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ABSTRACT

Cancer is the leading cause of death in economically developed countries and second leading cause of death in developing countries. 14,1 million new cancer cases were counted worldwide during 2012. The treatment possibilities of solid tumours nowadays include surgery, by means of resection of malignant cells, radiation, and pharmacological chemotherapy. A large percentage of the chemotherapeutic anti-cancer agents currently used, fall into the category of DNA-binding drugs. These agents interact with the DNA duplex by three binding modes: intercalation, groove binding and covalent DNA-crosslinking. However, our understanding of the relationships between DNA interaction, chemical structure, and biological activity, including genotoxicity, remains still incomplete. Devastating side effects often arise from poor specificity. It is expected that sequence specificity will be achieved more straightforwardly, since the opening of the DNA duplex to increased exposure to new interactions of the otherwise buried bases. This thesis reviews new approaches in DNA-interacting drug design and focus on advances in knowledge about gaining DNA-sequence specificity and ways to utilize this new knowledge for benefiting novel DNA-targeted anti-cancer drug design.

The development of new and improved DNA-binding drugs may come from slight variation of the classical binders: Several examples for this are: brostallicin and lexitropsins, which have been designed after the patterns of netropsin and distamycin, or pirarubicin and aclarubicin as new anthracyclines. Multi-anti-cancer activities via drug-combination have also been established, e.g., anthracycline conjugates with formaldehyde and telomerase inhibitors, respectively. Also altered DNA conformations or new binding modes are used to design alternative and trailblazing compounds. Thereby the idea of selectively targeting cancer cells via conjugation of anti-cancer drugs with new high-specificity compounds was investigated in a huge number of trails. For example hairpin polyamide-chlorambucil conjugates or liposomal formulations as carriers for anthracyclines have been developed. Another example provides the use of anti-cancer drugs connected to polyamine tails, which are potent ligands of the polyamine transporter system, overexpressed in many tumour types. Especially the latter type of conjugation shows great promise for novel specificity improved drug design.

CONTENTS

1. INTRODUCTION

Since the dawn of understanding about the cancer process some 5000 years ago, in the time of Imhotep, physician to the early Pharaoh Djoser, medical science has been mystified by the relentless growth and locally infiltrative nature of the primary tumour and humbled by its debilitating, frequently fatal effects upon the host. Physicians, including Hippocrates (~400 BC) and Galen (~150 BC) were aware of the poor prognosis of cancer patients, but so far as it can be determined, no record of a recognition oft the metastatic behaviour of malignant tumours existed, for over 4000 years of early cancer history. Not till the late 16th century, where French and Italian surgeons began to recognise the tendency of locally destructive primary cancerous lesions to cause hard expansive nodules in their environment. Simultaneously it was recorded that a subsequently excision of those satellites enables the patient even a low chance of survival. 200 years later the French gynaecologist J. Recamier recognised that a space-occupying brain-lesion which caused neurological syndromes in a patient with breast cancer, was a secondary tumour. He named the process cancer metastasis. He realised, that the tumour might spread along blood vessels. Finally Schwann and Virchwo defined the cellular body of tumours in the middle of the 17th century. It arose by reason of some painstaking microscopically studies that carcinomas arise from the epithelium and not, as former expected, from the connective tissue of an organ. This laid the foundation of the later realisation that tumours are not just balls of cancer cells but complex malign living entities, composed of interwoven non-neoplastic and neoplastic components[1].

1.1 Cancer – today: Global Cancer Statistics

About one in five of us will die of cancer[2]. So cancer is the leading cause of death in economically developed countries and second leading cause of death in developing countries. The global burden continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing

behaviours, including smoking, physical inactivity and “westernized diets”, in economically developing countries[3]. 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide. 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions[4].

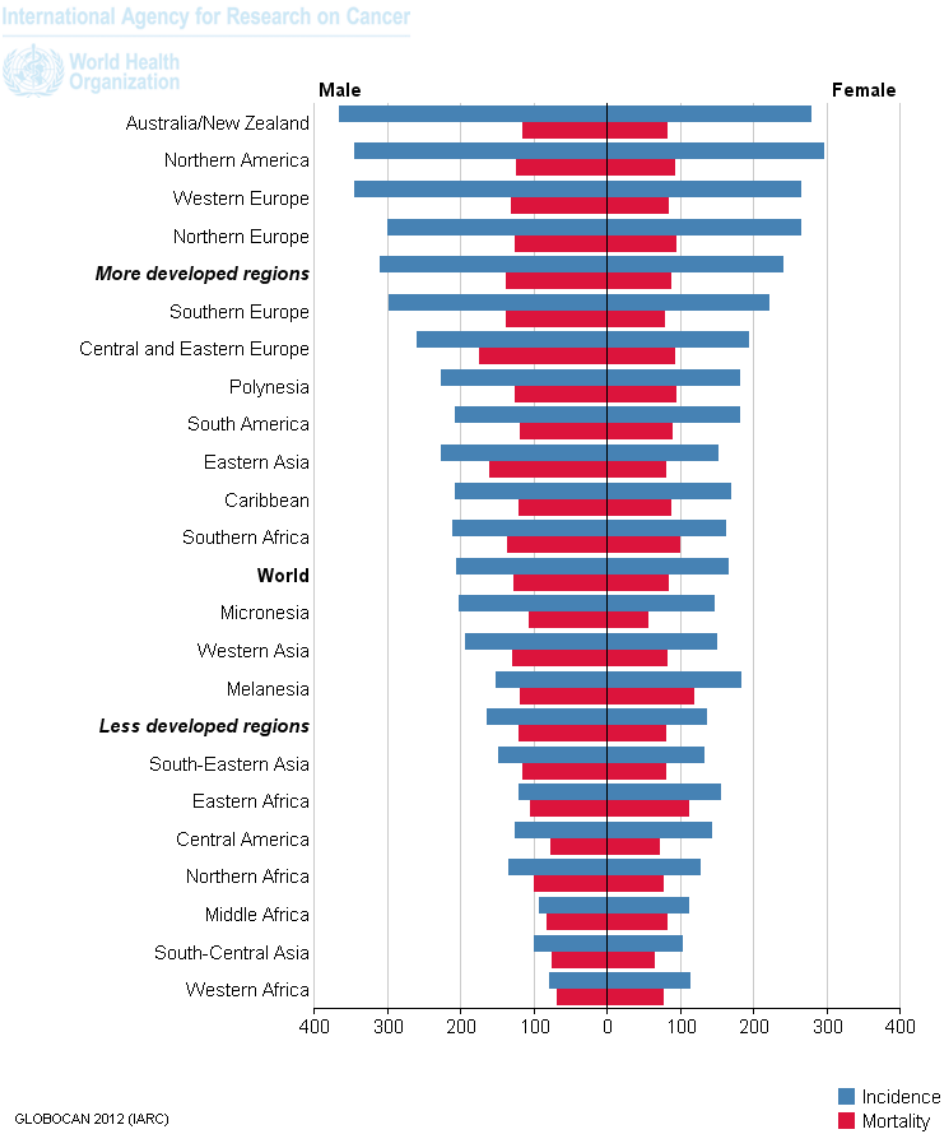


Figure 1: Incidence & Mortality Worldwide in 2012. Estimated age-standardised rates per 100,000. (Globocan 2012[4]).

The cancer incidence rate is almost 25% higher in men than in women. Male incidence rates vary almost fivefold across the different regions of the world, with rates ranging

from 79 per 100.00 in Western Africa to 365 per 100.00 in Australia/New Zealand (especially high rates of prostate cancer). There is less variation in female incidence rates (almost threefold) with rates ranging from 103 per 100.00 in South-Central-Asia to 295 per 100.00 in Northern America.

In terms of incidences, there is far more regional variability compared with the mortality rate. The rates are 15% higher in more developed than in less developed regions in men and 8% higher in women. The highest rate regarding men-mortality applies to Central and Eastern Europe (173 per 100.00). The lowest rate represents Western Africa (69). In contrast, the highest rates concerning women are Melanesia (119) and Eastern Africa (111). The Lowest rates regard Central America (72) and South-Central Asia (65)[4].

