ is not able to overcome a loss of the IIα isoform (Austin and Marsh, 1998). As the levels of this isoform are independent of the cell cycle, it might be responsible for the housekeeping functions of topoisomerase II (Heck *et al.*, 1988). Topoisomerase IIβ shows the same activity with negative and positive supercoils as this isoform is not involved in replication (McClendon *et al.*, 2005).

The two topoisomerase II isoenzymes show similarities in their primary structure. The N-terminal domain (first 670 amino acids) contains the site of ATP binding/hydrolysis, whereas the central domain (amino acids 671-1200) contains the active tyrosine (Y805) (Velez-Cruz and Osheroff, 2004).

In contrast to the other domains, the C-terminal domain shows high variability among the species and between the human isoforms. It is not only responsible for catalytic activity *in vitro* but also for nuclear localization and contains phosphorylation-sensitive sites (Shiozaki and Yanagida, 1992).The C-terminal domain is essential in recognizing the geometry of DNA (Corbett *et al.*, 2004). Figure 5 shows the linear organisation of human topoisomerase IIβ.

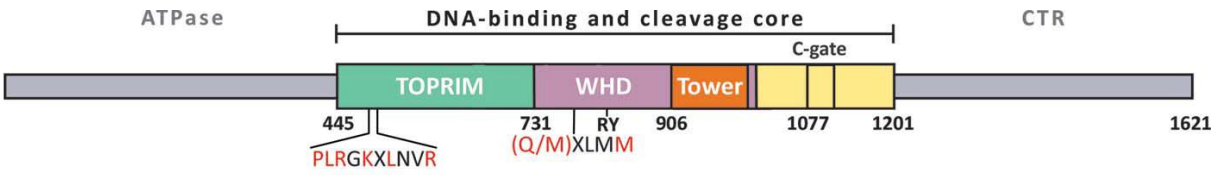


Figure 5: Linear domain organisation of human topoisomerase IIβ (Wu *et al.,* 2011).

3.1.4 Classification of topoisomerase II inhibitors

Topoisomerase II-targeting drugs can be divided into two classes. The first class contains agents stabilizing the enzyme/DNA-intermediate by the generation of a ternary complex. These compounds are clinically used and include e.g. etoposide, doxorubicin and mitoxantrone. Due to the stabilization of the protein/DNA-complex during the cleavage process, these agents are called **topoisomerase poisons**. The second class of topoisomerase II-targeting compounds inhibits the catalytic activity of the enzyme prior to DNA binding. Cell death is a possible consequence as the enzyme loses its essential activity. The agents are therefore called **catalytic inhibitors**. There are different steps in the enzyme cycle which can be affected by topoisomerase poisons or inhibitors. Figure 6 summarizes the effects of catalytic inhibitors and topoisomerase II poisons that are described in the following chapter 3.1.4.1. A competitive inhibition of ATP binding prevents the strand passage reaction. The inhibitor aclarubicin impairs the protein/DNA-binding and prevents the cleavage reaction. A suppression of the cleavage reaction *per se* is also possible as observed with the inhibitor merbarone. Apart from the cleavage, the re-ligation step can also be impaired. The topoisomerase II poison etoposide e.g. stops the catalytic cycle between DNA cleavage and re-ligation. Consequently, the enzyme recycling is blocked and further catalytic cycles cannot occur (Nitiss, 2009).

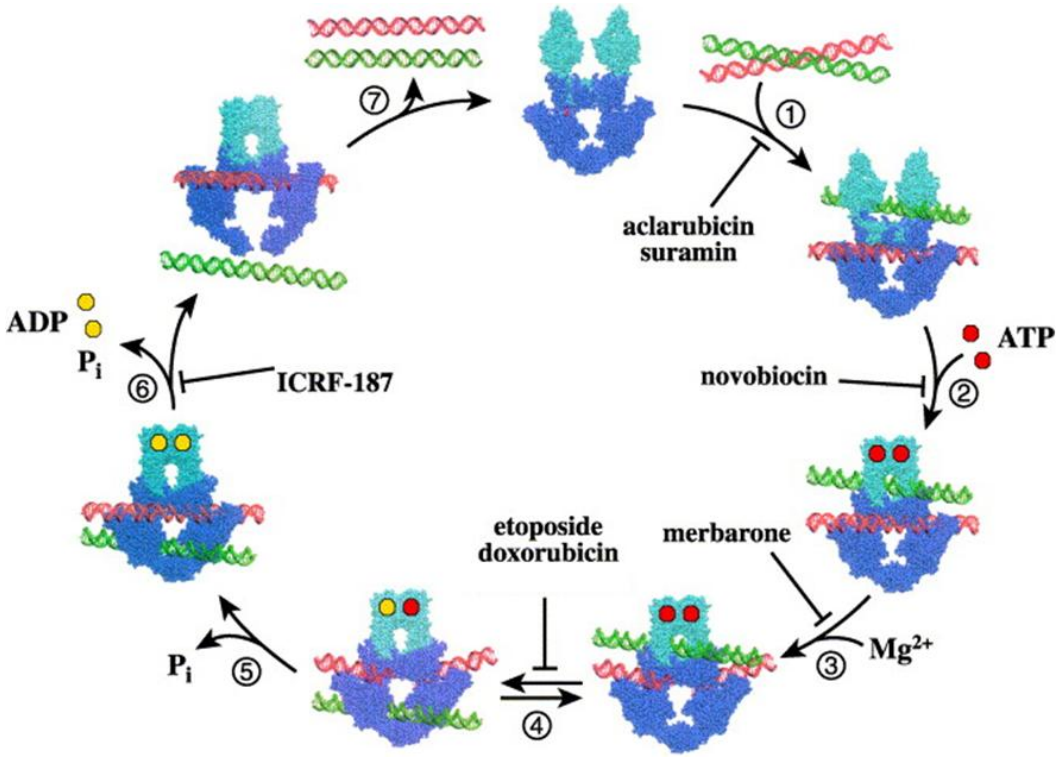


Figure 6: The catalytic cycle of DNA topoisomerase II (modified according to Larsen *et al.*, 2003).

3.1.4.1 Catalytic inhibitors

Inhibitors impair the catalytic activity of topoisomerase II by preventing the formation of transient enzyme/DNA-intermediates. Aclarubicin e.g. prevents the binding of topoisomerase II to DNA by intercalation (Sorensen *et al.*, 1992). Coumarin drugs, e.g. novobiocin, inhibit the catalytic activity of topoisomerase II by blocking the ATP binding site (Gormley *et al.*, 1996). By contrast, merbarone inhibits enzyme-mediated DNA cleavage with no influence on DNA-binding or ATP hydrolysis (Fortune and Osheroff, 1998).

Furthermore, oligomeric procyanins were shown to inhibit the catalytic activity of human topoisomerase IIβ (Fridrich *et al.*, 2007).The anthocyanidin delphinidin acts as a catalytic inhibitor of topoisomerase IIα without stabilizing the enzyme/DNA-intermediate (Esselen *et al.*, 2009). Also cyanidin was found to inhibit the catalytic activity of topoisomerase II without preferred interaction with one of the two isoenzymes (Habermeyer *et al.*, 2005).

Bisdioxopiperazines, like dexrazoxane (IRF-187), are classified as catalytic inhibitors although they stabilize the enzyme/DNA-association like topoisomerase-poisons (Roca *et al.*, 1994). As these compounds are highly specific for the enzyme, they are the most frequently used catalytic inhibitors of topoisomerase II in mammalian cells (Andoh and Ishida, 1998). Apart from their chelating activity, they block the catalytic cycle of topoisomerase II between strand passage and hydrolysis of the second ATP. Therefore, the enzyme is inhibited when it is associated to the cleaved DNA double strand (Andoh and Ishida, 1998). Except from bisdioxopiperazines, catalytic inhibitors show no specificity to topoisomerase II (Nitiss, 2009).

It is a common feature of catalytic inhibitors that they overcome the toxic effect of topoisomerase poisons. Therefore, it can be concluded that catalytic inhibitors and topoisomerase poisons differ completely in their mechanism of action (Jensen and Sehested, 1997). A recent study of our group showed that the anthocyanin cyanidin-3-glucoside (C3G) acts as an antagonist of camtothecin or doxorubicin. The compound impairs the poison-induced stability of the enzyme/DNA-intermediate (Esselen *et al.,* 2011). Furthermore, it was shown in *HT29* cells that delphinidin decreases the level of ternary topoisomerase IIα/DNA-complexes with etoposide and doxorubicin (Esselen *et al.*, 2009). The catalytic inhibitor suramin prevents the enzyme/DNA-interaction (Bojanowski *et al.*, 1992) and also inhibits the generation of ternary topoisomerase II/DNA/drug-complexes with the poison amasacrine (Lelievre *et al.*, 1995).

The structures of selected catalytic inhibitors are summarized in figure 7.

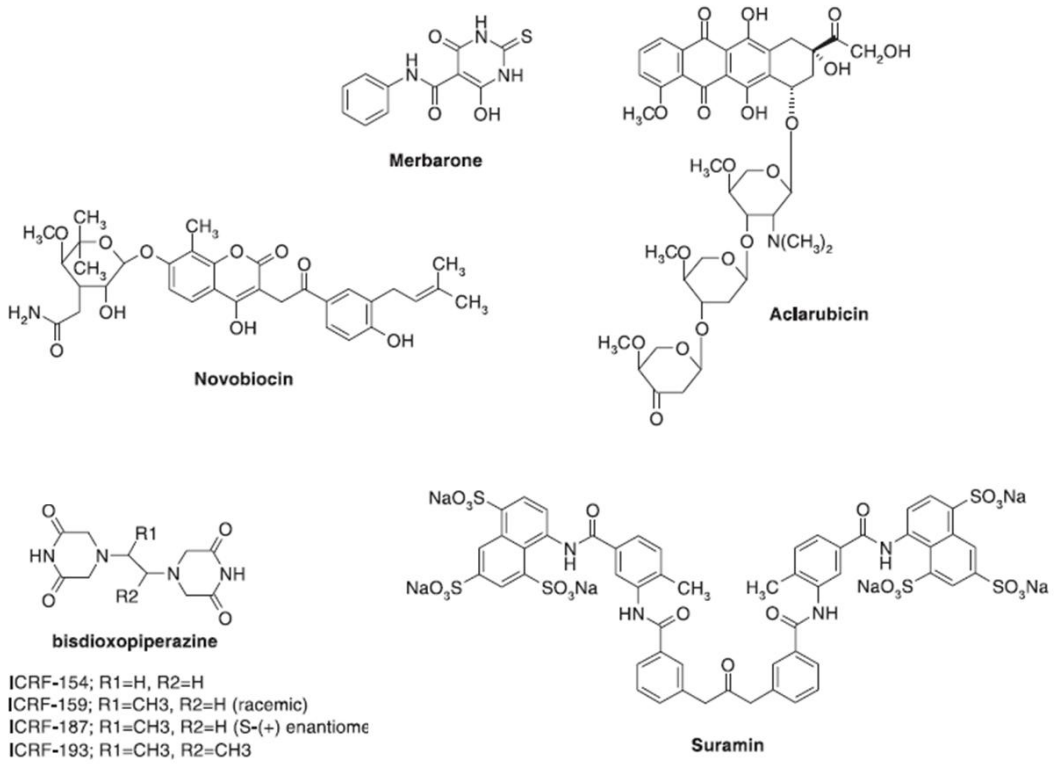


Figure 7: Catalytic inhibitors of topoisomerase II (modified according to Larsen *et al.*, 2003).

3.1.4.2 Topoisomerase II poisons

Compounds increasing the level of DNA/topoisomerase II-intermediates by creating a ternary complex are classified as **topoisomerase poisons.** By the formation of permanent instead of transient DNA double strand breaks, topoisomerase II might be shifted from an essential enzyme to a potent cytotoxic tool (Wilstermann and Osheroff, 2003).

Severe consequences occur with the increase in covalent enzyme/DNA-complexes. In addition to DNA damage, the transcription and replication machinery is disturbed by topoisomerase II poisons (Nitiss, 2009).

Generally, topoisomerase II poisons can act via two mechanisms. Some compounds prevent the enzyme from the re-ligation of the cleaved DNA segment and increase the lifetime of the covalent DNA/topoisomerase II-intermediates. Such poisons are e.g. etoposide, teniposide and the DNA intercalators doxorubicin and amasacrine. Another mechanism of action is the up-regulation of covalent enzyme/DNA-complex formation as performed e.g. by the natural flavonoid genistein. It is also possible to impair the DNA binding/dissociation equilibrium and therefore to increase the level of topoisomerase II-mediated cleavage (Fortune and Osheroff, 2000) (Figure 8).

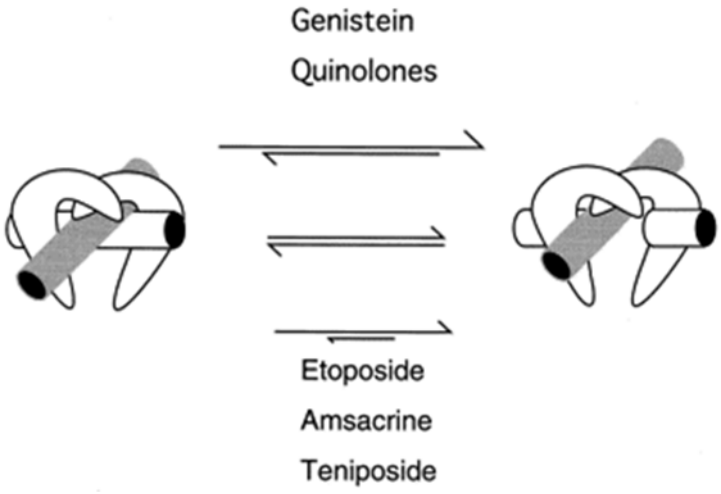


Figure 8: Effects of drugs on the cleavage/re-ligation equilibrium of topoisomerase II. Cleavage-enhancing-drugs are shown on the top of the figure, re-ligation-decreasing drugs are depicted on the bottom (Burden and Osheroff, 1998).

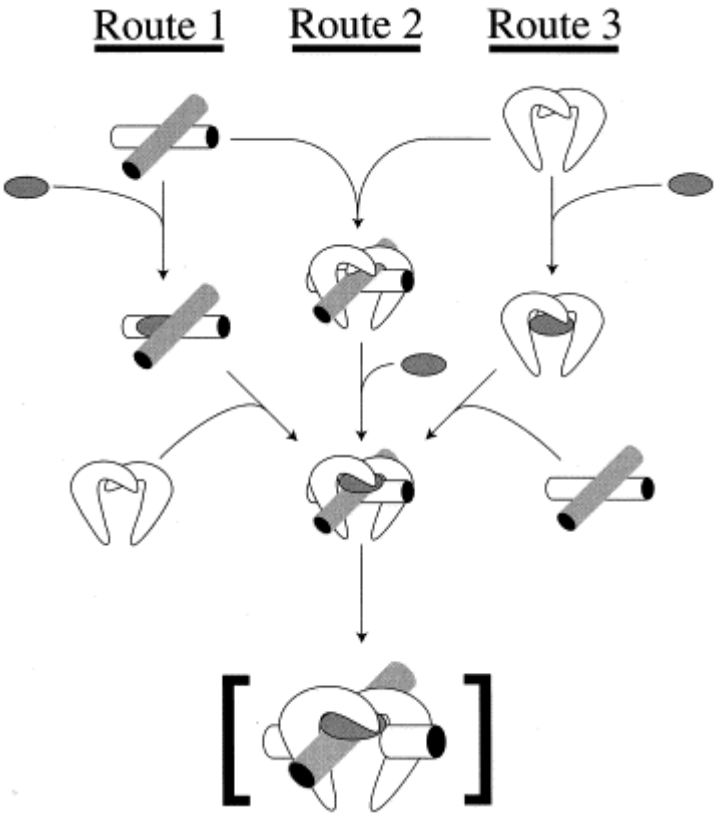


Figure 9: Routes of topoisomerase II/ /DNA/drug-complex formation (Burden and Osheroff, 1998).