In 2012 lung cancer remains the most common cancer in the world, regarding incidence and mortality. Breast cancer is the second most common cancer overall (1.68 million cases, 11.9 % of total) but ranks 5th as cause of death (0.52 million deaths, 6.4%)[5]. Although lung cancer is the most common cancer worldwide among men, it ranks second in developed regions, after prostate cancer[6].

Cancer tends to be diagnosed at later stages in many developing countries compared with developed countries and this, combined with reduced access to appropriate therapeutic facilities and drugs, has an adverse effect on survival[3].

1.2 THE CLASSIFICATION OF CANCER

The general term „cancer“ comprises a wide range of variable malignant growth and cell proliferations, which can be found in diverse sites inside our whole body[7]. Hence, there are over 100 types of cancer which affect virtually every organ or tissue[8].

Tumours can be divided by their benignity or malignity (Dignity) as well as by their source of origin (Histogenesis) and subsequently due to the present type of cell. According to the latter disposition, tumour-nomenclature determines to specify the different characteristics of neoplasms, where e.g. benign tumours of epithelial (Adenoma - if epithelium glands or the gastrointestinal mucous membrane are affected) or mes-

enchymal origin (Fibroma – in case of connective-tissue-neoplasm) receive the suffix "-om". Concerning malign tumours are related to the mesenchyme the two terms "Carcinoma" (e.g. Adenocarcinoma) and "Sarcoma" (Fibro sarcoma) are used[7].
An abnormal cell that grows and proliferates out of control will give rise to neoplasm. As log as the neoplastic cells do not become invasive, however, the tumour is benign whereas removing or destroying the mass usually achieves a complete cure. A tumour is considered as cancer only if it has the ability to invade. Invasiveness is an essential characteristic of cancer cells. It allows them to break loose, enter blood or lymphatic vessels, and form secondary tumours, called metastases, at other sites in the body. The more widely a cancers spreads, the harder it becomes to eradicate, so its generally metastases which kills a patient[2].

Single tumour stages, are defined by the WHO via TNM-Classification, which is an internationally accepted standard for cancer staging, published by the UICC – International Union Against Cancer for over 50 years. Cancer is classified by its anatomic disease extent, i. e. stage, which is the major determinant of appropriate treatment and prognosis. The "T category" represents the tumour growth, the "N category" describes the regional lymph node involvement and "M", as a further descriptor, informs about the presence or otherwise of distant metastatic spread[9].

1.2.1 The Character of Cancer Cells

Cancer cells break the most basic rules of cell behaviour. Our body operates as a society or ecosystem. The individual members are cells that synthesized by cell division and organize into tissues. Ultimately, all of the somatic cell lineages are committed to die: they leave no progeny and instead dedicate their existence to support of the germ cells, which alone have a chance of continued survival. To coordinate their behaviour, the cells send, receive, and interpret an elaborate set of extracellular signals that serve as "social controls", directing each other how to act. As a result, each cell behaves in a socially responsible manner – resting, growing, dividing, differentiating, or dying[2]. Thereby the cell growth-and-division-cycle, which is described within this chapter, en-

sure homeostasis and thus maintenance of tissue architecture and function[10]. In a human body with more than 10^{14} cells, billions of cells experience mutations every day, potentially disrupting the social controls. Most dangerously, a mutation may give one cell a selective advantage, allowing growing and dividing more vigorously and surviving more readily. Over time repeated rounds of mutation are the basic ingredients of cancer.

1.2.1.1 TUMOURIGENESIS

Most cancers originate from one single aberrant cell[2].

Tumourigenesis is a multistep process[11] in which cells with a growth advantage are selected in a step-wise fashion, resulting in a tumour. Carcinogenesis can be initiated by either a genetic or epigenetic event, as more detailed in the next chapter. Both, however, are ultimately necessary for the formation of a tumour[12].

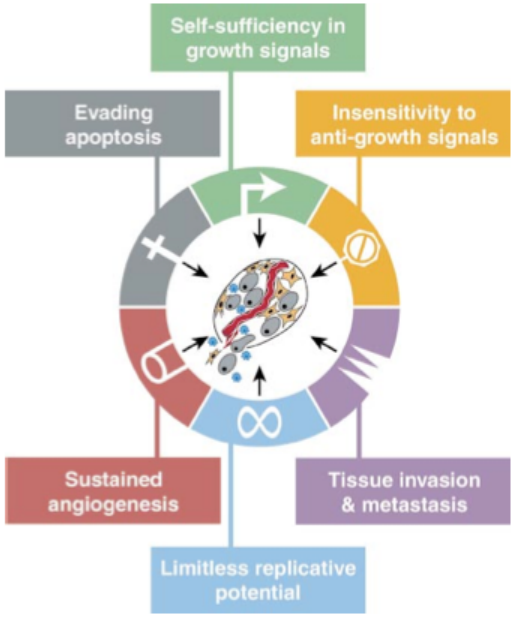


Figure 2: Acquired Capabilities of Cancer (Hanahan et all, 2000)[11]

As suggested from Hanahan et al.[11], cancer cells obtain six essential alterations in cell physiology that transform a normal cell into cancer (Figure 2): self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastases. These six capabilities are shared in common with most and perhaps all types of human tumours.

Each capability will be described in turn below.

1.2.1.1.1 Hallmarks of Cancer

Self-Sufficiency in Growth Signals

Normal cells require mitogenic growth factors (GFs) before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors[11], typically containing intracellular tyrosine kinase domains. The latter proceed to emit signals via branched intracellular signalling pathways that regulate progression through the cell cycle (figure 4) as well as cell growth. Often these signals influence yet other cell-biological properties, such as cell survival and energy metabolism[10].

Transmembrane receptors bind distinctive classes of signalling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. No type of cell can proliferate in absence of such stimulatory signals. Tumour cells, however, show a reduced dependency on these exogenously derived signals, which disrupts a very important homeostatic mechanism. While most soluble mitogenic GFs are made by one cell type in order to stimulate proliferation of another (heterotypic signalling)[11], cancer cells can acquire the capability to sustain proliferative signalling in a number of alternative ways: They may produce growth factor ligands themselves, to which they can respond via the expression of cognate receptors, resulting in autocrine proliferative stimulation[10].

Moreover, cell surface receptors, which transduce GFs, are a target of deregulation[11]. Hence, the levels of receptor proteins displayed at the cancer cell surface are elevated, rendering such cells hyperresponsive to otherwise-limiting amounts of GF[10], that normally would not trigger proliferation (e. g. the epidermal GF receptor EGF-R/erbB in stomach, brain and breast tumours or HER2/neu-receptor-overexpression in stomach and mammary carcinomas). Thus, as well as a structural alteration of receptors additionally can elicit ligand-independent signalling. Cancer cells can also switch the type of extracellular matrix receptors (integrins), favouring ones that transmit progrowth signals. Successful binding to specific moieties of the extracellular matrix (ECM) enables the integrin receptors to transduce signals into the cytoplasm, influencing the cell behaviour: quiescence in normal tissue to motility, resistance to apoptosis and entrance

into the active cell cycle. Both ligand-activated GF receptors and progrowth integrins can activate the SOS-Ras-MAP kinase pathway[11] (Figure 3).

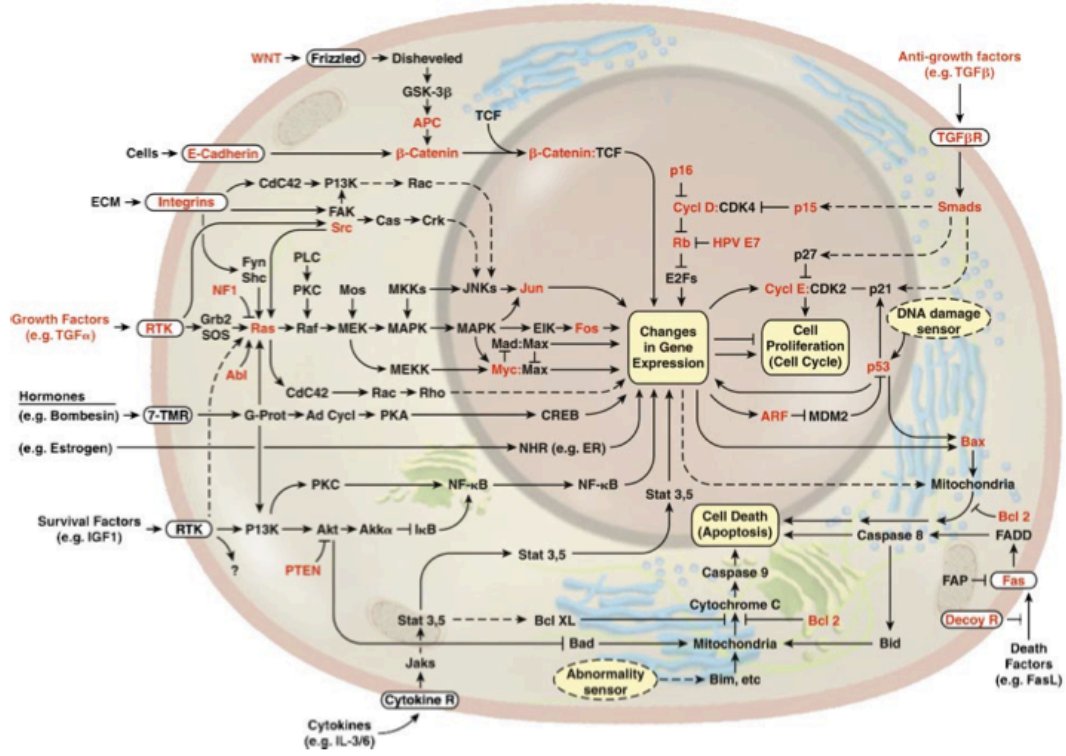


Figure 3: The Emergent Integrated Circuit of the Cell. As for the genetic reprogramming of this integrated circuit in cancer cells, some of the genes known to be functionally altered are highlighted in red. (Hanahan et al, 2000)[11]

Studies have revealed somatic mutations in certain human tumours that predict constitutive activation of signalling circuits usually triggered by activated GF receptors[10]. To give several examples: In 25% of human tumours, Ras proteins are structurally altered. So they can release a flux of mitogenic signals into cells, without ongoing stimulation by their normal upstream regulators. Within normal tissue, cells are largely instructed to grow by their neighbours. Nearly all tumour cells operate like this as well. So they co-opt their normal neighbours by inducing them to release abundant fluxes of GFs[11]. 40% of human melanomas contain such alteration in the structure of B-Raf protein, in turn resulting in constitutive signalling through the Raf to mitogen-activated protein (MAP)-kinase pathway. Similar events were shown within mutations of the catalytic subunit of phosphoinostide 3-kinase (PI3-kinase) isoforms, which again serves to hyperactivate the PI3-kinase signalling circuitry. The advantages to tumour cells of

activating upstream (receptor) versus downstream (transducer) signalling remain obscure. Figure 3 shows all present known examples for such type of alterations, highlighted in red. Contrary it was found that excessively elevated signalling by oncogenes such as RAS, MYC, and RAF provoke a wide range of counteracting cell-responses, specifically induction of cell senescence and/or apoptosis[10]. Accordingly, the relative intensity of oncogenic signaling in cancer cells may represent compromises between maximal mitogenic stimulation and avoidance of these antiproliferative defences. Alternatively some cancer cells may adapt to high levels of oncogenic signaling by disabling their senescence-or apoptosis –inducing circuitry[10].

INSENSITIVITY TO ANTIGROWTH SIGNALS

In addition to positive acting GFs, cancer cells must also circumvent powerful programs that negatively regulate cell proliferation[10].

Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. This signals can be either soluble growth inhibitors or immobilized inhibitors embedded in the ECM and surface of nearby cells. These signals are, like their positively acting counterparts, received by transmembrane cell surface receptors coupled to intracellular signalling circuits. There are two mechanisms for blocking: Cells can be forced out of the active proliferative cycle into the quiescent (G_0) state. Cancer cells must evade these antiproliferative signals. The cell cycle is responsible for the response of normal cells to these growth-stop signals, specifically the components governing the transit of the cell through the G_1 phase. During this cycle in healthy cells genome maintenance teams struggle to ensure that DNA sequence information remains intact. Mitosis is guaranteed by checkpoints that operate at critical times in the cell's life. Together, these system ensures that mutations rarely occur[11].

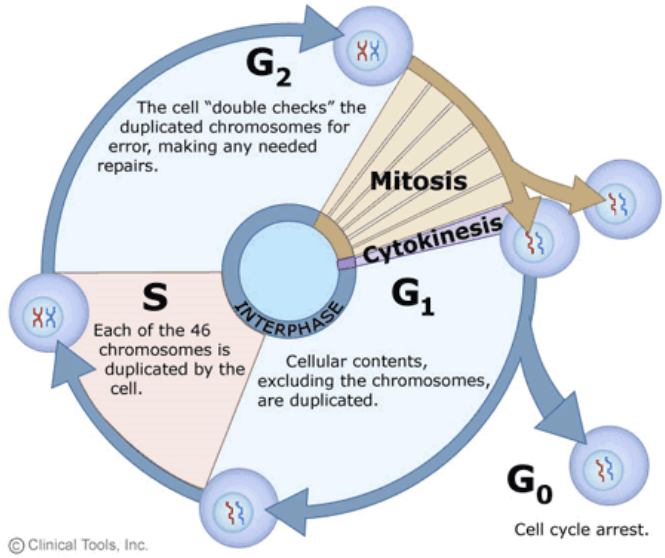


Figure 4: The Cell Cycle (University of Leicester)[150].

All somatic cells, are forced to pass through this circle (Figure 4). There are two phases: the interphase and mitotic phase. The interphase again consists of 3 parts: synthesis (S), where duplication of genetic constitution takes place. DNA-double helices get uncoiled and strands are separated to get subsequently tied up with their complementary bases. During the gap1- and gap2-phase (G_1 and G_2) no synthetically DNA-activity exists. While in G_1 organelles and other cell components are replicated, in G_2 enzymes and further proteins required for the mitotic phase are established. With G_0 the quiescent state during G_1 is meant, in which no more cell division takes place. After the interphase the mitotic phase is reached, which in turn is divided in pro-, meta-, ana-, and telo-phase, where chromosomes (mitosis), cell nucleus (karyokinesis) and finally the whole cell (cytokinesis) are split and an identical clone cell results[13].

Many of anti-growth programs depend on the actions of tumour suppressor genes. Huge amounts of tumour suppressors that operate in different ways to limit cell growth have been discovered. The two prototypical tumour suppressors encode the retinoblastoma (pRb) and tumour suppressor 53 (p53) proteins; they operate as central control points that handle the decisions of cell to proliferate or activate senescence and apoptotic programs. The pRB integrates signals from diverse extracellular and intracellular sources and responds with the decision wheter or not a cell should proceed through its cell-clock-cycle[10]. In a hypophosphorylated state, pRb blocks proliferation by sequestering and altering the function of E2F transcription factors that control the progression from G_1 into S phase. A disruption of pRb subsequently liberates E2Fs and thus allows cell proliferation, rendering cells insensitive to anti-growth factors that normally operate along this pathway to block advance through the G_1 phase of the cell cycle.