There are three possible routes by which the ternary enzyme/DNA/drug-complex can be generated, as shown in figure 9. The first route implies the drug binding to DNA and the enzyme binding to this complex. According to the second route, the enzyme binds to DNA and the drug associates to the enzyme/DNA-complex. The third route implies the enzyme/drug-interaction followed by DNA binding (Burden and Osheroff, 1998) which was shown to be the preferred route by many anticancer drugs (Burden *et al.*, 1996).

Topoisomerase II is reported to be the most important cytotoxic target of anticancer drugs although no isoform-specific compounds have been developed (Kaufmann, 1998). Table 2 summarizes topoisomerase II poisons used in cancer chemotherapy and table 3 illustrates investigational topoisomerase II poisons.

Table 2: Clinical use of topoisomerase II poisoning anti-cancer agents (Hande, 1998).

Doxorubicin	Adriamycin®	Lymphomas*
	Rubex®	Breast cancer*
	Doxil®	Sarcomas*
		Kaposi's sarcoma
		Leukaemias
Daunorubicin	Cerubidine®	Acute myelogenous leukaemia*
		Acute lymphocytic leukaemia
Idarubicin	Idamycin®	Acute myelogenous leukaemia
Mitoxantrone	Novantrone®	Acute leukaemia
		Breast cancer
		Lymphomas
Etoposide	VePesid®	Testicular cancer*
	Etopophos®	Small cell lung cancer*
		Lymphomas
		Ewing's sarcoma
		Kaposi's sarcoma
		Ovarian cancer
Teniposide	Vumon®	Poor prognosis acute lymphoblastic leukaemia*

*Drug of first choice for treatment of these diseases.

Table 3: Investigational agents which poison topoisomerase II (Hande, 1998).

Amonifide	Genistein
Amsacrine	Intoplicine
Anthrapyrazoles	Makaluvamines
Aza IQD	Merbarone
Azatoxin	Menogaril
Bis (2,6-dioxopiperazine)	Naphthoquinones
Bulgarein	Nitroimidazole
Distamycin	Saintopine
Ditercalinium	Streptonigrin
Elliptinium	Suramin
Epirubicin	Whitangulatin
Ethidium bromide	

Non-covalent topoisomerase II poisons interact at the interface between the DNA segment and the active site of the enzyme. It is supposed that the compounds enter the complex by the interaction with topoisomerase II (Leroy *et al.*, 2001). These topoisomerase II poisons are used for chemotherapy (McClendon and Osheroff, 2007). The compounds show differences in their binding properties. Etoposide, genistein and quinolones such as CP-115.953 are non-intercalative topoisomerase II poisons (McClendon and Osheroff, 2007).

Very early, etoposide was found to induce single and double strand breaks *in vitro* (Loike and Horwitz, 1976). Now, etoposide is one of the most commonly used anti-cancer-drugs and is applied against lung-cancer, lymphomas or germ-line malignancies (DeVore *et al.*, 1992); (Belani *et al.*, 1994). A complication of this kind of cancer treatment is the possibility of secondary malignancies resulting from translocations. It was shown that topoisomerase IIβ is responsible for etoposide-induced acute myeloid leukaemia (Nitiss, 2009).

A recent X-ray structure of etoposide in the ternary complex with topoisomerase IIβ is shown in figure 10.

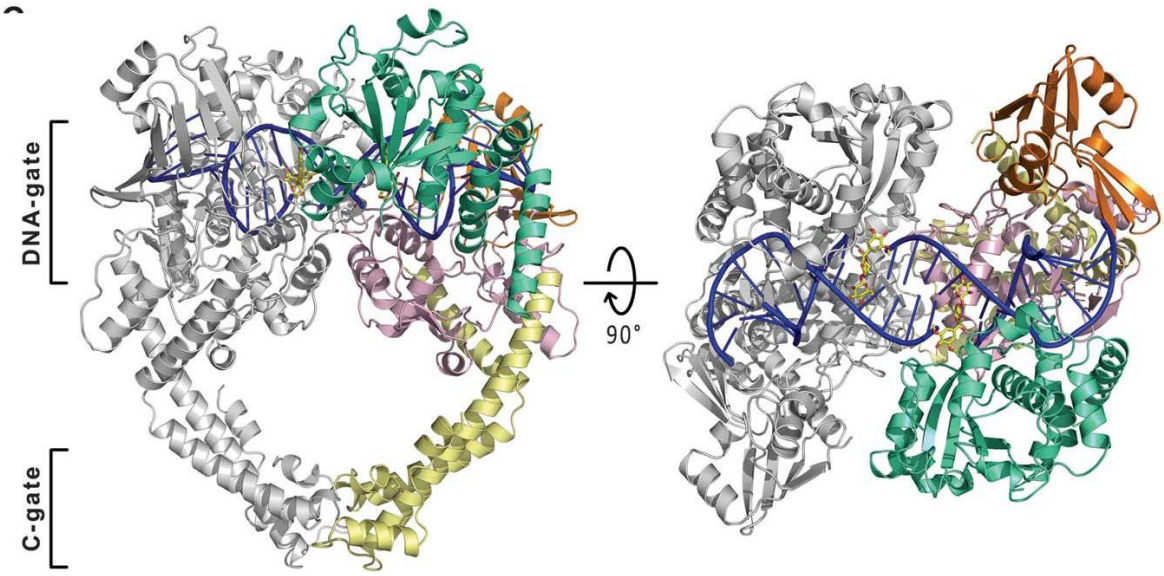


Figure 10: Orthogonal views of the ternary topoisomerase IIβ/DNA-complex with etoposide. DNA is shown in blue. One enzyme monomer is illustrated in grey, the other follows the scheme shown in figure (Wu *et al.,* 2011).

The etoposide-analogue teniposide shows similar potential in inhibiting topoisomerase II but with higher cytotoxicity due to the better cellular uptake (Stahelin and von Wartburg, 1991).

DNA intercalation is also possible as shown by amasacrine as well as by doxorubicin and mitoxantrone showing a very high DNA affinity (McClendon and Osheroff, 2007). Anthracyclins are intercalative topoisomerase II poisons as the planar aglycon moiety can insert between base pairs of DNA (Bachur *et al.*, 1992). In clinic, the most frequently used anthracyclin is doxorubicin (Goebel *et al.*, 1996). An anthracyclin analogue with an improved tolerability profile is e.g. the anthracenedione mitoxantrone (Faulds *et al.*, 1991).

With non-intercalative drugs, the rate of the ternary complex formation with enzyme/DNA is not influenced by the DNA substrate (McClendon, Osheroff, 2007). By contrast, intercalative drugs show a “bell-shaped” curve in the ternary complex with negatively supercoiled DNA substrates (McClendon, Osheroff, 2007) and the cleavage rate decreases with higher drug concentrations. With positively supercoiled DNA, the cleavage rate remains high (McClendon, Osheroff, 2006).

Bioflavonoids play an important role as phytoestrogens and are contained in many vegetables, fruits, legumes and plant leaves (Kurzer and Xu, 1997). Genistein contained in soy products may play an important role in chemoprevention as shown by the low incidence of breast, prostate and colon cancer in the Asian population (Barnes *et al.*, 1995). The isoflavone genistein was shown to be the most active topoisomerase poison amongst other bioflavonoids, flavones, flavonols and isoflavones acting mainly by inhibiting enzyme-mediated DNA ligation. Daidzein, myricetin, quercetin, kaempferol and luteolin, for instance, were also shown to inhibit both isoenzymes in cultured human cells (Bandele and Osheroff, 2007). Furthermore, the green tea catechin (-) -epigallocatechin-3-gallate was also found to act as topoisomerase II poison (Suzuki *et al.*, 2005).

The structures of selected non-covalent topoisomerase II poisons are shown in figure 11.

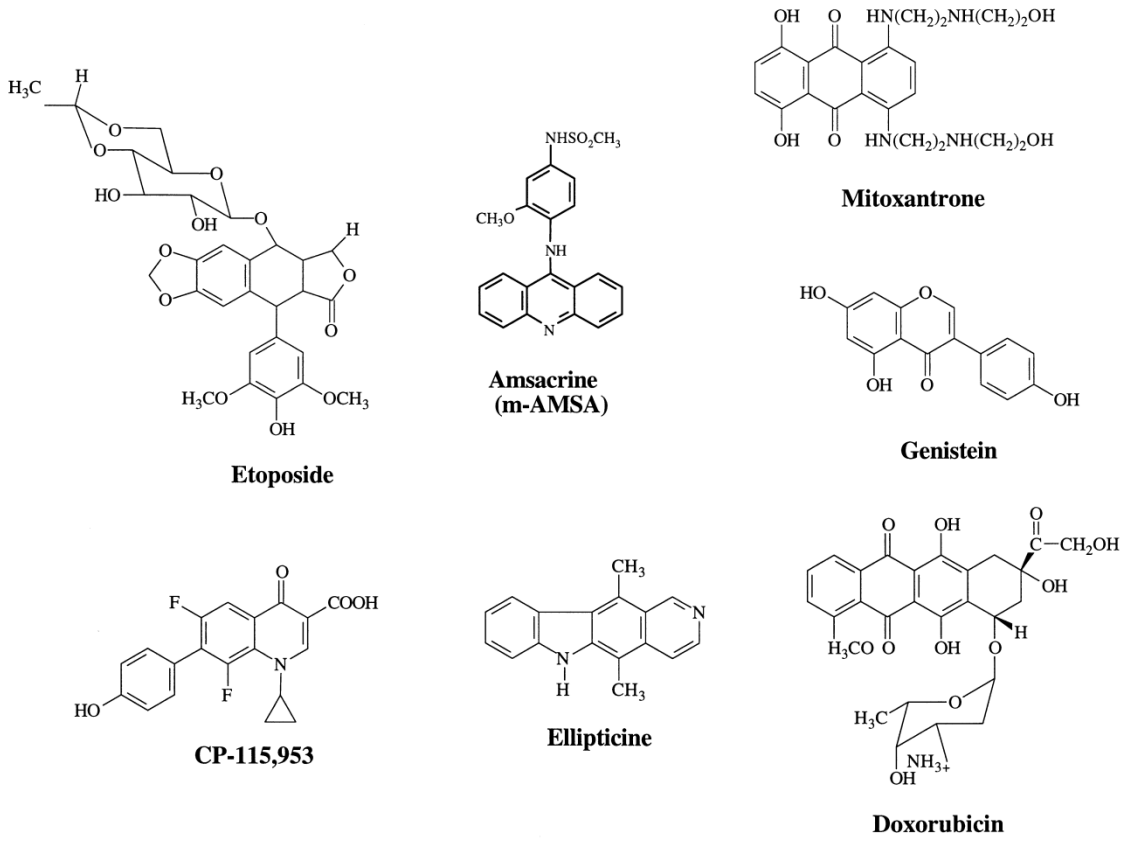


Figure 11: Structures of selected non-covalent topoisomerase II poisons (Burden and Osheroff, 1998).

Covalent topoisomerase II poisons contain sulfhydryl-reactive compounds e.g. quinones (Wang *et al.*, 2001). Interestingly, in the presence of a nucleic acid substrate, quinones act as topoisomerase II poisons, whereas in the absence of DNA they inhibit the catalytic activity of the enzyme. It was concluded that they might influence the enzyme during the cleavable complex formation. This mechanism might be based on the cross-linking of the enzyme´s N-terminal gate while DNA is enclosed within the active site. The entry of the DNA segment, which is not yet captured by the enzyme, is prevented (Bender *et al.*, 2006). Quinones prefer the inhibition of topoisomerase IIα (Lindsey *et al.*, 2004).

Camptothecins are intercalative topoisomerase I poisons as they integrate into the ternary complex between the -1 and +1 bases. The drug is associated to the protein by hydrogen bonding, which also stabilizes the ternary enzyme/DNA/poison-complex. Furthermore, stacking interactions between the compound and the bases are involved in ternary enzyme/DNA/drug-complex stabilization. The same mechanism of interfacial inhibition can be used to explain the intercalation of topoisomerase II poisons. As poisons alter the geometry of DNA, no re-ligation after the strand passage reaction can occur (Nitiss, 2009).

However, this is an explanation for topoisomerase II poisons blocking the re-ligation of the DNA double strand break and cannot be applied to cleavage stimulation agents (Robinson *et al.*, 1991).

In cells treated with topoisomerase II poisons, the two subunits of the homodimer strongly bind to DNA. Therefore, proteolytic or nucleolytic processing might be additionally required to induce DNA double strand cleavage. When the collision with a tracking system occurs, the ternary enzyme/DNA/drug-complex becomes irreversibly and this problem can only be solved by inducing repair or apoptosis. In order to repair the drug-induced DNA damage, the ternary topoisomerase II/DNA/drug-complex has to be differentiated from an active enzyme that releases DNA within a normal catalytic cycle. Successful repair has to release the covalently bound enzyme from the DNA substrate and has to re-ligate the double strand break. The recognition mechanism might involve the tracking system, which is prevented from performing transcription or replication. A possible repair mechanism is the nucleolytic excision of the DNA lesion within the enzyme/DNA-complex (Nitiss, 2009).

A second possibility to get rid of the enzyme bound to the DNA is the degradation of the enzyme by proteolysis. It was shown that topoisomerase IIβ was degraded by ubiquitinilation. Therefore, it is supposed that topoisomerase IIβ degradation functions as a signal for drug-induced DNA damage (Mao *et al.*, 2001). Recently, this mechanism was also observed with topoisomerase IIα but the degradation occurs to a lower extent compared to the β isoform (Fan *et al.*, 2008).

Two pathways might be responsible for the processing of the drug-induced DNA damage. On the one hand there might exist a transcription-induced pathway with proteasome-mediated processing, and on the other hand a replication-dependent pathway with no proteasome involvement is supposed (Nitiss, 2009). Both pathways lead to the loss of topoisomerase and to the generation of DNA double strand breaks, which might be repaired by a common mechanism. Non-homologous end-joining might be such a mechanism, as cells lacking this ability react hypersensitive towards topoisomerase II-mediated DNA damage (Nitiss, 2009). An excess of permanent double strand breaks can lead to apoptotic cell death (Kaufmann, 1998).

As mentioned before, topoisomerase II is one of the main targets in anticancer therapy. The level of topoisomerase II expression shows a direct proportionality to the drug sensitivity (Nitiss and Beck, 1996). Therefore, a targeted regulation of topoisomerase II levels can induce resistance to a wide range of topoisomerase poisons (Nitiss, 2009).This coherence was also proved *in vivo* (Burgess *et al.*, 2008).

Both isoenzymes of topoisomerase II show an interaction with poisons but the α isoform is the preferred cytotoxic target, as it is highly present in proliferating cells (Sullivan *et al.*, 1987).

That means for anticancer therapy that the treatment of tumours with topoisomerase-targeting drugs might be very efficient if they express a high level of the enzyme (Nitiss, 2009).

3.2 Red wine polyphenols

3.2.1 General features

Resveratrol (3, 4`, 5-trihydroxystilbene) is a phytoalexin belonging to the class of stilbene compounds. Resveratrol acts both as a natural antibiotic and as a proliferation inhibitor. In *Vitis vinifera*, it is produced during infection and blocks the proliferation in *Botrytis cinerea*. Furthermore, the stilbene is generated in response to environmental stress like climate, ozone and UV-radiation (Langcake, 1976 and 1979).