One of the best documented of those antigrowth factors is TGF-ß, which acts in a number of ways, most elusive, to prevent the phosphorylation that inactivates pRb, i.e., TGF-ß blocks advance through G_1 and finally, functional pRb may be lost through gene mutation. In addition, cancer cells can also turn off expression of integrins and other cell adhesion molecules that send anti-growth signals, favouring instead those that convey progrowth signals. The bottom line is, that the antigrowth circuit (figure 3) converging onto Rb and the cell division cycle is disrupted in a majority of human can-

cers, defining the concept and a purpose of tumour suppressor loss in cancer[11]. Whereas Rb transduces anti-growth signals that originate usually from outside of the cell, p53 receives inputs from stress and abnormality sensors that operate within the cell's intracellular processes: if the damage degree of the genome is excessive, or if amount of nucleotide pools, GFs, glucose or oxygenation are suboptimal, p53 can call a stop to further cell-cycle progression until these condition reach acceptable levels again. In case of alarm signals indicating excessive or irreparable damage, p53 can trigger apoptosis. Accordingly, the effects of p53 are complex and highly context dependent, varying by cell type and by different conditions of cell stress and genomic damage.

Recent results have also highlighted the important role of negative feedback loops that normally operate to dampen various types of signalling and thereby ensure homeostatic regulation of the release of signals coursing through the intracellular circuitry. Defects of this feedback are capable of enhancing proliferative signalling. The prototype of this regulation type involves the Ras oncoprotein: the oncogenic effects of Ras do not result from its hyperactivation but the oncogenic mutations affecting Ras genes compromise Ras GTPase activity, which causes an intrinsic negative-feedback mechanism that normally ensures that active signal transmission is transitory. Analogous of those negative-feedback mechanisms operate at different points within the proliferative siganling circuitry. Famous examples involve PTEN phosphatase, which counteracts PI3-kinase by degrading its product, phospatdylinositol (3,4,5) trisphosphate (PIP_3). Lost function due to mutations in PTEN enhance PI3K signaling and promote tumourigenesis. Another example: the mTOR kinase, a coordinator of cell growth. Thus, when mTOR is inhibited in such cancer cells (such as by the drug rapamycin), the associated loss of negative feedback results in increased activity of PI3K and its effector Akt/PKB, hereby blunting the antiproliferative effects of mTOR inhibition. So it is likely that compromised negative-feedback-loops play an important role by which cancer cells can achieve proliferative independence. Moreover, distortion of such attenuating signaling ways may contribute to the development of adaptive resistance toward drugs which target the mitogenic signaling ways[10].

EVADING APOPTOSIS

The ability of tumour cell populations to expand is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death – apoptosis - represents a major source of this attrition. Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. Apoptotic program is present in latent form in virtually all cell types throughout the body. During the process the cellular membranes are disrupted, the cytoplasmic and nuclear skeletons are broken down, the cytosol is extruded, the chromosomes are degraded, and the nucleus is fragmented, all in a span of 30 – 120 min. In the end, the shrivelled cell corpse is engulfed by nearby cells in a tissue and disappears, typically within 24 hr[11].

The apoptotic machinery can be broadly divided into two classes of components – upstream regulators and downstream effectors. The regulators, in turn, are divided into two circuits, one receiving and processing extracellular death-inducing signals (the extrinsic apoptotic program including Fas ligand/Fas receptors), and the other sensing and integrating a variety of signals of intracellular origin (the intrinsic program)[10]. Both are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. Each culminates in activation of the ultimate effectors of apoptosis include the two "gatekeeper" caspases, - 8 and -9. Mostly activated by death receptors such as FAS or cytochrome C release. They trigger the activation of a dozen or more effector caspases that execute the death program, i.e., regulators handle the effectors of apoptotic death. The sentinels include cell surface receptors that bind survival or death factors. Examples of these ligand/ receptor pairs include survival signals conveyed by IGF-1/ IGF-2 through their receptor, IGF-1R, and by IL-3 and its cognate receptor, Il-3R. Death signals are conveyed by the FAS ligand binding the FAS-R. and by TNFα binding TNF-R1. Intracellular sensors monitor the cell's well-being and activate the death pathway in response to detecting abnormalities, including DNA damage, signalling imbalance provoked by oncogene action, survival factor insufficiency, or hypoxia. Further, the life of most cells is in part maintained by cell-matrix and cell-cell adherence-based survival signals whose abrogation elicits apoptosis. Many of the signals that elicit apoptosis converge on the mitochondria[11]. The apoptotic trigger that conveys signals between regulators and effectors is controlled by counterbalancing properties of the members

of the Bcl-2 family of regulatory proteins which have either proapoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W, Mcl-1, A1) function. Inhibitors of apoptosis, acting in large part by binding to and thereby suppressing Bax and Bak, two proapoptotic proteins which are embedded in the mitochondrial outer membrane. When relieved of inhibition by their anti-apoptotic relatives, Bax and Bak disrupt the integrity of the outer mitochondrial membrane, causing a release of proapoptotic signals – cytochrome C - the most famous of them. Thus, in turn activates a cascade of caspases, which work as catalysts for apoptosis. Bax and Bak share protein-protein interaction domains, thermed BH3 motifs, with their antiapoptotic Bcl-2-like relatives. Every single of those BH3 motifs, is coupled to sensors of cellular abnormality; these "BH3-only" proteins in turn act either by disrupting antiapoptotic or inducing proapoptotic proteins. Most notable of such abnormality sensors is a DNA-damage sensor that functions via the TP53 tumour suppressor. The p53 protein can elicit apoptosis by upregulating expression of proapoptotic Bax and its relatives, in response to sensing DNA damage[10].

Altering components of the apoptotic machinery can dramatically affect the dynamics of tumour progression. Resistance to apoptosis can be acquired by cancer cells through a wide range of strategies. The most commonly occurring loss of a proapoptotic regulator through mutation involves the p53 tumour suppressor gene. The resulting functional inactivation p53 is seen in the majority of human cancers. Additionally, tumours may achieve similar ends by increasing expression of antiapoptotic regulators, e. g. Bcl-2, Bcl-XL, as well as an elevation of survival signals such as IGF-1/2 (insulin-like-growth factor) or IL-3 (interleukin-3), or by downregulation of proapoptotic factors (Bax, Bak, Bim, Puma). Another option is to short-circuiting the extrinsic ligand induced pathway[10], as for instance an upregulation of nonsignaling decoy FAS receptor, which takes death-inducing signals away from FAS death receptors[11]. Recent advances in research in this field have involved other forms of cell death[10]:

In human tumour cells, apoptosis, necrosis, autophagy and senescence are all found to contribute to overall cell death. Conversely, autophagy can promote both cell survival and cell death and may result in a non-apoptotic form of programmed cell death. (PCD): autophagic cell death (ACD) occurs primarily when homeostatic maintenance needs massive cell elimination. This is also observed in cancer[14]. Autophagy normally

enables cells to break down cellular organelles, such as ribosomes or mitochondria, allowing the resulting catabolites to be recycled and thus, used for biosynthesis and energy metabolism[10]. Reduction of these autophagic sequestration occurs in the early steps of carcinogenesis, which is caused by the fact that the tumour growth requires higher levels of protein synthesis than protein catabolism[14].

Necrosis, however, release proinflammatory signals into the surrounding tissue. Activated inflammatory cells can drive tumour promotion. Accordingly invasive and metastatic tumours may gain an advantage by tolerating some level of necrosis leading to the recruitment of tumour-promoting inflammatory cells and its GFs[10].

LIMITLESS REPLICATIVE POTENTIAL

Growth signal autonomy, insensitivity to antigrowth signals, and resistance to apoptosis lead to an uncoupling of a cell's growth program from signals in its environment.

However, does not ensure proliferative tumour growth. Many and perhaps all types of mammalian cells carry an intrinsic, cell-autonomous program that limits their multiplication. This program operates independently of the cell-to-cell signaling pathways described above (Figure 3). Cultured cells have a finite replicative potential[11]. Repeated cycles of cell division first cause induction of senescence and then, for those cells surviving this barrier, a crisis phase eliminates great majority of the population[10]. During the multistep progress of tumourigenesis, evolving premalignant cells exhaust their endowment of allowed doublings and can only complete their tumorigenic schedule by reaching unlimited replicative potential – immortalization.

Normal human cell types have the capacity for 60–70 doublings[11]. Multiple investigations indicate that a protection facility of the ends of chromosomes, called telomeres, which are composed of thousand repeats of a very short sequence element of 6 base pairs, are centrally involved in the ability for unlimited proliferation[10]. Due to a progressive shortening of telomeres, replicative generations are counted by the 50–100 base pair loss of telomeric DNA from the ends of every chromosome during each cell cycle. This results in an inability of DNA polymerases to completely replicate the 3 ends of chromosomal DNA during each S phase. This protects the ends of chromosomal DNA and as resulting in an end-to-end chromosomal fusion, which in turn causes cell death[11]. Accordingly, the length of telomeric DNA in a cell dictates how many cell gen-

erations its progeny can pass through before telomeres are largely eroded and have consequently lost their protective functions, triggering entrance into crisis[10]. The maintenance of telomeres is evident in almost all types of malignant cells - 85%–90% of them upregulate the expression of the telomerase enzyme, which adds hexanucleotide repeats onto the ends of telomeric DNA[11]. Telomerase is almost absent in nonimmortalized cells, thus, suppression of telomerase activity leads to telomere shortening and activation of senescense and/or crisis. Concerning senescence now, it was found that there is not only triggering by various proliferation-associated abnormalities, including high levels of oncogenic signaling but also subcritical shortening of telomeres which can activate the initiation of senescence[10]. Alternatively to this, cancer cells have invented a way of activating a mechanism (ALT), which appears to maintain telomeres through recombination-based interchromosomal exchanges of sequence information. Both mechanisms cause telomere maintenance at a length above a critical limit. This again permits unlimited multiplication of descendant cells[11].

SUSTAINED ANGIOGENESIS

Like normal tissues, tumour survival is required in form of nutrients and oxygen als well as an ability to evacuate metabolic wastes and carbon dioxide[10] supplied by our vasculature, obligating nearly all cells in a tissue to reside within 100 µm of a capillary blood vessel. Due to this dependence on nearby capillaries, it would be plausible that proliferating cells within a tissue would have an intrinsic ability to stimulate blood vessel growth. The tumour-associated neovasculature, generated by the process of angiogenesis, addresses this needs. During embryogenesis, the development of the vasculature involves the birth of new endothelial cells and their assembly into tubes (vasculogenesis) in addition to the sprouting (angiogenesis) of new vessels from existing ones. Following to this, the normal vasculature becomes largely quiescent. In the adult, as part of physiologic processes such as wound healing and female reproductive cycling, angiogenesis is turned on, but only transiently. In contrast, during tumour progression, an “angiogenic switch” is almost always activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths[10]. Counterbalancing either positive or negative signals encourage/discourage angiogenesis[11]. Some of these angiogenic regulators are signalling pro-

teins that bind to stimulatory or inhibitory cell-surface receptors displayed by vascular endothelial cells. A well-known prototype of angiogenesis inducer is vascular endothelial growth factor-A (VEGF-A). The VEGF-A gene encodes ligands that are involved in orchestrating new blood vessel growth during embryonic and postnatal development, homeostatic survival of endothelial cells, as well as in physiological and pathological situations in adults. VEGF signalling via three receptor tyrosine kinases (VEGFR-1–3) is regulated at multiple levels, reflecting this complexity of purpose[10].

As an example for angiogenesis inhibitors, thrombospondin-1, which binds to CD36, a transmembrane receptor on endothelial cells coupled to intracellular Src- like tyrosine kinases. Integrins follow the same mechanism. Quiescent vessels express one type of integrins, while sprouting capillaries express another. Interference with signalling from the latter class of integrins can inhibit angiogenesis. So there is an important necessity of angiogenesis for an explosive growth of tumour explants[11]. The last decade has seen reports of another dozen such agents. Most are proteins, and many are derived by proteolytic cleavage of structural proteins that are not themselves angiogenic regulators. The data suggest that such endogenous angiogenesis inhibitors serve under normal circumstances as physiologic regulators that modulate transitory angiogenesis during tissue remodelling and wound healing[10].

It is indicated that neovascularization is a requirement to the rapid clonal expansion associated with developing macroscopically tumours. To activate the angiogenic switch, tumours chance the balance of angiogenesis inducers and countervailing inhibitors. One common strategy is a shift in gene transcription. Many increase their expression of angiogenesis inducers (e.g., VEGFs) compared to their normal tissue counterparts. In others, expression of endogenous inhibitors such as thrombospondin-1 or -interferon is dimed. Both alterations may occur, and indeed be linked, in some tumours.

As an example for downshifting, the inhibitor thrombospondin-1 has been found to positively regulated by the p53 tumour suppressor protein in some cell types. Subsequently, the loss of p53 function, which occurs in most human tumours, can cause thrombospondin-1 levels to fall, i.e. liberating endothelial cells from its inhibitory signals. Also proteases, which are able to control the bioavailability of angiogenic activators and inhibitors, can be influenced by alteration. Different types of tumour cells use

distinct molecular strategies to activate the angiogenic switch. This switch will play a very important role of target based tumour treatment in the next decade[11].

TISSUE INVASION AND METASTASIS

Sooner or later primary tumours spawn pioneer cells that move out, invade neighbouring tissues, and travel to distant sites where they may succeed in founding new colonies. These distant settlements of tumour cell, metastases, are the cause of 90% of human cancer deaths[11]. Metastasis is a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and haematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumours (colonization)[10]. Like the formation of the primary tumour mass, successful invasion and metastasis depend upon all of the other five acquired hallmark capabilities[11]. Associated cancer cells typically develop alterations in their shape, in their attachment to other cells as well as to the extracellular matrix (ECM)[10].

One best characterized alteration involves the loss by carcinoma cells of E-cadherin, a key cell-to-cell interaction molecule ubiquitously displayed on epithelial cells[11]. By forming connection between adjacent cells, E-cadherin helps to assemble epithelial cell sheets and maintain the quiescence of the cells within these sheets[10]. Moreover a transmission of antigrowth and other signals occurs via cytoplasmic coupling with ß-catenin to intracellular signalling circuits. Thus, E-cadherin function is lost in a majority of epithelial cancers, by mechanisms which contains mutational inactivation of E-cadherin or ß-catenin genes, transcriptional repression, or proteolysis of the extracellular cadherin binding site[11]. E-cadherin serves as an antagonist of invasion and metastasis, whereas reduction of its expression was known to potentiate these phenotypes, as it happens in carcinomas. Conversely, N-Cadherin an adhesion molecules, normally associated with the cell migrations during embryogenesis and inflammation, are often upregulated. e.g., which is usually expressed in migrating neurons, is upregulated[10]. Moreover malignant cells facilitate invasion by shifting their expression of integrins from those that favour the ECM to other integrins that preferentially bind the degrad-

ed stromal components produced by extracellular proteases – a second general parameter of the invasive and metastatic properties. Protease genes are upregulated, protease inhibitor genes are downregulated, and inactive zymogen forms of proteases are changed into active enzymes. Docking of active proteases on the cell surface can facilitate invasion by cancer cells into nearby tissue, across blood vessel walls, and through normal epithelial cell layers[11].

It is increasingly apparent that crosstalk between cancer cells and cells of the neoplastic stroma is involved in the acquired capability for invasive growth and metastasis. Such signalling may affect on carcinoma cells and act to alter their hallmark capabilities as suggested above. For instance mesenchymal stem cells present in the tumour stroma, secrete CCL5/RANTES in response to signals released by cancer cells; CCL5 then acts reciprocally on the cancer cells to stimulate invasive behaviour. Macrophages at the tumour periphery, activated by IL-4 produced by the cancer cell, foster local invasion by supplying matrix-degrading enzymes such as metalloproteinase and cysteine cathepsin proteases.