Resveratrol is synthesised from the precursor phenylalanine catalyzed by the enzyme stilbene synthase. The compound is contained in more than 70 plant species out of 32 genera (Figure 12). Resveratrol and its natural analogues piceatannol and pterostilbene were found in peanuts (*Arachis spp*) and berries (*Vaccinium sp*). High concentrations are present in red wine and grape juice, as one gram of fresh grape skin contains about 50-100 mg of resveratrol (Harikumar and Aggarwal, 2008).

Figure 12: Structure and sources of resveratrol (Harikumar and Aggarwal, 2008).

The structural features of resveratrol are two phenole rings connected by a styrene double bond determining cis or trans conformation, with the trans form as the more stable isomer (Harikumar and Aggarwal, 2008).

As the trans form is converted to the cis isomer upon UV-light exposure, isolation was performed in dark conditions (Lin *et al.*, 2007). Protected from light and high pH buffers, resveratrol is stable for several months. It shows the highest solubility in ethanol and DMSO (Harikumar and Aggarwal, 2008).

In Mediterranean nutrition, moderate red wine consumption as well as the intake of fruits, nuts, vegetables and legumes play an important role. In this context, the term “French Paradoxon” was defined, as a lower prevalence of coronary heart disease has been observed in the French population, despite their high fat- and cholesterol-rich diet. Several studies have confirmed that red wine shows beneficial effects concerning the incidence of cardiovascular diseases (Estruch, 2000; Di Castelnuovo *et al.*, 2002).

Indeed, many *in vivo* studies have shown that resveratrol treatment is of benefit against a variety of age-associated diseases like cancer, diabetes, Alzheimer´s disease, cardiovascular and pulmonary diseases. Resveratrol has been demonstrated to be involved in a wide range of pathways and interacts with a variety of cell-signaling molecules as summarized in figure 13.

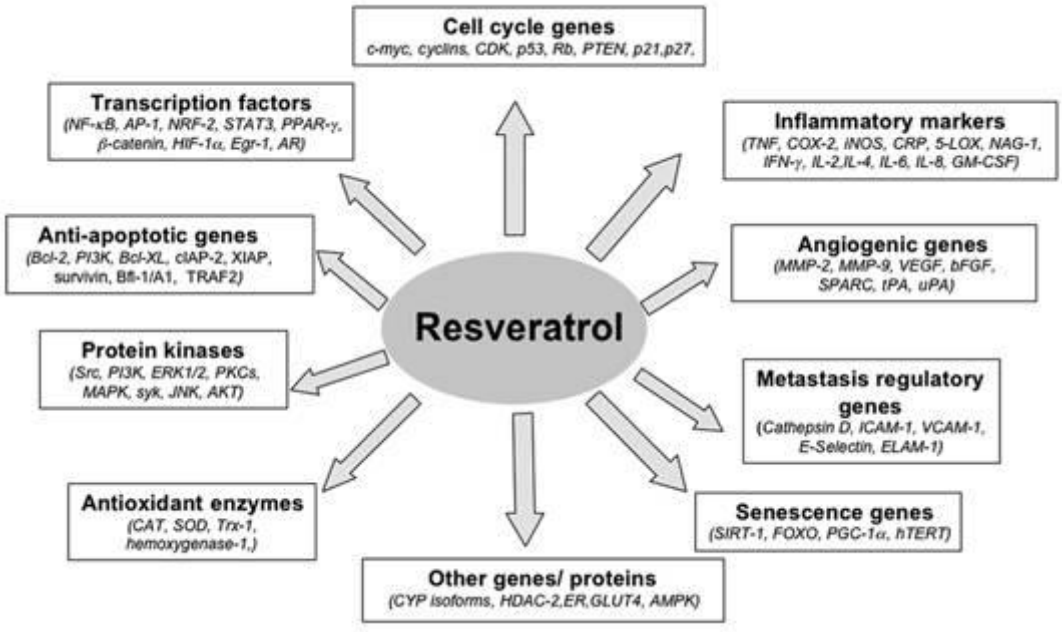


Figure 13: Various gene products that are modulated by resveratrol (Harikumar and Aggarwal, 2008).

Vineatrol®30

The grapevine shoot extract vineatrol®30 was developed by the French firm Actichem and BRECO GmbH and was brought on the market. The extract was produced by water/ethanol extraction from the early sprouts of *Vitis vinifera* in Bordeaux. Vineatrol®30 contains not only a high ratio of resveratrol but also a high portion of resveratrol oligomers and –polymers (Table 4).

Table 4: Resveratrol-oligomers contained in vineatrol®30 and the content [%] in the extract.

Resveratrol monomers and oligomers	content in vineatrol®30 [%]
ε-viniferin	14,6
trans-resveratrol	7,7
ampelopsin A	3,4
miyabenol C	2,5
r-viniferin	2,5
iso-*trans*-ε-viniferin	2,4
hopeaphenol	1,8
r-2-viniferin (visitin A) 1	1,6
trans-piceatannol	0,6
total ratio resveratrol-monomers and oligomers	37,1

Resveratrol-oligomers, contained in vineatrol®30, are e.g. the tetramers hopeaphenol (Figure 14a) and r-2-viniferin (Figure 14b) as well as the dimers ε-viniferin (Figure 14c) and ampelopsin A.

Similar to resveratrol, the oligomers exhibit a variety of health supporting effects. The dimer ε-viniferin showed an anti-oxidative (Privat *et al.*, 2002) as well as an anti-inflammatory (Zhang *et al.*, 2004) effect. Furthermore, it is anti-carcinogenic (Kim *et al.*, 2002) and neuroprotective (Yanez *et al.*, 2006). The tetramer r-2-viniferin showed an anti-oxidative effect and in this context a benefit for the cardiovascular system was concluded (Huang *et al.*, 2005). Furthermore, it also acts neuroprotective (Jang *et al.*, 2007).

The tetramer hopeaphenol was shown to have an anti-carcinogenic potential (Sahidin *et al.*, 2005), an anti-inflammatory effect as well as an anti-microbial effect (Zgoda-Pols *et al.*, 2002).

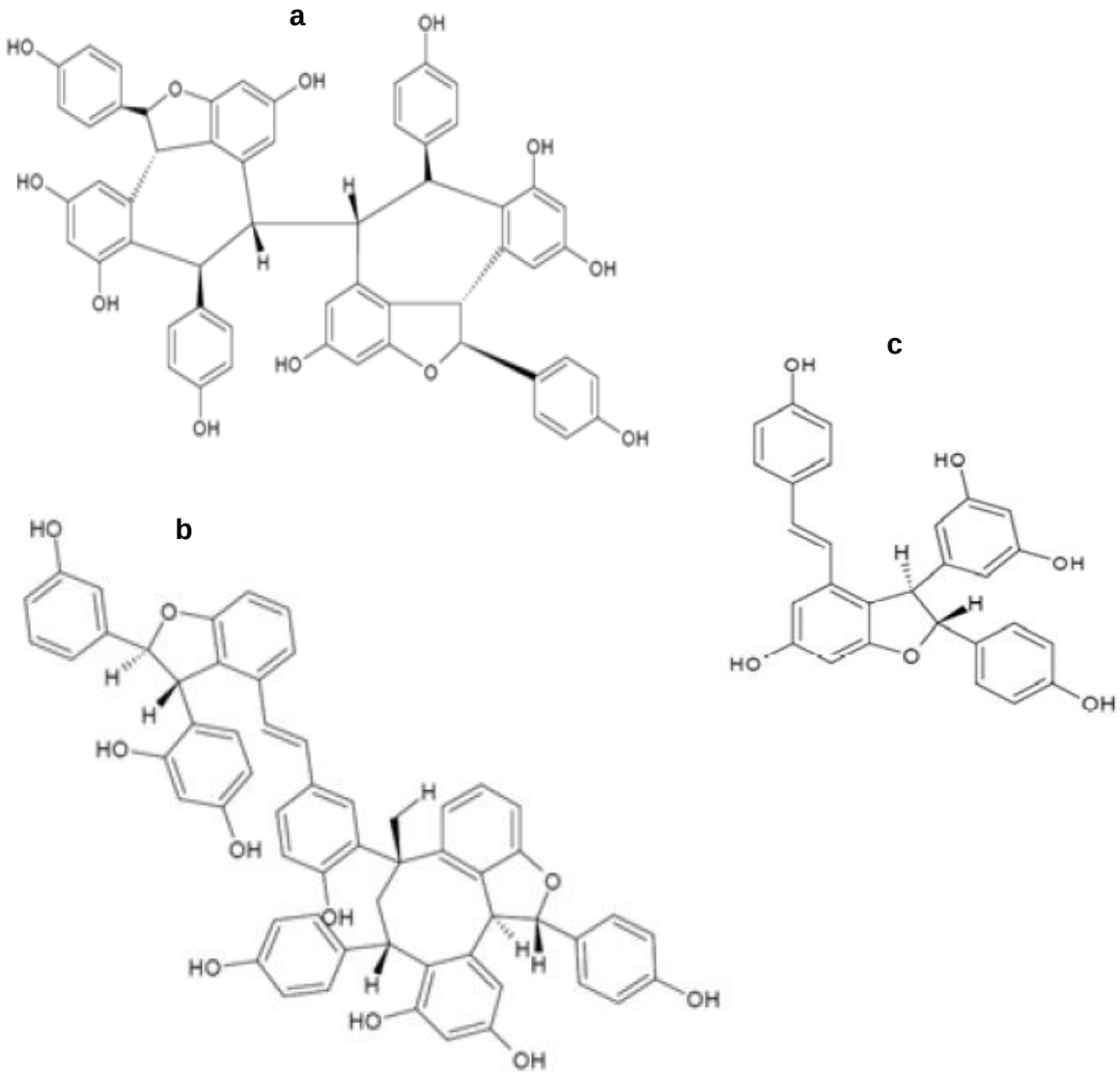


Figure 14: Structures of hopeaphenol (a), r2-viniferin (b) and ε-viniferin (c).

The extract was shown to be a free-radical-scavenger and a potent antioxidant at non-cytotoxic concentrations (Muller *et al.*, 2009).

3.2.2 Influence on DNA integrity

Resveratrol was shown to induce the histone H2AX phosphorylation. DNA double strand breaks might be induced due to a poisoning effect on human topoisomerase IIα (Leone *et al., 2010*). This result might indicate an effect of resveratrol in terms DNA double strand break induction.

A previous study (Cho *et al.*, 2000) defined resveratrol as one of the potent chemo-preventive agents with an effective inhibition of the catalytic activity of mammalian topoisomerase II. Resveratrol showed a 50% inhibition of topoisomerase II at a concentration of 65.72 µM. Jo *et al.*, 2005, showed a dose-dependent enzyme inhibition with an IC_{50} at a resveratrol concentration of 78.86 µM.

Resveratrol treatment for four hours resulted in a chromosome displacement and in the induction of micronuclei (Schmitt *et al.*, 2002). Furthermore, it was shown that the stilbene induced structural chromosome aberrations, particularly chromatid breaks and sister chromatid exchange (Matsuoka *et al.*, 2001). Resveratrol treatment resulted in an enhancement of nuclear size indicating that apoptotic cell alterations proceed (Delmas *et al.*, 2003). Concerning DNA integrity, an inter-nucleosomal fragmentation resulting from a cleavage in the linker region between nucleosomes was detected, upon resveratrol exposure at a range of concentrations between 20-100 µM for 24 hours (Woo *et al.*, 2007).

Several studies investigated the strand breaking potential of resveratrol in the presence of Cu(II) and under oxidative conditions. Mobilization and reduction of endogenous Cu(II) ions by resveratrol induces DNA scission via the generation of ROS (Hadi *et al.*, 2010). The presence of catalase inhibited the copper-catalyzed DNA cleavage effect of resveratrol indicating a requirement of H_2O_2 in the reaction (Fukuhara and Miyata, 1998). As cancer cells were shown to exhibit elevated levels of endogenous copper, this effect implicates the possibility of a tumour-cell-specific DNA cleavage (Ebadi and Swanson, 1988). Although 200 µM of resveratrol promote DNA breakage without the addition of Cu(II) in human peripheral lymphocytes, it was shown that intracellular copper ions are involved in the DNA breakage reaction (Hadi *et al.*, 2010).

Additionally, in the presence of antioxidants, like ascorbic acid and glutathione, resveratrol did not influence the DNA cleavage rate (Burkitt and Duncan, 2000). By contrast, resveratrol acts as a scavenger of hydroxyl and superoxide radicals (Leonard *et al.*, 2003). These data indicate that resveratrol shows a Janus face concerning oxidative activity, as it can switch between an anti-oxidative and a pro-oxidative effect.

Furthermore, resveratrol was shown to be involved in DNA double strand repair mechanisms as it activates ATM and/or ATR (Gatz *et al.*, 2008). This aspect is very important due to the phosphorylation of H2AX and will be discussed later in chapter 5.1.

4 Aim of the Master´s Project

Recently, resveratrol was shown to inhibit the catalytic activity of human topoisomerase IIα. γH2AX foci were detected indicating a DNA damaging effect of the substance (Leone *et al., 2010*).

Therefore, the first aim of the current study was to confirm the effect of resveratrol on topoisomerase IIα and on DNA integrity. By the use of the Comet assay, the DNA strand breaking potential of the polyphenol was investigated in the vulva carcinoma cell line *A 431*. Conducting a cell-free Decatenation assay, the potency of resveratrol to inhibit the catalytic activity of topoisomerase IIα was examined.

The second aim was to examine three selected constituents of the extract vineatrol®30, as well as the investigation of the extract *per se*. The grapevine-shoot extract vineatrol®30, in particular, contains resveratrol oligomers and polymers. As the extract has been admitted as a dietary supplement, it is essential to understand the mechanisms underlying the possible anti-cancer potential of vineatrol®30 and its constituents. It was suggested, that resveratrol oligomers might be even more effective than the reference substance. Using the Decatenation assay we investigated whether the extract, as well as the substances inhibit the catalytic activity of human topoisomerase IIα. As the reference substance resveratrol showed potent catalytic inhibition of topoisomerase IIα and might induce DNA strand breaks (Leone *et al., 2010*), a poisoning of the enzyme by resveratrol was suggested. Using the ICE-bioassay the poisoning effect of the extract vineatrol®30 on topoisomerase II was examined in the vulva carcinoma cell line *A 431,* as these cells showed over-expression of human topoisomerase (Meyer *et al.*, 1997).

By the combination of the Decatenation assay and the ICE-bioassay, it was distinguished between catalytic inhibition and topoisomerase II poisoning. Cancer therapy is only effective when the underlying enzyme-drug-interaction is understood. Therefore, the anti-cancer potential of vineatrol®30 and the three selected constituents must be estimated concerning the effect on human topoisomerase II.

5 Results and Discussion

5.1 Effect of resveratrol on DNA integrity

In order to investigate the strand breaking potential of resveratrol, the Comet assay was carried out in the vulva carcinoma cell line *A 431*. We performed the Comet Assay under alkaline conditions according to Singh *et al.*, 1988, to detect both DNA single and double strand breaks. During the experimental setup, the cells were treated with formamido pyrimidine glycosylase (fpg) to examine the presence of the formamidopyrimidines 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), major DNA lesions caused by UV radiation, photosensitization or H_2O_2/metal ions (Dizdaroglu *et al.*, 2008). Therefore, these compounds result from oxidative induced DNA damage (Dizdaroglu, 1992). As they derive from purines, they can be regarded as purines with an opened imidazole ring, although these compounds are pyrimidines. The major difference to other pyrimidines is that they are linked to desoxyribose not through N1, but through the amino group attached to C6 (Dizdaroglu *et al.*, 2008). Using the repair enzyme fpg, these specific DNA-lesions can be detected as an additional outcome.