Metastasis can be divided into two major phases; the physical dissemination of cancer cells from the primary tumour to tissues in the distance, and the adaptation of these cells to foreign tissue microenvironments that results in successful colonization as for instance the growth of micro metastases into macroscopic tumours. Thus, colonization is not strictly coupled with physical dissemination, as evidenced by a lot of patients of a huge amount of micro metastases that have successfully disseminated but never process to macroscopic metastatic tumours. Moreover there are some types of cancer, which render such micro metastases in a quiescent state, as revealed clinically by explosive metastatic growth soon after resection of the primary growth. In other cases, however, macroscopic metastases may erupt decades after a primary tumour has been surgically removed of destroyed pharmacologically: these metastatic tumours arise from dormant micro metastases.

PROGRAMMING OF HALLMARK CAPABILITIES

The way cells become malignant is very variable. Mutations in certain oncogenes and tumour suppressor genes can occur early in some tumour progression pathways and late in other ones (Figure 5). Subsequently, the acquisition of biological capabilities such as resistance to apoptosis, sustained angiogenesis, and unlimited replicative potential can appear at different times during these various progressions. It still remains independent, however, how the steps in this pathways are arranged. The hallmark capabilities of cancer will prove to be shared in common by all types of tumours[11].

An additional dimension of complexity was gained due to investigation during the last decade, involving considerable interconnections and crosstalk between the individual subcircuits. Certain oncogenic events can affect multiple capabilities (e.g. mutant ras, upregulated myc) such as proliferative signalling, energy metabolism, angiogenesis, invasion and survival. Future renditions of this integrated circuit will encompass further subcircuits and associated hallmark capabilities that knower days remains unknown[10].

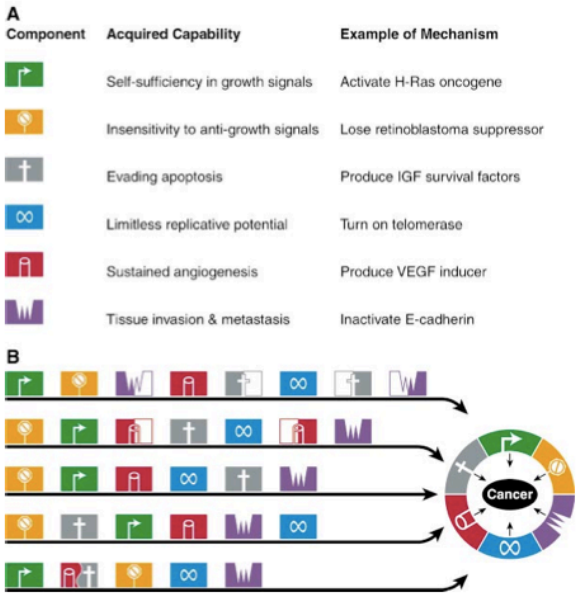


Figure 5

While supposed that virtually all cancers acquire the same six hallmark capabilities (A), their intention of doing so will vary significantly in both mechanistically and chronologically ways (B).In other tumors, a capability may only be acquired through the collaboration of two or more distinct genetic changes, thereby increasing the total number necessary for completion of tumor progression. Thus, in the eight-step pathway shown, invasion/metastasis and resistance to apoptosis are each acquired in two steps. (Hanahan et al. 2000)[11].

1.2.1.1.2 EMERGING HALLMARKS

The hallmarks of cancer are defined as acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate; this acquisition is made possible by two enabling characteristics. Most prominent is the development of genomic instabil-

ity in cancer cells, which generates random mutations including chromosomal rearrangements; among these are the rare genetic changes that can orchestrate hallmark capabilities. A second enabling characteristic is the inflammatory state of premalignant and frankly malignant lesions that is driven by cells of the immune system. Furthermore circumvention of immune destruction plays an important role in tumourigenesis.
Our immune system plays an important role in resisting or eradicating malignant neoplasia. Solid tumours that do appear have somehow managed to avoid detection by the distinct arms of the immune system or have been able to limit the extent of immunological killing, thereby evading eradication. However, the great majority of shrinking tumours caused by the immune system includes virus-induced cancers, suggesting that avoiding this class of cancer depends on reducing viral burden through eliminating virus-infected cells. It is suggested that our immune system operates as nothing less than a significant barrier to tumour formation and progression, at least in some forms of non-virus-induced cancer.
When mice genetically engineered to be deficient for various components of the immune system were assessed for the development of carcinogen-induced tumours, it was established that tumours arose more frequently and/or grew more rapidly in the immunodeficient mice relative to immunocompetent controls. Deficiencies in the expression or function of $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$, T_h1 helper T cells, or natural killer (NK) cells each led to increases in tumour incidence. Additionally clinical epidemiology supports the existence of anti-tumoural immune responses in some cancer forms. For example, patients with colon of ovarian tumours that are heavily infiltrated with CTLs and NK cells have a better prognosis than those that lack such abundant killer lymphocytes. There are still arguments against the importance of immune surveillance as an effective barrier to tumourigenesis, based on the epidemiology of chronically immunosuppressed patients that does not indicate significantly increased incidences of nonviral human cancer. Highly immunogenic cancer cells may well evade immune destruction by disabling components of the immune system that have been dispatched to eliminate them, e.g., paralyzing infiltrating CTLs and NK cells, by secreting TGF-ß or other immunosuppressive factors. The generality of immunoevasion as a core hallmark capability remains to be firmly established.

1.3 RISK FACTORS OF CANCER

Cancer is caused by both intrinsic factors (such as inherited mutation, hormones, and immune conditions) and environmental/acquired factors. Such acquirements includes in the first line tobacco as primary cause of lung cancer. Compared to non-smokers, male smokers are 23 times and female smokers 17 times more likely to develop cancer. Tobacco contains at least 50 carcinogens, which can alter a large number of cell signalling pathways. Further risk factor includes alcohol and diet like heavy consumption of red meat. How both diet and alcohol exactly contributes to cancer is not fully understood. Studies suggest co-carcinogenesis of ethanol and carcinogens in food such as nitrates, nitrosamines, pesticides and dioxins, either from food itself, food additives or from cooking. Obesity also plays an important role in cancer development. Studies have shown that the common denominators between obesity and cancer include neurochemicals; hormones such as insulin like growth factor 1 (IGF-1), insulin, leptin, sexual steroids; adiposity; insulin resistance; and inflammation and along with them all their associated pathways are involved too. Moreover infectious agents like viruses are able to initiate cancer. Among these viruses are e.g. Human papillomavirus, Epstein Barr virus, HIV, etc. Additionally, there is environmental pollution which is linked with cancer due to outdoor air pollution by carbon particles associated with polycyclic aromatic hydrocarbons (PAHs), food pollution, carcinogenic metals, pharmaceutical medicines and cosmetics. 10% of total cancer cases may be induced by radiation, both ionizing and nonionizing, typically from radioactive substances and UV exposure caused primarily by the depletion of the ozone layer in the stratosphere. Also low-frequency electromagnetic fields, as found e.g. in high voltage power lines, can cause clastogenic DNA damage[15].

1.4 Cancer Treatment – Present and Future

The treatment possibilities of solid tumours nowadays include surgery, by means of resection of malignant cells, radiation, and pharmacological chemotherapy. Thus, all out of these methods lead to vast side effects caused by reason of a lack of specificity and selectivity [16].

Surgery is most effective in the treatment of early stage localized primary tumour and associated regional lymphatics. When used as a single treatment, surgery cures more patients than any other individual form of cancer therapy, according to the fact that surgery operates in a way in which 100% of excised cells are killed[17]. However, huge damage of healthy tissue is unavoidable[16]. With the advent of radiation therapy in the 1920s and chemotherapy after 1940s, cancer surgery has become conservative. In contrast to surgery, however, chemotherapy and radiation therapy are only capable of killing a certain part of tumour cells by each treatment. Both processes are acting complementary and thus often are used in combination. Approximately 45% of new cancer cases will receive radiotherapy (RT)[17], acting in damaging chromosomes for avoiding cell proliferation[16]. Thereby RT is responsible for 40% of cancer cure[17], however as well affects surrounding healthy tissue[16]. In cases of early stage head and neck tumours as well as prostate cancer RT is used as single treatment either in terms of external beam and/or brachytherapy[17]. Very common is the combination of RT with chemotherapeutics and/or surgery:

Adjuvant therapy: chemotherapeutics used after surgery and/or radiation. Neoadjuvant therapy: chemotherapeutics used before surgery and/or radiation, for shrinking tumour mass and dilution[16].