Resveratrol was tested in a range of concentrations between 0.1 and 200 µM. UV-radiation was used as positive control as it induces double strand breaks in the S-phase (Marti *et al.*, 2006). Figure 15 shows the results of the Comet Assay performed with resveratrol.

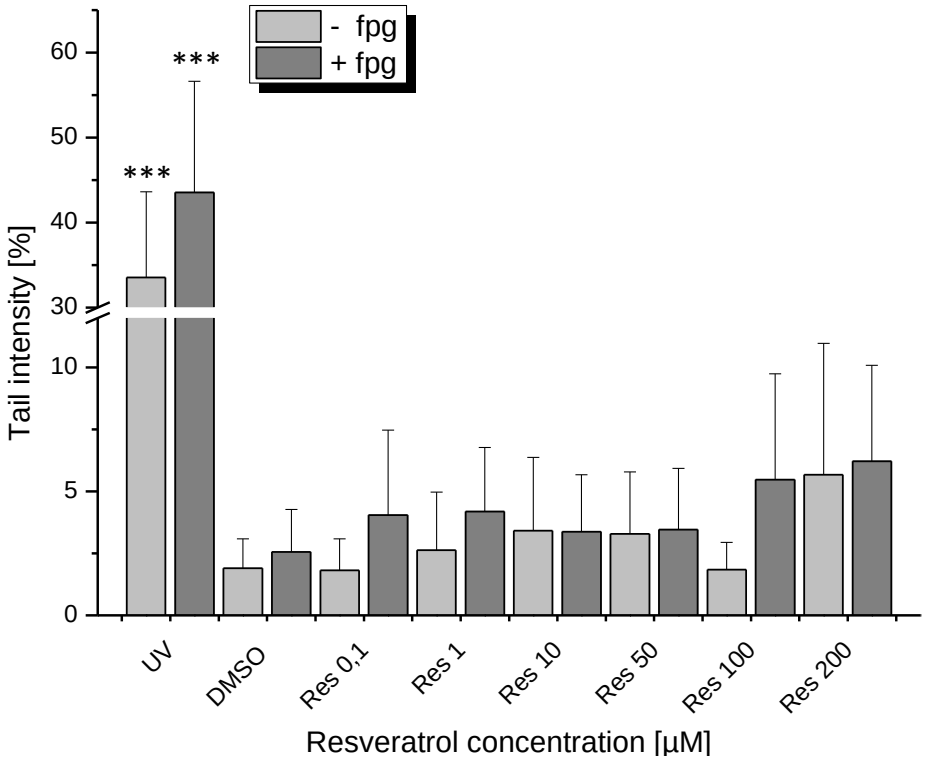


Figure 15: The Comet assay was performed with resveratrol in *A 431*-cells. Incubation time was 1 h at 37 °C with the addition of catalase [100 U/ml] and FCS [10 %] to the incubation media. DMSO [0.1 %] was used as the negative control, UV-radiation was used as the positive control and fpg-treatment was also carried out. The means ± SD of 5 replicas are shown. Outliners were excluded using the Nalimov test. The significances are related to the negative control (*** = $p < 0.001$).

Figure 15 demonstrates that resveratrol does not significantly induce DNA strand breaks, even in the highest tested concentration of 200 µM. After incubation with resveratrol, no fpg-sensitive sites can be observed, therefore no oxidative DNA-lesion was found.

The positive control, UV-radiation, shows no significant difference between fpg-treatment and no enzyme addition. Possibly, UV-exposure caused such a high extend of DNA damage, that the additional strand breaks, caused by fpg, did not influence the Comet tail readouts. As a result, the reliability of the graph and the five replicas are weakened. However, the possibility, that resveratrol might show an effect at least in the highest tested concentrations cannot be excluded. The resveratrol concentrations of 100 and 200 µM show a tendency concerning the generation of fpg-sensitive sites, although the effect is not significant. At the resveratrol concentration of 200 µM, a tendency in the induction of DNA strand breaks is observed. Furthermore, the high standard deviation of the five replicas exacerbates the interpretation of the results. Further investigations must be conducted in order to better understand the DNA strand breaking potential of resveratrol.

Cancer cells were shown to exhibit elevated levels of endogenous copper (Ebadi and Swanson, 1988). In previous studies resveratrol was shown to induce DNA double strand breaks in the presence of Cu(II) and under pro-oxidative conditions. The presence of catalase prevented the copper-catalyzed DNA cleavage effect of resveratrol, indicating the need for H_2O_2 within the reaction (Fukuhara and Miyata, 1998). It was concluded that resveratrol mobilizes and reduces endogenous copper, whose re-oxidation generates a variety of ROS and induces DNA strand scission (Hadi *et al.*, 2010).

In order to investigate the DNA strand breaking potential of resveratrol without the influence of additional oxidative stress, we added catalase [100 U/ml] to the incubation media. The addition of the enzyme avoided the induction of ROS by pro-oxidant H_2O_2. The use of catalase resulted in a reduction of H_2O_2 in the incubation media and prevented the formation of artefacts. According to figure 15, there is no significant difference between samples treated with or without fpg, which might be a consequence of the catalase addition reducing the oxidative stress and decreasing the number of fpg sensitive sites. It would be interesting to perform the Comet assay without the addition of catalase which might lead to a significant increase in fpg sensitive sites.

Leone *et al., 2010*, used γH2AX-antibodies to detect DNA double strand breaks, and reported the occurrence of γH2AX-foci in the human glioblastoma cell line *U87-MG* after a 48-hour-incubation with 80µM of resveratrol.

The histone H2AX belongs to the H2A family organizing eukaryotic DNA into chromatin (Bonner *et al.*, 2008). Chromatin modifications are involved in DNA damage recognition and repair (Groth *et al.*, 2007). The rapid phosphorylation of H2AX at Ser-139 produces γH2AX (Redon *et al.*, 2002). These so called γH2AX foci are seen as quantitative and very sensitive markers of DNA double strand breaks (Rothkamm and Lobrich, 2003). The presence of γH2AX is a very early indicator of DNA damage as it is detected within minutes after DNA damaging irradiation (Sedelnikova *et al.*, 2002). When the repair process is completed, γH2AX foci vanish by either de-phosphorylation or removal from chromatin (Bonner *et al.*, 2008). Resulting from a double strand break in DNA, activated PI3K-like kinases ATM, ATR and DNA-PK phosphorylate H2AX and other DNA double strand repair (DDR) proteins (Bonner *et al.*, 2008).

The detection of DNA double strand breaks using γH2AX as a marker is an indirect method of detection, and as such has its intrinsic limitations as it detects activated pathways induced for DNA double strand break repair. Even if the strand break is repaired by NHEJ and HRR, γH2AX foci may persist in some tumour cells (Kinner *et al.*, 2008). In this context, it was shown that resveratrol (≥ 5μM) activates the PI3Ks ATM and/or ATR (Gatz *et al.*, 2008). As ATM phosphorylates H2AX, resveratrol might modulate DNA double strand break repair pathways and may not be involved in the induction of the initial DNA double strand breaks.

The study of Leone *et al., 2010*, demonstrates an effect of 80 μM resveratrol on DNA strand break induction. According to our results, resveratrol exposure does not induce significant DNA damage but a tendency in the induction of DNA strand breaks around 200 μM is observed. However, the experimental setup was different in our study and the question arises whether the two distinct results can be compared. The incubation time of 48 hours is in contrast to our incubation time of 1 hour. Furthermore, Leone *et al., 2010*, detected DNA double strand breaks with immunohistochemistry staining of nuclear γH2AX foci, while we conducted single cell gel electrophoresis (Comet assay).

It cannot be concluded that our results support the findings of Leone *et al., 2010*. Further experiments are required to reveal the effect of resveratrol on DNA integrity.

5.2 Influence of resveratrol on human topoisomerase II

In order to investigate the effect of resveratrol to inhibit human topoisomerase IIα, we conducted the cell-free Decatenation assay. Kinetoplast DNA (kDNA) networks failed to migrate into an agarose gel (catenated lane). Upon incubation with topoisomerase II decatenated minicircles moved rapidly into the gel due to their small size (decatenated lane). Figure 16 shows the results of the Decatenation assay performed with resveratrol.

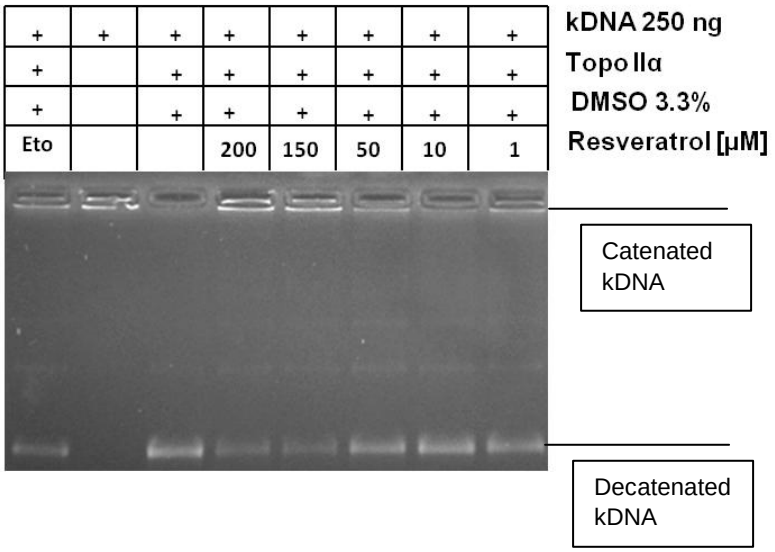


Figure 16: Resveratrol inhibits topoisomerase IIα. Etoposide (Eto) [50 µM] was used as the positive control, DMSO [3.3 %] was used as the negative control and kDNA was used as the substrate. Decatenation reactions were performed with 1 U of human topoisomerase IIα at 37°C for 1 h of incubation. 4-8 replicas were conducted.

The compound was tested in the concentration range between 1 and 200 µM. As figure 16 shows, human topoisomerase IIα is inhibited above a concentration of 50 µM resveratrol.

To further define the extent of inhibition, the decatenated kDNA lane was quantified and the results are shown in Figure 17.

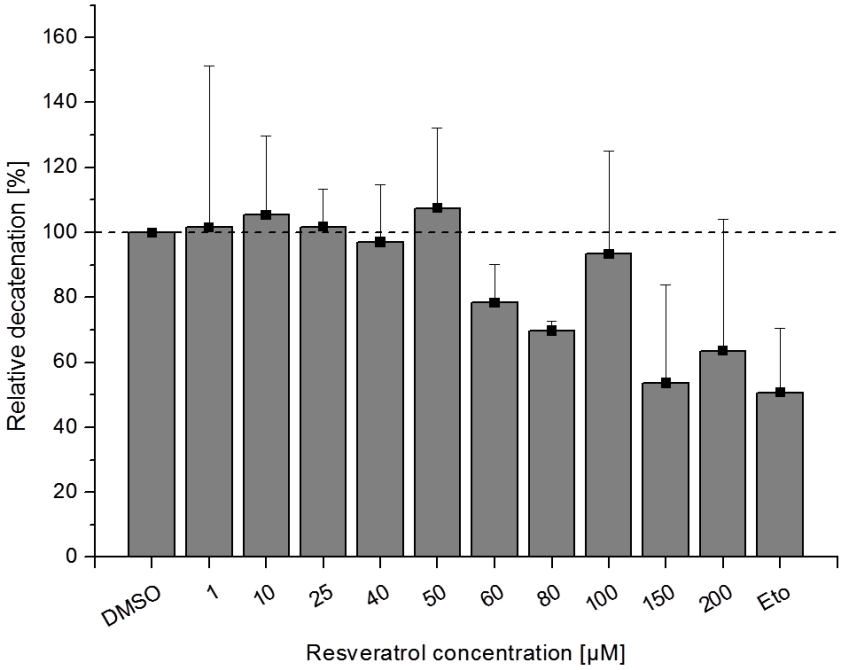


Figure 17: Quantification of the decatenated kDNA after incubation with resveratrol. Etoposide (Eto) [50 µM] was used as the positive control and DMSO was used as the negative control. The means ± SD of 4-8 replicas are shown. The values were referred to the negative control. Outliners were excluded using the Nalimov test.

According to Figure 17, a 40 % inhibition of the catalytic activity of topoisomerase IIα is observed at the highest tested concentration of 200 µM resveratrol. Topoisomerase IIα is not inhibited at a concentration below 50 µM of resveratrol. Due to the high error bars, a tendency of the inhibitory potential can be observed.

Leone *et al, 2010,* showed that resveratrol inhibits the enzyme almost completely at a concentration of 80 µM. As the figure of the representative agarose gels shows (Figure 16), our results do not contradict the results of Leone *et al., 2010.* A previous study (Cho *et al.*, 2000) defined resveratrol as one of the potent chemo-preventive agents with an effective inhibition of the catalytic activity of mammalian topoisomerase II. Resveratrol showed a 50% inhibition of topoisomerase II at a concentration of 65.72 µM in concordance with a study by Jo *et al.*, 2005, showing a dose-dependent inhibition of the enzyme with an IC_{50} at a resveratrol concentration of 78.86 µM.

Taken together, these previous results indicate a more potent inhibitory effect of resveratrol compared to our observations. However, the mentioned studies did not conduct the Decatenation assay as it was performed in our study. It turns out, that the Relaxation assay, the Cleavage assay and Decatenation assay might not lead to comparable results.

In our study, resveratrol was shown to be an inhibitor of topoisomerase IIα above a concentration of 50 µM. At the highest tested concentration of 200 µM, resveratrol induces a 40 % inhibition of topoisomerase IIα. This data indicate that resveratrol inhibits human topoisomerase IIα, either as an inhibitor of the catalytic activity or as a topoisomerase II poison. In order to investigate whether the substance acts as topoisomerase II poison, the ICE-bioassay should be conducted.

5.3 Influence of vineatrol®30 on human topoisomerase II

In order to investigate the potential of vineatrol®30 to inhibit human topoisomerase IIα, we conducted the Decatenation assay as a cell-free-system. The results are shown in figure 18.

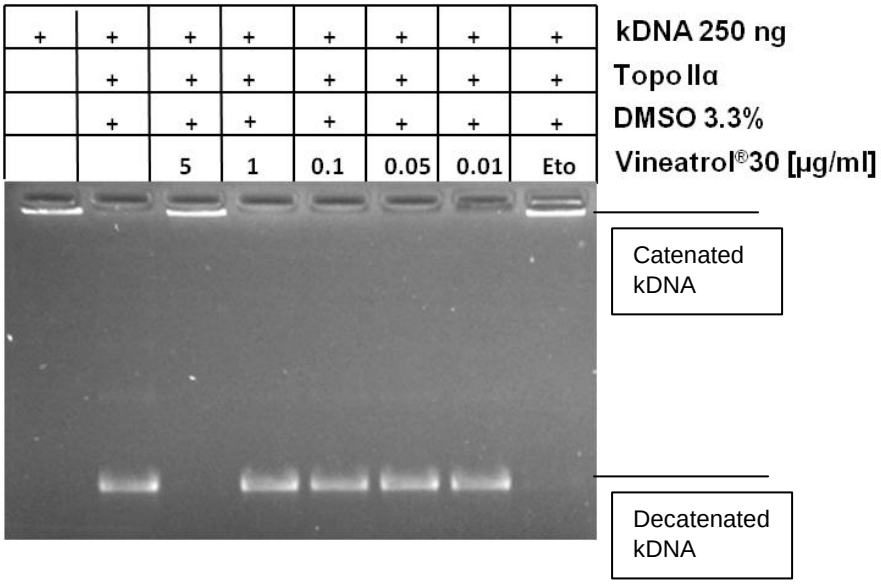


Figure 18: Vineatrol®30 inhibits human topoisomerase IIα. Etoposide (Eto) [50 µM] was used as the positive control, DMSO [3.3 %] was used as the negative control and kDNA was used as the substrate. Decatenation reactions were performed with 1 U of human topoisomerase IIα at 37°C for 1 h of incubation. 3 replicas were conducted.

As figure 18 shows, the grapevine-shoot extract vineatrol®30 strongly inhibits the enzyme at a concentration of 5 µg/ml, whereas no inhibition was observed at 1 µg/ml.

The Decatenation assay alone cannot distinguish between catalytic inhibitors and topoisomerase poisons. In order to investigate the mechanism of vineatrol®30 to inhibit human topoisomerase II, the ICE-bioassay was performed. As this assay can directly determine the amount of covalent topoisomerase/DNA-complexes, it can be examined whether vineatrol®30 acts as a poison generating ternary enzyme/DNA/vineatrol®30-complexes. Figure 19 shows the results of the ICE-bioassay, performed after the incubation with vineatrol®30 in *A 431*-cells for one hour.

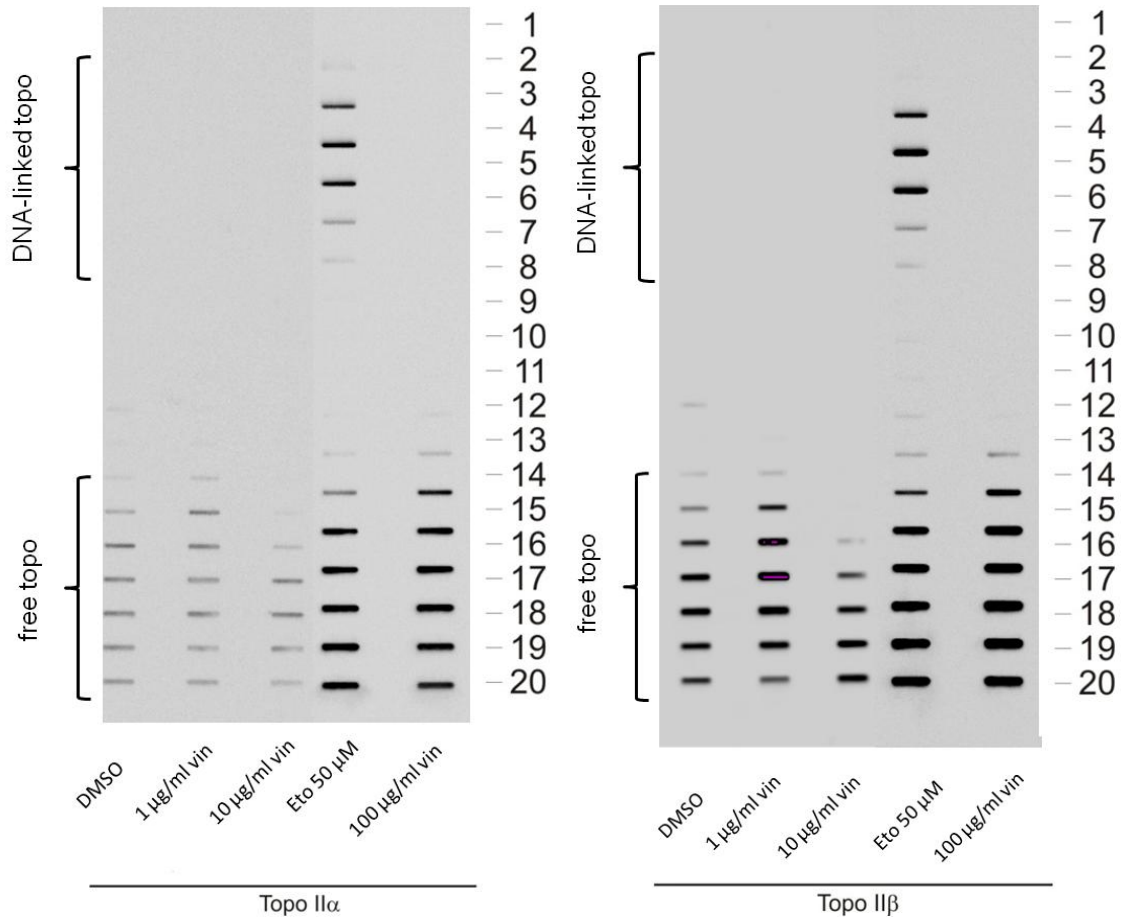


Figure 19: The ICE-bioassay was performed with vineatrol®30 in *A 431*-cells. Incubation time was 1 h at 37°C with the addition of catalase [100 U/ml] and FCS [10 %] to the incubation media. Etoposide (Eto) [50 µM] was used as the positive control and DMSO as the negative control. The experiment was performed once. The numbers indicate fractions taken from the gradient.

As Figure 19 indicates, the positive control Eto shows ternary topoisomerase II/DNA/Eto-complexes, contained in fraction 4 to 8, and free enzyme within fraction 13 to 20. This contrasts with the negative control DMSO that only shows free topoisomerase II. This result was expected as Eto is known as a potent topoisomerase II poison (chapter 3.1.4.2). Vineatrol®30 was tested within the range of concentrations from 1 to 100 µg/ml and does not poison topoisomerase IIα and β. In fraction 4 to 8, no covalent enzyme/DNA-complexes are detected, but free enzyme is observed in fraction 13 to 20. Combining the results of the ICE-bioassay and the Decatenation assay, vineatrol®30 acts as a strong inhibitor of the catalytic activity of human topoisomerase IIα. Within intact cells, the extract is not capable to generate ternary enzyme/DNA/vineatrol®30-complexes, but interacts with topoisomerase II before the DNA-binding-step.

According to Wu et al, 2011, the most drug-interacting residues are conserved between topoisomerase IIα and β. However, they found a specific drug-contacting residue which is different in both isoenzymes, resulting in a polarity change. As there are substances acting isoenzyme-specific, it is important to investigate both isoforms of human topoisomerase II. With the Decatenation assay as cell-free-system, it cannot be excluded, that the extract shows a preference for one topoisomerase isoform within intact cells. The results of the ICE-bioassay show that vineatrol®30 has the same effect on both human topoisomerase II α and β.

In order to determine the ratio of topoisomerase II covalently bound to DNA and to obtain a scaling constant, the DNA content of the samples was measured. The DNA content of the negative control DMSO is shown in figure 20.

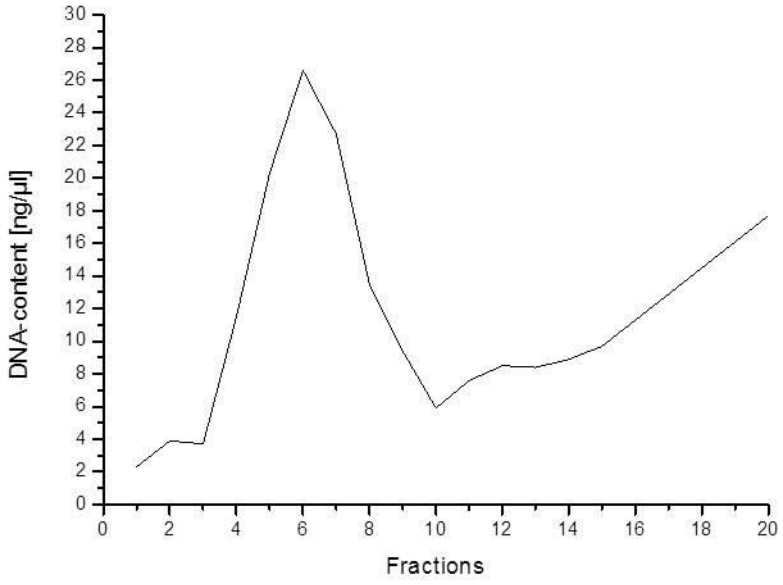


Figure 20: DNA content of the fractions of the negative control DMSO [0.1 %].

The graph indicates that the fraction 6, containing 28 ng/µl, shows the highest DNA content. The cells showed no sufficient confluence leading to the measured low DNA content. As a result, the reliability of the experiment is weakened as a DNA content above 80 ng/µl would be sufficient to differentiate between a strong and a weak poisoning effect. As the experiment was performed once, it functions as an initial orientation concerning the effect of the extract on topoisomerase II in *A 431*-cells. At least two repetitions are required to confirm the effect of vineatrol®30 on human topoisomerase II.

5.4 Influence of resveratrol oligomers on human topoisomerase II

5.4.1 Hopeaphenol

As the extract vineatrol®30 showed inhibition of the catalytic activity of human topoisomerase IIα, selected resveratrol-oligomers, contained in the extract, were examined. Figure 21 illustrates the results of the Decatenation assay performed with the resveratrol-tetramer hopeaphenol.

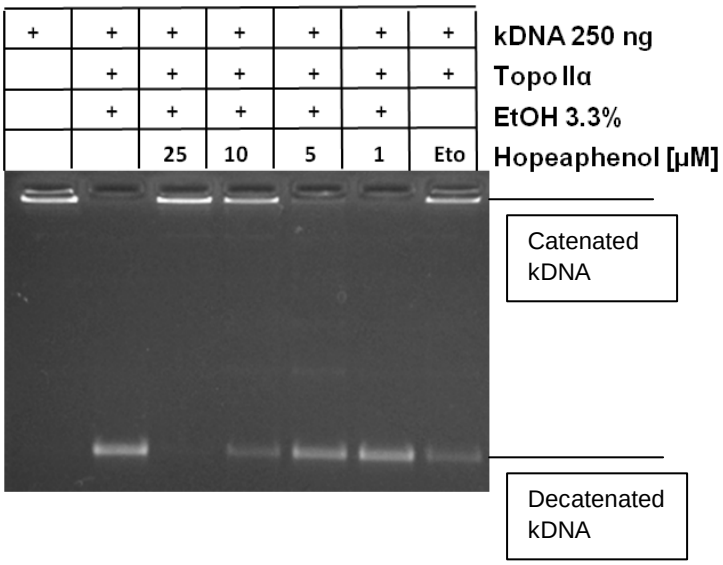


Figure 21: Hopeaphenol inhibits human topoisomerase IIα. Eto [50 µM] was used as the positive control and was dissolved in DMSO. Ethanol (EtOH) was used as the negative control and kDNA as the substrate. Decatenation reactions were performed with 1 U of human topoisomerase IIα at 37°C for 1 h of incubation. 4 replicas were conducted. The 5 µM concentration was tested once to further define the threshold of inhibition and no effect.

According to figure 21, hopeaphenol strongly inhibits the catalytic activity of topoisomerase IIα at a concentration above 25 µM. No inhibition is observed at 1 µM of hopeaphenol. The quantification of the decatenated kDNA lane is shown in Figure 22.

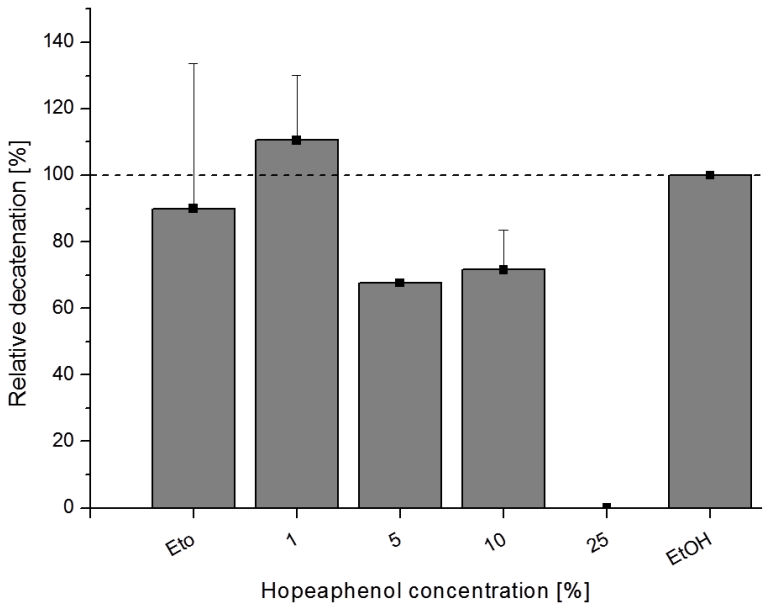


Figure 22: Quantification of the decatenated kDNA after incubation with hopeaphenol. Etoposide (Eto) [50 µM] was used as the positive control and DMSO as the negative control. The means ± SD of 4 replicas are shown. The values were referred to the negative control ethanol.

For the positive control, the graph (Figure 22) shows a high error bar as the enzyme was only inhibited in one replica. In agreement with the gel picture (Figure 21), the results of the quantification show a strong topoisomerase IIα inhibition at a concentration above 25 µM. 10 µM of hopeaphenol resulted in a 30 % inhibition, meaning a relative decatenation effect of 70 %. The catalytic activity of the enzyme is not inhibited at a hopeaphenol concentration of 1 µM, whereas 5 µM of hopeaphenol resulted in a 32 % inhibition.

5.4.2 r-2-Viniferin

A further tested constituent of the extract vineatrol®30 was the resveratrol-tetramer r-2-viniferin. The results of the Decatenation assay are shown in Figure 23.

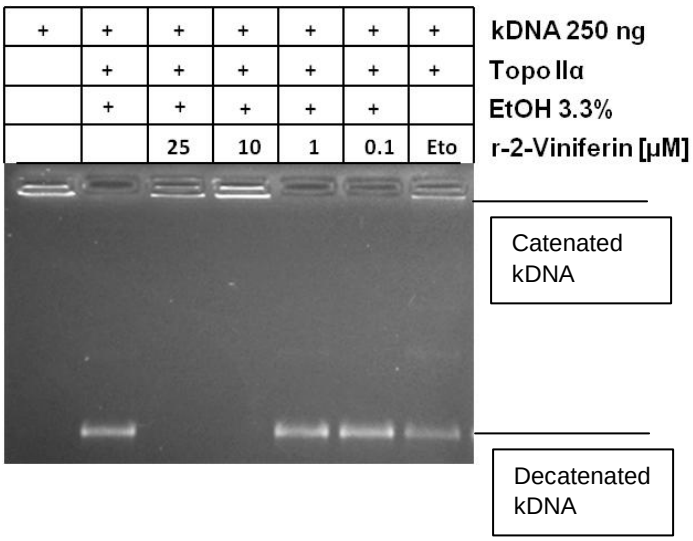


Figure 23: r-2-Viniferin inhibits human topoisomerase IIα. Eto [50 µM] was used as the positive control and was dissolved in DMSO. Ethanol (EtOH) was used as the negative and kDNA was used as the substrate. Decatenation reactions were performed with 1 U of human topoisomerase IIα at 37°C for 1 h of incubation. 4 replicas were conducted.

r-2-Viniferin was tested in a range of concentrations between 0.1 and 25 µM. Figure 23 indicates that a strong inhibition of the catalytic activity of topoisomerase IIα is induced above a concentration of 10 µM r-2-viniferin. At the concentration of 1 µM the enzyme is not inhibited.

5.4.3 ε-Viniferin

The resveratrol-dimer ε viniferin, contained in the extract vineatrol®30, was also tested in the Decatenation assay and the results are shown in Figure 24.