Since the early 1980s target-based drug design has been playing a more and more important role in cancer research. Increased specificity by less side effects, lower dosage and higher power provide the most intense requirements for prospective cancer treatment.

1.4.1 CHEMOTHERAPY

The era of chemotherapy began in the 1940s with the first uses of nitrogen mustard, as an alkylating intermediate, and antifolate drug. Since then, cancer drug development has transformed from low-budget, government-supported research effort to a high-stakes, multi-billion dollar industry. The targeted-therapy revolution has arrived, but the principles and limitations of chemotherapy discovered by the early researchers still apply[18].

All effects of chemotherapeutics are favouring primarily rapidly proliferating tissues and thus, both malignant and normal cells are affected the same toxic way and so the entirety of anti-cancer drugs are very effective in fighting very aggressive against just now dividing tumour cells but on the other hand, however, they are attended to a lot of enormous side effects on physiologically constantly new regenerating cells like the bone marrow (aplasia), intestinal mucosa (mucositis) and hairy cells (alopecia). Moreover liver and kidney, as eliminating organs, and the heart are damaged. Chemotherapy can persue distinct goals. Thus, there are the possibilities of either a curative therapy, where healing is aimed to be achieved or a palliative therapy, where simply an abatement of pain or prolonging of life is desired to be obtained. In almost every case healing turns out to be difficult. This occurs amongst others due to the development of resistance and thus, a loss of effectiveness of anti-cancer drugs. This can be caused by several conditions such as low-dose administration, when the effect of the utilized drug is insufficient but an increase of dosage would lead to too hard side effects, or it depends on the pharmacokinetic, if the cytostatic drug isn't able to reach the affected site. Furthermore, malignant neoplasm can acquire cellular mechanisms for developing resistance, as followed:

- Inactivation of absorption into the cell, e.g. of methotrexate
- Inactivation of cytostatic function of the drug, e.g. through an increase of aldehyde-dehydrogenase, inactivating cyclophosphamides
- Decrease of prodrug-activation, which happens very often by antimetabolites

- Increase of DNA repair concerning DNA damaging drugs like alkylating agents, cisplatin or topoisomerase-II-inhibitors
- Alteration of the drug target, where drug binding is prevented
- Alteration of drug specificity
- Overexpression of the glycoprotein P-170, which under physiologically condition works as a transport system for foreign substances through the cell membrane, can cause an exfiltration of the drug and thus, decreased effectiveness.

To conquer chemoresistance several strategies have been developed. Amongst others the combination of anti-cancer drugs with RT and surgery, local application of high-dose agents, and combination among therapeutics with different effecting ways[16].

1.4.1.1 Anti-cancer Agents

To give a small insight of the present agents for tumour treatment, the single compounds are summarized and classified through their point of action within this chapter.

1.4.1.1.1 Molecular Pathway Target-Therapy

The introduction of mechanism-based targeted therapies (TAT) to treat human cancers has been heralded as one of the fruits of three decades of remarkable progress of research into the mechanisms of cancer pathogenesis[10]. TATs refer to drugs with particular mechanisms that specifically act on a well-defined target or biologic pathway that, when activated or inactivated, causes regression or destruction of the malignant process. Relative to conventional surgery, radiotherapy, and common chemotherapy, TATs utilize cutting edge translational research findings either from the unique characteristic of molecules, antibodies, proteins, and peptides or from structures, metabolisms, and other phenotypic properties of cancer to destroy cancer cells more precisely and therefore may improve cancer treatability[17]. At present the number of targeted

therapeutics is rapidly growing according to their effects on one or more hallmark capabilities.[10]

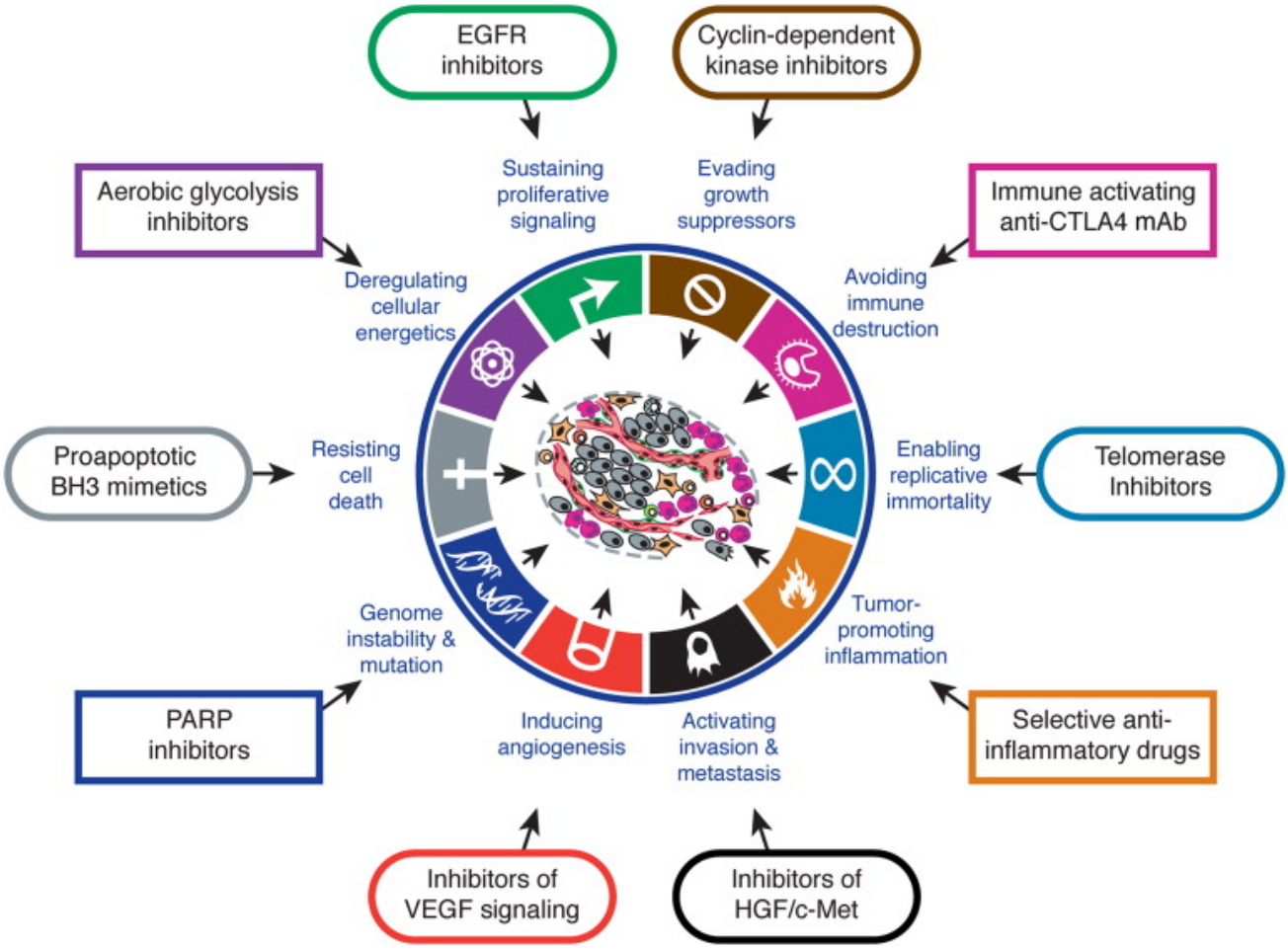


Figure 6 Therapeutic Targeting of the Hallmarks of Cancer

Drugs that interfere with each of the acquired capabilities necessary for tumour growth and progression have been developed and are in clinical trials or in some cases approved for clinical use in treating certain forms of human cancer. The drugs listed are but illustrative examples; there is a deep pipeline of candidate drugs with different molecular targets and modes of action in development for most of these hallmarks (Hanahan et all., 2011)[10].