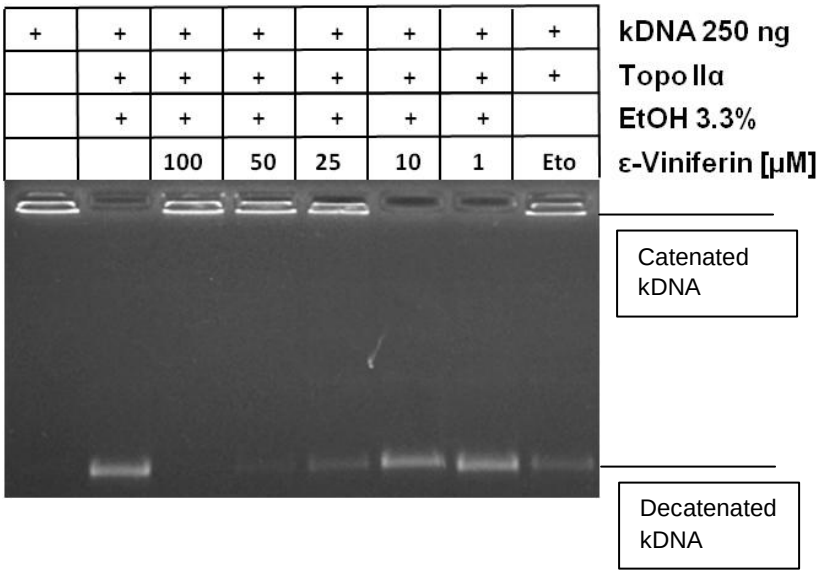


Figure 24: ε-Viniferin inhibits human topoisomerase IIα. Eto [50 µM] was used as the positive control and was dissolved in DMSO. Ethanol (EtOH) was used as the negative control and kDNA as the substrate. Decatenation reactions were performed with 1 U of human topoisomerase IIα at 37°C for 1 h of incubation. 4 replicas were conducted.

ε-Viniferin was tested in the range of concentrations from 1 to 100 µM. Figure 24 demonstrates that ε-viniferin strongly inhibits topoisomerase IIα at a concentration of 50 µM. No inhibition is observed with 10 µM, whereas a almost complete inhibition is shown with 25 µM of the substance.

Consistent with the Decatenation assay results of the other substances, the mechanism of inhibition cannot be concluded and the ICE bioassay must be performed to examine the presence of covalent enzyme/DNA-complexes. Although the extract showed no poisoning, the pure constituents might induce the formation of ternary enzyme/DNA/poison-complexes in higher concentrations.

From the results of the Decatenation assay the question arises, which test substance shows the highest potency to inhibit human topoisomerase IIα. Figure 25 illustrates the concentrations of the tested constituents of the extract vineatrol®30, as well as the minimal inhibition concentration (MIC) to completely inhibit topoisomerase IIα.

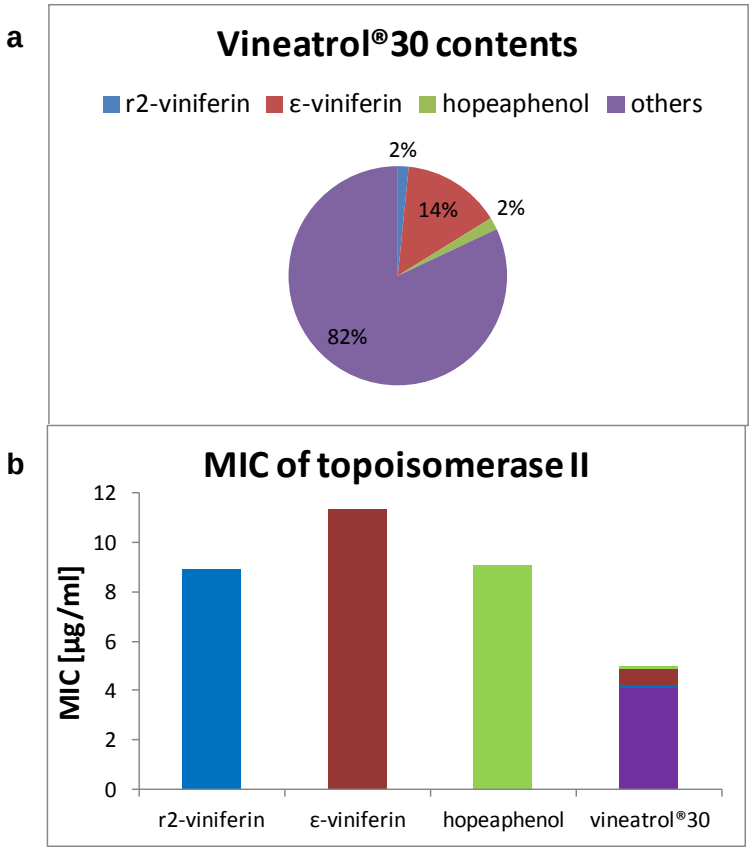


Figure 25: Content of the test substances in the extract vineatrol®30 (a) and their minimal inhibition concentration (MIC) to inhibit human topoisomerase II (b). In the comparison, the MICs were shown in µg/ml, and not in the µM-concentrations as in the gel pictures. The vineatrol®30 bar shows the composition of the extract in relation to the test substances.

As displayed in figure 25a, ε-viniferin exhibits the highest content in the extract vineatrol®30.

According to figure 25b, the potency (inverse to MIC) of the pure single compounds is lower than the potency of the extract vineatrol®30. However, the three tested constituents may act in synergy. Co-incubation experiments could be carried out to assess the synergy effects. The extract shows a high potential to inhibit human topoisomerase IIα. The effect is 2-fold higher than the effect of the pure constituents. As figure 25b indicates, the effect of ε-viniferin, r-2-viniferin and hopeaphenol on the inhibition of the enzyme is similar. Comparing the gel pictures (Figure 21, 23 and 24), the resveratrol-tetramer r-2-viniferin shows the lowest MIC and the therefore the highest potency (Figure 23).

6 Conclusion

In conclusion, it was shown, that resveratrol might not be capable of significantly inducing DNA strand breaks, although the tendency is observed with the highest tested concentration of 200 µM. No fpg-sensitive-sites were shown (Figure 15). Furthermore, resveratrol inhibits the catalytic activity of human topoisomerase IIα at a concentration above 50 µM, in the line with Leone *et al., 2010* (Figure 16).

Moreover, it was shown in figure 19 that the grapevine-shoot extract vineatrol®30 does not poison both isoforms of human topoisomerase II, but acts as an inhibitor of the catalytic activity of the enzyme with the MIC of 5 µg/ml (Figure 18).

The resveratrol oligomers hopeaphenol, r-2-viniferin and ε-viniferin, contained in the extract vineatrol®30, were shown to act as inhibitors of the catalytic activity of human topoisomerase IIα. Comparing the gel pictures, r-2-viniferin showed the lowest MIC of 10 µM and the highest potency (Figure 23) Hopeaphenol and ε-viniferin completely inhibit topoisomerase IIα above a concentration of 25 µM (Figure 21 and 24). The potency of the extract to inhibit topoisomerase IIα is 2-fold higher than the potency of the tested constituents (Figure 25) suggesting that synergy effects of the constituents occurred. When compared to the reference substance resveratrol, resveratrol oligomers showed a stronger potential to inhibit topoisomerase II.

Further examinations are required to confirm that vineatrol®30 does not act as a topoisomerase II poison. Whether the extract as well as the pure constituents hopeaphenol, r-2-viniferin and ε-viniferin are capable of significantly inducing DNA strand breaks, is also of interest. Although the extract might show no poisoning effect on human topoisomerase II, it cannot be excluded that one of the pure constituents induces the generation of ternary complexes with topoisomerase II/DNA at higher concentrations and the ICE-bioassay should be conducted.

It was shown that the grapevine-shoot extract vineatrol®30 acts as a strong inhibitor of topoisomerase II, but might not induce enzyme-mediated DNA-damage by the generation of permanent instead of transient DNA double strand breaks, topoisomerase-poisoning, as the most effective topoisomerase II-targeting drugs applied in cancer therapy.

7 Materials and Methods

7.1 Cell culture

7.1.1 Materials

A431 media

MEM + 1 % L-glutamine, 10 % or 20 % FCS + 1 % penicillin (10.000 units/ml penicillin-6-sodium)/streptomycin (10000 µg/ml streptomycin sulphate).

FCS

FCS was defrosted slowly, inactivated in the water bath at 56°C for 30 minutes and frozen in aliquots at – 20 °C.

PBS (10x)

NaCl 1.71 M, KCl 2.7 mM, KH_2PO_4 1.5 mM, Na_2HPO_4 8.1 mM, adjusted to pH 7.4.

Trypsin/EDTA solution

Trypsin 0.021 mM, EDTA 0.86 mM, PBS (10x) 10 % (v/v), stirred on ice over night, adjusted to pH 7.0-7.4, sterile filtrated and frozen in aliquots at – 20 °C.

7.1.2 Sterile working procedure

The media, PBS and trypsin/EDTA were pre-warmed in a water bath at 37 °C and the airflow in the hood was stabilized for about 10 minutes. The required pipettes were flamed and an aspirating pipette was attached to vacuum. All the materials including tubes and flasks were cleaned with 70 % ethanol. The hands were covered with gloves and cleaned with ethanol.

7.1.3 Mycoplasma test

A sterile microscope slide was transferred into a dish filled with culture media. The cells were trypsinized and 3-4 drops of the cell suspension were applied to the slide surface. After 24-72 hours, when a cell confluence of 50-70 % was observed, the culture media was replaced by – 20 °C cold methanol. In order to fix the cells, the slide was transferred into a Coplin chamber with – 20 °C-cold methanol for at least 30 minutes. Then, three drops of 4',6-diamidino-2-phenylindole (DAPI)/SR 101 staining-solution [1 µg/ml] were applied to the slide. The detection was performed using fluorescence microscopy with an excitation wave length of 340 nm and an emission wave length of 488 nm. The experiments were performed exclusively with mycoplasma-free cells (Lindl and Gstraunthaler, 2008).

7.1.4 Cell line *A 431*

The epithelial-like *A 431* cell line was established from the solid tumour of an 85-year-old woman. When adherence is achieved, *A 431* cells grow in monolayers with a doubling time of about 80-100 hours. Therefore, cell passaging should be constantly performed depending on the cell confluence (DSMZ, Braunschweig).

7.1.5 Cell culture conditions

A 431 cells were cultured in MEM containing 1 % L-glutamine, 10 % FCS as well as 1 % P/S. The cells were cultured in monolayers in T flasks at the incubator conditions of 37 °C, 5 % CO_2 and 95 % relative humidity.

7.1.6 Changing media

With increasing confluence, the conditions change constantly in a batch culture in terms of raising metabolites concentration as well as decreasing nutrients. These continuous variations influence not only the pH-value, but also the redox-state of the cells. Cell lines growing as monolayers in dishes or T flasks, require the continuous change of the media for their growth and viability (Lindl and Gstraunthaler, 2008).

7.1.7 Passaging cells

It was confirmed under the microscope that the cells in the T-flask showed a confluence of 90 % - 100 %. The cells were washed with 10 mL of PBS and the buffer was aspirated from the T-flask. Then, 2.5 ml of trypsin/EDTA was added and the cells were incubated at 37 °C for 8 minutes. The T-flask was removed from the incubator and the side of the T-flask was tapped on the hand for several times. During this procedure, it was checked if cell clusters were dispersed. In order to stop the trypsin reaction, 10 ml of culture media was added to the T flask and the cells were carefully re-suspended. Depending on the experimental setup, a certain volume of the cell suspension was transferred into a T-flask filled with media and put back into the incubator (Lindl and Gstraunthaler, 2008).

7.1.8 Counting cells and seeding

The density of initially inoculated cells influences the growth rate of adherent growing cells. It has to be found a balance between too confluent cells inhibiting growth and too low cell density slowing growth. The cell number was determined using the Neubauer cell counting chamber which is a precision instrument. The grid of a cell counting chamber is shown in Figure 26.

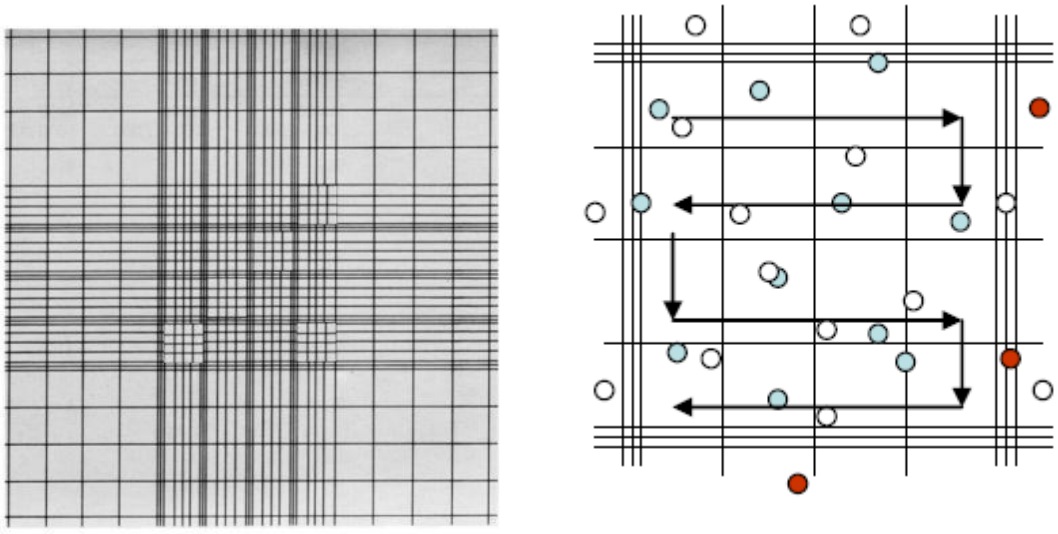


Figure 26: Illustration of the Neubauer cell counting chamber. a) The grid of the cell counting chamber is depicted. b) In this picture dead cells are shown in white, viable cells are illustrated in blue. The red cells are not counted (modified according to Lindl and Gstraunthaler, 2008).

After trypsination, the cell suspension was stained with trypan blue (dilution 1:10). By applying a drop of the cell suspension, a specified volume was drawn into the grid by capillary forces. The cells in the four quadrants were counted. The cell number per milliliter of the cell suspension was calculated by multiplication of the average cell number in each quadrant with the dilution factor as well as with the volume factor 10^4. Depending on the designated cell number, a certain volume was inoculated into the media and equitable applied to the dishes (Lindl and Gstraunthaler, 2008).

7.1.9 Determination of the cell viability

In order to differentiate vital from dead cells, the trypan blue test was used. As an acidic dye trypan blue can bind to proteins in its anionic state. The principle of this test lies in the fact that the dye does not accumulate in the intracellular of vital cells as it is transported out of the cell. By contrast, dead cells are stained because their metabolism is disturbed.

Viability means the ratio of vital cells to the total cell number and is calculated with the following formula:

$$\%\ \text{viability} = \frac{\text{unstained cells}}{\text{stained cells} + \text{unstained cells}} \times 100$$

(Lindl and Gstraunthaler, 2008)

7.1.10 Defrosting cells

The *A 431* cell stocks stored in a cryo tube at – 80 °C were quickly transferred into a water bath at 37 °C. The cells were defrosted in the water bath and immediately transferred into 10 ml pre-warmed culture media containing 20 % of FCS. Then, the cell suspension was centrifuged at 250 x g at RT for five minutes. After discarding the supernatant, the cell pellet was re-suspended in 5 ml of the media and transferred to a T-flask (25 cm^2). Another 5 ml of culture media was added. The cells were not touched during the following twelve hours. After two passages, the FCS-concentration was decreased to 10 %
(Lindl and Gstraunthaler, 2008).

7.2 Test substances

The grapevine-shoot extract vineatrol®30 and the pure constituents hopeaphenol, ε-viniferin as well as r-2-viniferin were provided by Prof. Pablo Steinberg from the Institute of Food Toxicology and Analytical Chemistry of the Veterinary University Hannover. Resveratrol was purchased from Sigma. The molecular weight of the test substances is summarized in Table 5.

Table 5: Molecular weight of the test substances.

test substance	molecular weight [g/mol]
vineatrol®30	Extract
ε-viniferin	454.5
r-2-viniferin (visitin A) 1	890.8
hopeaphenol	906.9
trans-resveratrol	228.25

Hopeaphenol, ε-viniferin and r-2-viniferin were dissolved in ethanol. Vineatrol®30 and resveratrol were dissolved in DMSO. The stock solutions were stored at – 80 °C.

7.4 Single cell gel electrophoresis: Comet assay

In the Comet assay, established by Ostling and Johanson, 1984, the cells are embedded in agarose on a microscope slide. The cells form a cavity in the gel and most of the biomolecules diffuse through the agarose gel into the lysis buffer. Only DNA stays in the cavity due to its high molecular weight. When applying an electric field, DNA fragments migrate towards the anode, is released from the cavity and forms a “tail”, whereas intact DNA shows no or less migration. Strand breaks enhance the migration of DNA towards the anode due to the changes in DNA topology. Therefore, strand breaks can be quantified according to the tail intensity. Figure 27 shows the experimental setup of the Comet assay.

Because the Comet assay according to Ostling and Johanson, 1984, is performed under neutral conditions only DNA double strand breaks can be detected. Singh *et al.*, 1988, developed the Comet assay under alkaline conditions. Due to the alkaline conditions, not only cellular RNA disturbing the quantification is degraded, but also the DNA double strands are separated. Therefore, both single- and double-stranded DNA-breaks as well as other alkali-sensitive-sites can be detected. Additional treatment with specific enzymes can detect common DNA lesions.

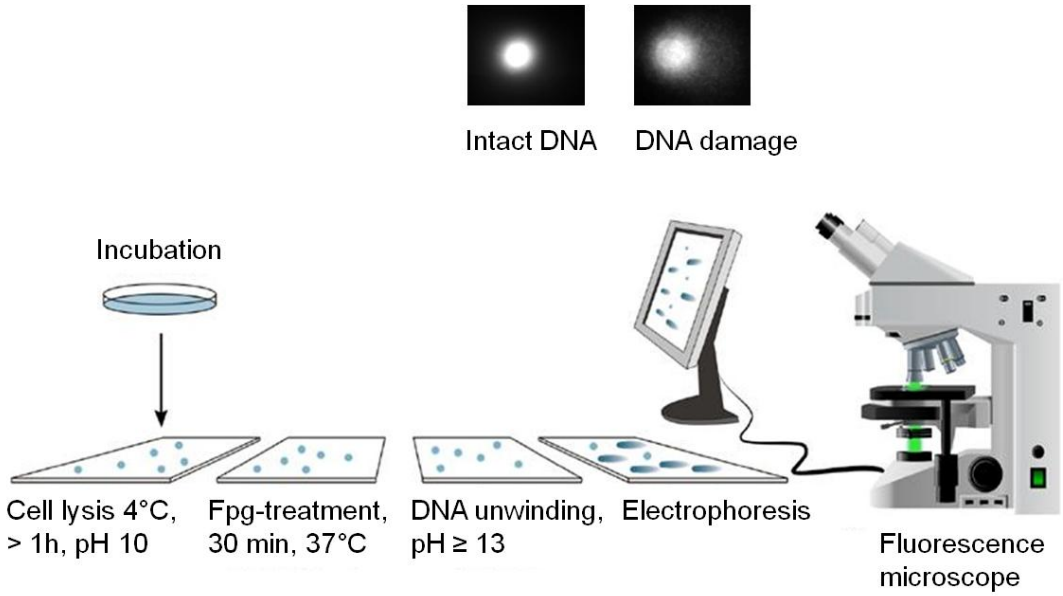


Figure 27: Experimental Setup of the Comet assay.

Incubation

A 431 cells were seeded at a density of 3x 10^5 cells/dish in culture media for 48 hours. At a confluence of 50-60 %, the cells were exposed to resveratrol [0.2-200 µM[for one hour. DMSO [0.1 %] was used as solvent. In order to compensate H_2O_2 generation, catalase [100 U/ml] was added. UV-light exposure for two minutes was used as the positive control whereas DMSO [0.1 %] was used as the negative control.

Preparation of the slides

Two slides for each incubated dish were prepared meaning one slide additionally for the fpg-treatment.

Melted 0.8 % normal-melting agarose (NMA) (40 µl) was coated on the matted side of the slide. After drying, the slides were coated for a second time in order to achieve a better grip for the pads. The pads consisting of NMA (65 µl) were applied to the coated slides and coverslipped free from air bubbles. The slides were dried on an ice-cold surface to allow solidification of agarose for at least 30 minutes and stored in a wet slide box at 4 °C.

Preparation of the cells

After incubation, the media was discarded and the cells were washed twice with pre-warmed PBS (2x 2 ml). The cells were trypsinised at 37 °C for eight minutes. The trypsin reaction was stopped with FCS-containing culture media (0.5 ml) and the cell suspension was transferred into ice-cold tubes. In order to collect all cells, the dish surface was rinsed with culture media (0.5 ml) and pooled with the first fraction.

Viability test

The cell suspension was stained with trypan blue (1:2) in order to maintain the ratio of dead to vital cells. For the exclusion of cytotoxic effects, the cell viability should be higher than 80 %.

Embedding of cells in the agarose pads

The cell suspension was separated into four aliquots containing 30,000 cells each and transferred into a tube. After centrifugation at 2,000 rpm at 4 °C for ten minutes the supernatant was discarded.

For blind testing, the prepared cover slides were encoded. The cells were re-suspended in 0.8 % low-melting agarose (LMA) (65 µl), applied to the NMA-pads and covered with a cover slip free from air bubbles. The slides were dried on an ice-cold surface for at least 15 minutes.

Cell lysis

Cover slips were removed and the slides were put in a Coplin chamber containing cold lysis buffer (89 ml of lysis stock solution: NaCl 2.5 M, EDTA 100 mM, tris 100 mM , N-lauroylsarcosyl sodium salt 1 % (w/v), pH 10; 10 ml of DMSO, 1 ml of triton X-100). The cells were lysed at 4 °C for at least one hour. DNA unfolding occurred during the lysis process.

Fpg-treatment

Formamidopyrimidine-DNA-glycosylase (fpg) was used to detect the presence of FapyAde and FapyGua, which are common DNA lesions caused under oxidative conditions. The slides were washed with ice- cold 1x fpg-enzyme buffer (10x stock solution: HEPES 400 mM, KCl 1 M, EDTA 5 mM, BSA 2 g/l, pH 8) at 4 °C for 3x5 minutes. During the last washing step, 10 µl aliquots of fpg-enzyme were defrosted on ice. Then the aliquots were diluted (1:30) using ice-cold 1x fpg-enzyme buffer. The slides were covered with either 50 µl of the enzyme-buffer or 50 µl of the enzyme and incubated in a wet chamber at 37 °C for 30 minutes.

Alkalinization and electrophoresis

After removing the cover slips the slides were applied to an ice-cold electrophoresis chamber. The chamber was filled with electrophoresis buffer (NaOH 10 M, 3 % (v/v), EDTA 200 mM 0.5 % (v/v)). The slides were alkalized in the dark for 20 minutes. During this alkalization step, DNA unwinding occurred. The required amperage was adjusted by addition or removal of electrophoresis buffer. Then electrophoresis ran at 25 V and 300 mA in the dark for 20 minutes.

Neutralization

In order to confer DNA in its double stranded state, a washing step with neutralization buffer (tris/HCl 400 mM, pH 7.5) was performed. With neutralization buffer also alkali and detergents which could interfere with ethidium bromide were removed. The slides were washed with neutralization buffer at 4 °C for 3x5 minutes.

DNA staining

Ethidium bromide solution ready to use (36 µl) (250x stock-solution: 1 µg/ml ethidium bromide in H_2O) was applied to the slides and covered with a cover slip.

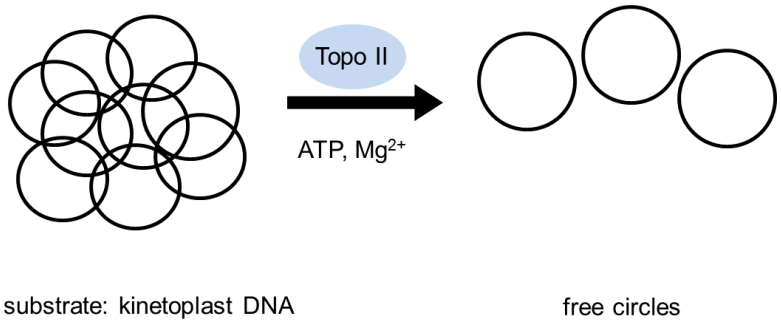
Analysis

The analysis was performed using a fluorescence microscope with a mercury vapour lamp as light source. Ethium bromide was excited at a wave length of 512 nm using a connected CY-3 filter resulting in an emission at a wave length of 590 nm. The image was taken with a CCD camera and analysed with the software Comet Assay IV-System (Perspective Instruments). For each gel pad 50 non-overlapping cells were analysed and the mean value of the tail intensities was calculated. After analyzing all slides, the encoding was abolished.

7.5 Inhibition of the catalytic activity of topoisomerase II α: Decatenation assay

With this cell-free assay, inhibition of catalytic activity of topoisomerase II can be examined. The catenated DNA substrate was prepared from the kinetoplast of the insect trypanosome *Crithidia fasciculata*. Kinetoplast DNA (kDNA) is an aggregate of connected DNA mini-circles (mostly 2.5 kb) generating large networks of high molecular weight. Consequently, these networks fail to migrate into an agarose gel. Upon incubation with topoisomerase II, which engages DNA in a double stranded breaking and reunion cycle, DNA minicircles are released (decatenated). The decatenated minicircles move rapidly into the gel owing to their small size (Figure 28).

a)



b)

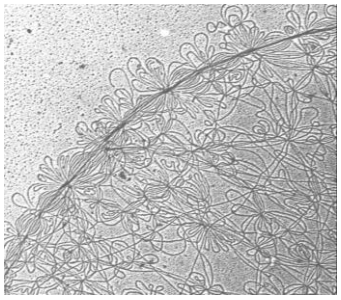


Figure 28: Experimental setup of the Decatenation assay. a) Release of minicircles from kDNA catalyzed by topoisomerase II b) Catenated DNA

Table 6: Pipette scheme of the Decatenation assay.

Solution	kDNA [µl]	kDNA + topo IIα + inhibitor [µl]
H_2O bidest.	18.95	17.40
KCl 1 M	3.6	3.6
Saltmix II ($MgCl_2$ 50 mM, DTT 5 mM EDTA 5 mM, ATP 10 mM, BSA 0.3 mg/ml	3	3
Tris/HCl 500 mM, pH 7.9.	3	3
kDNA 250 ng/µl	1	1
Substance or control		1
Topoisomerase IIα [1 U] (1:50 in dilution buffer, tris/HCl 50 mM (pH 7.5), NaCl 400 mM, DTT 1 mM, glycerol 50 % (v/v)	1	1
Proteinase K (1 mg/ml in 10 % (w/v) SDS-solution)	3	3
Loading dye (Bromine phenole blue 0.25% (w/v), glycerol 30% (v/v))	5	5

Incubation and detection

The reaction mix was pipetteted according to table 6 and incubated at 37 °C for 60 minutes Etoposide (50 or 100 µM) was used as the positive control and the specific solvent, DMSO or ethanol (3.3 %) was used as the negative control.

As the enzyme might influence the migration of the DNA through the gel, it was denatured by proteinase K. Incubation with proteinase K was performed at 37°C for 30 minutes. Loading dye was added and 17 µl of the samples each were applied to the gel (agarose 1 % (v/v) in 1x TAE buffer (TAE buffer 50x: tris 2 M, acetic acid 5.71% (v/v), EDTA 0,5 M (pH 8.0) 10%, pH 8.5).

Electrophoresis was performed at 60 V for three hours in 1x TAE buffer. The gel was stained with ethidium bromide (1 µg/ml in H_2O) for 20 minutes and washed in H_2O for five minutes. The lanes were detected using LAS 4000 at a fluorescence wavelength of 560 nm.

7.6 ICE (*in vivo* complex of enzyme to DNA)-bioassay

Antibodies

Primary antibody

Anti-topoisomerase IIα (*rabbit*, Santa Cruz): 1:333 in blocking buffer

Anti-topoisomerase IIβ (*rabbit*, Santa Cruz): 1:333 in blocking buffer

Secondary antibody

Anti-*rabbit* HRP-IgG-conjugated (Santa Cruz): 1:2000 in blocking buffer

According to its name, this assay can detect covalent complexes of DNA and topoisomerases. Therefore, topoisomerase inhibitors can be examined concerning their mechanism of action. As only topoisomerase poisons can stabilize covalent enzyme/DNA-complexes they can be distinguished from catalytic inhibitors.

The principle of this assay lies in a separation according to densities using a CsCl gradient. Covalent DNA-topoisomerase-complexes gravitate during ultracentrifugation. By contrast, non-covalent protein-DNA-interactions dissociate in the density gradient. Free topoisomerase can be detected in the upper part of the centrifugation tube. Fractions of the gradient are taken and plotted on nitrocellulose. Topoisomerase II α and IIβ can be detected by the use of specific antibodies (Figure 29).

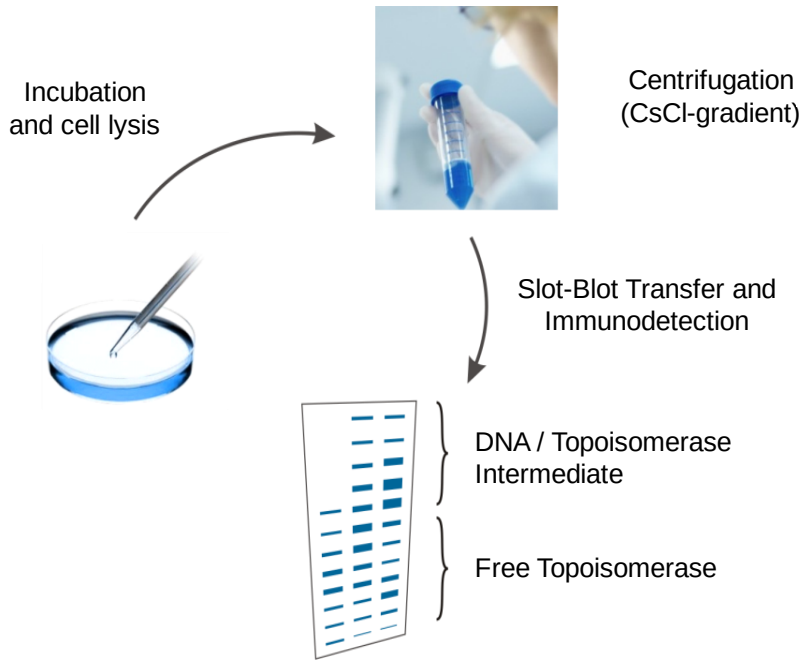


Figure 29: Experimental setup of the ICE-bioassay.

Sample preparation

A 431 cells were seeded at a density of 3x 10^6 cells/dish in culture media for 72 hours. At a confluence of 80-90 %, the cells were exposed to vineatrol®30 [0.1 µM-250 µM] for one hour. In order to compensate H_2O_2 generation, catalase [100 U/ml] was added.

Cells were washed with 2x4 ml 1x PBS each and lysed by the addition of 6 ml of lysis buffer (sarcosinate 1 % (w/v) in TE-buffer). Cells were harvested by abrading and transferred into 15 ml tubes. DNA was sheared using an insulin syringe and 4 ml of the cell lysate was layered onto a CsCl gradient in polyallomer tubes.

Generation of the CsCl gradient and ultracentrifugation

Each of the polyallomer tubes was layered carefully with solution A-D (2ml each) with a decreasing density from the bottom to the top.

A: 1850 µl CsCl solution (120 g CsCl in 70 ml TE-buffer) + 150 µl TE buffer (tris/HCl 10 mM**,** EDTA 1 mM, pH 8)

B: 1600 µl CsCl solution + 400 µl TE buffer

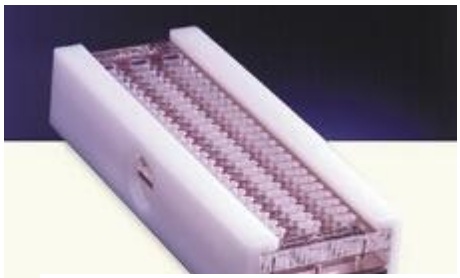
C: 1100 µl CsCl solution + 900 µl TE buffer

D: 900 µl CsCl solution + 1100 µl TE buffer)

The solution was covered with mineral oil in order to tare the ultracentrifuge and to exclude oxygen. Ultracentrifugation was performed using the swing rotor TST 41.14 at 23,000 rpm (100,000 x g) and 20 °C for at least 22 hours.

Fractionation and blotting

a)



b)

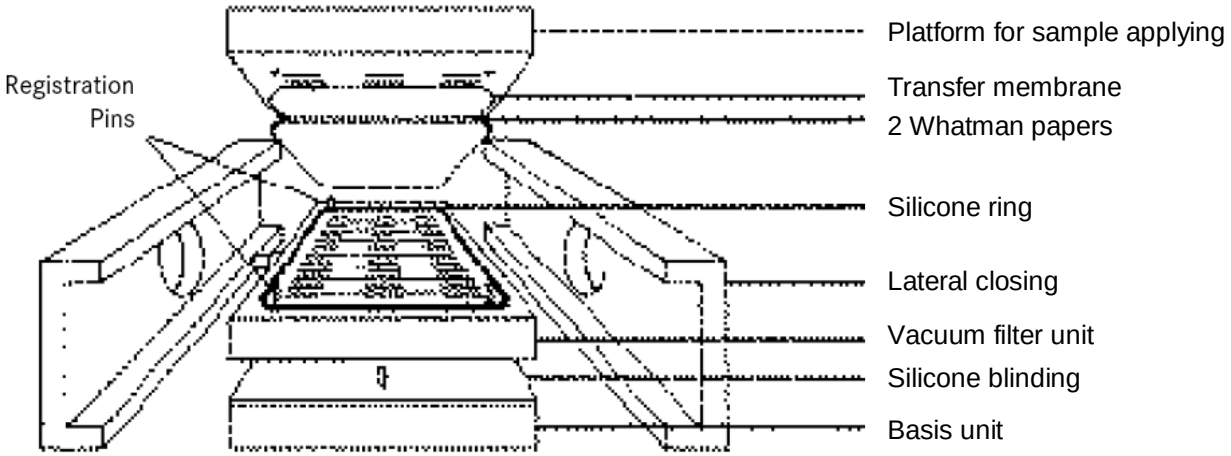


Figure 30: a) Slot blot apparatus b) Assembly of the slot blot apparatus.

The nitrocellulose membrane as well as the Whatman papers (2 for each membrane) were equilibrated in 25 mM phosphate buffer (phosphate buffer 1 M: Na_2HPO_4 1 M, NaH_2PO_4 1 M, mixed 1:1) at 4°C.

The gradient was fractionated from the bottom of the polyallomer tube using insulin syringes. 20 fractions (300 µl each) were gathered and vortexed with 25 mM phosphate buffer (300 µl).

The cell components of the sample solution (200 µl) were blotted on a nitrocellulose membrane using the slot-blot-system (Figure 30).

Blocking and incubation with primary antibody

The membranes were equilibrated in 1x TBST buffer (10x TBST buffer: tris 200 mM , NaCl 1.37 M,tween-20 1% (v/v), pH 7.5) for ten minutes. Unspecific binding sites of the antibody were blocked (blocking buffer: milk powder 5 % (w/v) in 1x TBST) at RT for three hours. Incubation with topoisomerase IIα or β antibody (1:333 in blocking buffer) was performed at 4 °C over night.

Incubation with secondary antibody and detection

The membranes were washed in 1x TBST for 3x 5 minutes to remove non-bound antibody. Incubation with HRP-conjugated anti-rabbit antibody was performed at RT for 2 hours. The membranes were washed (3x 5 minutes). Chemiluminescence reaction was started by LumiGlo and detected using LAS-3000 (Image Reader, Fujifilm).

Determination of the DNA content

The DNA concentration was measured using nanodrop®-spectrophotometer at a wavelength of 260 nm. Due to its high density DNA is expected in the initial fractions. As blank 2 µl of H_2O were used. The detection was performed using ND 1000 V 3.2.1 software.

Analysis

The DNA-rich fractions of the negative control DMSO are taken as reference.

7.7 Chemicals

Table 7: List of chemicals.

Substance	Manufacturer
Adenosine triphosphate (ATP)	Sigma
Acetic acid	Roth
Agarose	Invitrogen
Bovine serum albumine (BSA)	Roth
Bromine phenole blue	Sigma
Catalase	Sigma
Cesium chloride	Roth
4',6-diamidino-2-phenylindole (DAPI)/SR 101	Partec
1,4-Dithiothreitol (DTT)	Fluka
Dimethyl sulfoxide (DMSO)	Roth
Ethylene diamine tetraacetic acid (EDTA)	Roth
Ethanol	Roth
Ethidium bromide	Roth
Etoposide	Sigma
Fetal calf serum (FCS)	Invitrogen
Glycerol	Roth
HEPES	Roth
Hydrochloric acid	Merck
Kinetoplast DNA (kDNA)	Topogen
lauroylsarcosyl sodium salt	Roth
L-glutamine (L-Glu)	Sigma
LumiGLO	Santa Cruz
Methanol	Merck

Magnesium chloride	Roth
Mineral oil	Sigma
Minimum essential media (MEM)	Invitrogen
Milk powder	Roth
Phosphate buffered saline (PBS)	Gibco
Penicillin/Streptomycin (P/S)	Sigma
Proteinase K	Sigma
Potassium chloride	Roth
Potassium hydrogen phosphate	Roth
Sodium dodecyl sulfate (SDS)	Roth
Sodium chloride	Roth
di-sodium hydrogen phosphate	Roth
Sodium hydroxide	Roth
Tris	Roth
Triton X-100	Sigma
Trypan blue	Sigma
Trypsin	Invitrogen
Tween-20	Sigma

7.8 Materials

Table 8: List of materials.

Material	Manufacturer
Cover slips	Menzelgläser
Eppendorf tubes	Sarstedt AG, Co
Glass pipettes	VWR
Culture flasks	Sarstedt AG, Co
Pipette (blue, yellow, bright)	Sarstedt AG, Co
Pipette 5ml	VWR
Pasteur pipettes	Carl Roth GmbH, Co. KG
Dishes (d= 5 or 20 cm)	Sarstedt AG, Co
Tubes (v=13, 15, 50 ml)	Sarstedt AG, Co
Whatman nitrocellulose transfer membrane	Pall Life Sciences
Whatman papers	Whatman Inc.
Cell abrading scraper, 25 cm,	Sarstedt AG, Co

7.9 Equipment

Table 9: List of equipment.

Equipment	Manufacturer
Autoclave Systec DX-150	Systec GmbH
Bio freezer VipTM Series	Sanyo
Burner Fuego Basi	WLD-TEC
Incubator Heracell 240 i, CO2 Incubator	Thermo Fisher Scientific
Ice machine MF 46	Scotsman Frimont
Gel documentation machine	LAS-4000 Luminescent Image Analyser, Fujifilm, software: Image Reader LAS 4000; detection: Multi Gauge V3.2
Gel electrophoresis	Mini-PROTEAN® Tetra System, Bio Rad Modell 45-2010-i, Peqlab Biotechnology GmbH
Fridge/freezer	Liebherr Comfort (4 °C, -20 ° C), Liebherr
Magnetic stirrer	IKA® RCT basic, safety control, IKA
Microscope	Inversmikroskop Axiovert 40C, Zeiss
Neubauer cell counting chamber	Marienfeld
pH-meter	Mettler Toledo Seven Easy, Mettler,Toledo
Pipettes, multipipettes	Eppendorf Research P 10 –P 5000, Eppendorf
Pipettetus accu	Hirschmann Laborgeräte
Power station electrophoresis	Power Supply EV231, PeQlab
Vacuum pump	Laboport®, IBS Integra biosciences
Over head shaker	Roto-Shake-Genie, Scientific Industries
Spectral photometer	Nanodrop 2000c Spectrophotometer, Peqlab Biotechnology GmbH
Sterile bench	Hera Safe KS 18Thermo Fischer Scientific
Thermocycler	MJ Research PTC-200, Peltier Thermal Cycler

Drying chamber	Binder, ATP lineTM ED (E2), Binder GmbH
Vortex	Bio Vortex V1, Peqlab Biotechnologies Vortex Genie 2 Scientific Industries
Scales	Scale New Classic MF, ML204/01 Mettler Toledo Scale Micro, Sartorius AG Scale New Classic MF, ML6001/01Mikro 200, Mettler Toledo GD 100, Grant
Water bath	GD 100, Grant
Centrifuges	Table centrifuge, Mikro 220R, Hettich Zentrifugen Table centrifuge, Mikro 200, Hettich Zentrifugen Table centrifuge, Galaxy MiniStar, VWR Ultra centrifuge, Optima L-100XP, Beckmann Coulter

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9.1 Abstract

Resveratrol (3, 4`, 5-trihydroxystilbene) has shown to exhibit beneficial effects in the prevention of different stages of carcinogenesis. The grapevine-shoot extract vineatrol®30, admitted as a dietary supplement, contains a high amount of resveratrol as well as resveratrol oligomers and –polymers. Recently, it was shown that resveratrol induces DNA double strand breaks and inhibits the catalytic activity of human topoisomerase IIα (Leone *et al., 2010*).

The aim of the current study was firstly to confirm the effect of resveratrol on human topoisomerase IIα and DNA integrity. Secondly, it should be determined whether the extract vineatrol®30 acts as an inhibitor of the catalytic activity of human topoisomerase II, or as a poison by the generation of ternary complexes with the enzyme and DNA. Thirdly, the bioactive constituents in vineatrol®30 concerning the effect on topoisomerase II should be identified.

Using the Comet assay, it was shown that resveratrol exposure (0.1-200 µM) for one hour might not be capable of inducing DNA strand breaks in the human vulva carcinoma cell line *A 431* although the tendency is observed with the highest tested concentration. Furthermore, resveratrol inhibits the catalytic activity of human topoisomerase IIα with a minimal inhibition concentration (MIC) of 50 µM as tested with the cell-free Decatenation assay.

The extract vineatrol®30 was shown to inhibit the catalytic activity of topoisomerase IIα with a MIC of 5 µg/ml. Selected extract-constituents (hopeaphenol, r-2-viniferin and ε-viniferin) inhibited the catalytic activity of the enzyme within the range of MICs between 10 and 25 µM with r-2-viniferin as the most potent test-substance in a cell-free system. Furthermore, it was concluded that vineatrol®30 does not poison human topoisomerase IIα and IIβ in *A 431* cells as no ternary enzyme/DNA/vineatrol®30-complexes were detected using the ICE-bioassay.

In conclusion, it was shown that resveratrol might not be capable of significantly inducing DNA strand breaks in the tested concentrations, but inhibits the catalytic activity of human topoisomerase IIα. The grapevine-shoot extract vineatrol®30 acts as strong inhibitor of the enzyme, but might not induce enzyme-mediated DNA-damage by the generation of permanent instead of transient DNA double strand breaks, topoisomerase-poisoning, as performed by the most effective topoisomerase II-targeting drugs applied for cancer therapy.

9.2 Abstract German

Resveratrol (3, 4`, 5-Trihydroxystilben) wirkt präventiv in unterschiedlichen Stadien der Karzinogenese. Der Weinrebensprösslingsextrakt Vineatrol®30, der bereits als Nahrungsergänzungsmittel zugelassen ist, besteht zu einem Großteil aus Resveratrol sowie Resveratrol Oligo-und Polymeren. In einer aktuellen Studie wurde gezeigt, dass Resveratrol sowohl DNA-Doppelstrangbrüche induziert, als auch die katalytische Aktivität der humanen Topoisomerase IIα hemmt (Leone *et al., 2010*).

Ziel der vorliegenden Arbeit war es, die Wirkung von Resveratrol auf humane Topoisomerase IIα sowie auf die Integrität der DNA zu bestätigen. Außerdem sollte untersucht werden, ob der Extrakt Vineatrol®30 die katalytische Aktivität der humanen Topoisomerase IIα hemmt, oder ob dieser die Bildung von ternären Komplexen mit dem Enzym und der DNA induziert, und somit als Topoisomerase-Gift wirkt. Des Weiteren sollten die bioaktiven Inhaltsstoffe bezüglich des Effekts auf Topoisomerase II in Vineatrol®30 identifiziert werden.

Mittels Comet Assay konnte gezeigt werden, dass Resveratrol in den Konzentrationen von 0,1 bis 200 µM mit einer Inkubationszeit von einer Stunde keine DNA-Strangbrüche in der humanen Vulvakarzinomzelllinie *A-431* induziert, obwohl die Tendenz dazu in der höchsten Testkonzentration feststellbar war. Des Weiteren hemmte Resveratrol die katalytische Aktivität der humanen Topoisomerase IIα mit einer minimalen Inhibierungskonzentration (MIC) von 50 µM, wie mit dem zellfreien Dekatenierungsassay gezeigt werden konnte.

Der Extrakt Vineatrol®30 hemmte die katalytische Aktivität der humanen Topoisomerase IIα mit einer MIC von 5 µg/ml. Ausgewählte Inhaltsstoffe des Extrakts (Hopeaphenol, r-2-Viniferin und ε-Viniferin) hemmten die katalytische Aktivität des Enzyms in einem MIC-Bereich von 10 bis 25 µM, wobei sich r-2-Viniferin als die potenteste Testsubstanz in einem zellfreien System herausstellte. Außerdem wirkt Vineatrol®30 wohl nicht als Topoisomerase II-Gift in *A-431*-Zellen, da keine ternären Enzym/DNA/ Vineatrol®30 - Komplexe mittels ICE-Bioassays nachgewiesen werden konnten.

Abschließend konnte gezeigt werden, dass Resveratrol wohl keine signifikanten DNA-Strangbrüche in den getesteten Konzentrationen induziert, aber die die katalytische Aktivität der humanen Topoisomerase IIα durch diese Substanz gehemmt wird. Der Weinrebensprösslingsextrakt Vineatrol®30 wirkt als starker Inhibitor des Enzyms, obwohl keine enzymvermittelte DNA-Schädigung, als Folge der Bildung permanenter anstatt transienter DNA-Doppelstrangbrüche, der Topoisomerase-Giftung, festgestellt werden konnte, wie es bei den stärksten Topoisomerase II-Krebstherapeutika der Fall ist.

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

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9.6 CV

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