Since the outset of TAT, distinct anti-cancer mechanisms where developed, including anti-cancer antibodies, especially conjugated with cytotoxic drugs, radioisotopes or poisons seeking out and kill malignant cells bearing the target antigens. Small molecular inhibitors of protein kinases, like imatinib for inhibition of Brc-Abl fusion protein in the chronic leukaemia pathway or gefitinib in blocking activation of epidermal growth factor receptor in the Ras pathway, have also emerged viable drugs for TATs. Moreo-

ver, multifarious agents such as pro-apoptotic agents, PARP-inhibitors, vascular disrupting agents, angiogenesis inhibitors, radiolabeled peptides, immunoconjugates, as well as antisense strategies, immunologic therapies and many more, shown in figure 6, can selectively target cancer cells[19].

1.4.1.1.2 Intervention of Cell Metabolism & DNA Synthesis

Common anti-cancer drugs used in the very past and present are shortly described within this chapter.

Antimetabolites

Antimetabolites act in the way to replace natural metabolic components (metabolites), leading to the production of functional macromolecules or block enzymes through complex formation. Thus they lead to distortion of metabolism and division of the cancer cell. Compounds of this group are folic acid antagonists (intervention of carbon transport essential for nucleic acid production) and antagonist of the purine and pyrimidine bases (avoiding DNA synthesis)[16].

Alkylating Agents

This term summarizes reactive, mostly bifunctional cytostatic drugs which phase-unspecific effect primarily based on alkylation of nuclei acids. After their activation to carbocations the substances react except for proteins also with guanine base out of desoxyribonucleic acids, causing multiple DNA alterations including DNA-cross-linking, abnormal base pairing, and DNA chain-breaks. Thus, both DNA reduplication and cell division are interrupted. Alkylating agents involve nitrogen mustard derivatives, aziridines, n-nitroso-carbamide derivatives and platinum complexes – all acting alkylating different phases of DNA development[16].

Topoisomerase Inhibitors

To be placed in the nucleus of a cell, DNA has to be twisted to reach a much smaller size to fit right. Therefore enzymes, called topoisomerases, contemporary make breaks

in DNA strands to uncoil DNA for replication, followed by reconnecting them again after the process. Topoisomerase inhibitors form a complex with the enzyme and the DNA, which again results in uncontrolled double or single strand breaks (topo I and topo II, respectively), carried out by the enzyme[16].

Microtubule Inhibitors

Vinca-alkaloides, taxanes and epitholones, all with the origin of natural sources or a derivative of them, are mitotic poisons, which are able to block the development of the mitotic spindle apparatus essential for successful division of the cell. All of these 3 categories are bearing on tubulin, a protein of the cytoskeleton of a cell. Whereas vinca-alkaloides inhibit the polymerisation of tubulin to intact microtubule, taxanes and epitholones stabilize the microtubule which in turn prohibits depolymerisation[16].

Intercalating Agents

The cytotoxic mechanism of these group remains still discussed controversially. It seems that several strategies are involved[20]. More details about the function and single compounds of DNA intercalating agents are reviewed within chapter 2 of this thesis.

Further anti-tumoural substance classes include platinum analogous, hormones, enzymes, immunotoxines, cytokines and histone-deacylase inhibitors. Additional description of such was renounced within this thesis.

2. DNA AS A TARGET FOR CANCER THERAPY

2.1 NUCLEIC ACIDS – STRUCTURE & ORGANISATION

DNA (desoxyribonucleic acid) is a biomolecule that contains the genetic instructions specifying the biological development of all cellular forms of life (and many viruses). It is often referred to as the molecule of heredity, as it is responsible for the genetic propagation of all traits[21]. When cell division occurs, DNA is duplicated and both identically copies are divided into two daughter cells (replication). Newly established cell ware able to activate certain parts of its DNA and transcribes it into ribonucleic acids (transcription). These certain part of DNA is termed “gene” and codes for proteins, whereas the cell uses information out of its ribonucleic acids (RNA) to translate it into a protein (translation).

The structure of DNA is a very simple one: Two antiparallel strands are coiled around each other to form a DNA double helix. The helix consists of desoxyribonucleotides, which include 3 components: base (thymine (T), cytosine (C), adenine (A), guanine (G)), sugar (desoxyribose), and phosphate residues. Each of the two purine bases A and G and pyrimidine bases T and C are connected to each of its complementary base[22], i.e., interactions between TA and GC can be observed (Watson-Crick base pairs)[21]. Recent Developments in the Chemistry of Desoxyribonucleic Acid (DNA) Intercalators: Principles, Design, Synthesis, Applications and Trends, i.e., C-G/A-T base pairs are established. All of them are linked by means of a N-glycosidically bound. Thereby the connection between one base and one sugar molecule is termed nucleoside, further the bound of one phosphate residue to such nucleoside component is called nucleotide. The 4 bases are placed in the central of the helix while the remaining sugar-phosphate residues built the shell. Due to the interaction between purine and pyrimidine base pairs stability of the entire helix is guaranteed[22].

The DNA double helix comprises two distinct grooves, which can be differentiated through their size. The major groove is wider compared to the minor groove which

results in two different possibilites for DNA binding agents to do so. Moreover there are 3 differnet forms of DNA:

- A-DNA:
    - Shorter, broader helix than B-DNA
    - Deep, narrow major groove not easily accessible to proteins
    - Broad, shallow minor groove
    - Clockwise orientation
- B-DNA:
    - Most common DNA conformation in vivo
    - Narrower, more elongated helix than the A-form
    - Broad major groove easily accessible to proteins
    - Narrow minor groove
    - Clockwise orientation
- Z-DNA:
    - More elongated and taller than the other forms
    - Counterclockwise orientation[22]

2.2 THE MODE OF BINDING

A large percentage of the chemotherapeutic anti-cancer agents currently used, fall into the category of DNA-binding drugs. These agents interact with the DNA duplex by any of the three general binding modes, which are described within the following chapter. Small molecules that bind to DNA are extremely useful as biochemical tools for the visualization of DNA. Additionally, the clinical significance of DNA-binding compounds can hardly be overstated as many anti-cancer regimes include compounds that bind to and/or modify DNA. A large percentage of chemotherapeutic anti-cancer drugs are agents that interact with DNA directly or indirectly by preventing the proper relaxation of DNA (through inhibition of topoisomerases)[23].

In principle there are six ways for reversible interactions of molecules with double-helical DNA: electrostatic interactions with the anionic sugar-phosphate backbone of DNA, interactions with the DNA major groove, interactions with the DNA minor groove, intercalation between base pairs via the DNA major groove, intercalation between base pairs via the DNA minor groove and a threading intercalation mode. Depending on structural features of both the molecule and DNA, many molecules show more than a single interaction mode with DNA[24].

Main Drug-DNA interactions can be classified into three major categories: intercalation, groove binding and covalent DNA-crosslinking[25] (Figure 16).

2.2.1 INTERCALATION

DNA intercalation is a noncovalent interaction in which a molecule, or a portion thereof, inserts between adjacent DNA base pairs[26], which requires an uncoiling of the DNA[27] resulting in a decrease in the DNA helical twist and lengthening of the DNA. Unless a covalent interaction subsequently occurs, the molecule is free to move on and off across the DNA, controlled primarily by van der Waals forces coupled with electrostatic (e.g., hydrogen bonding) interactions[23]. Intercalators are typically planar heterocycles of approximately the size and shape of a DNA base pair. The most feasible coverage, intercalating agents can bind to DNA, is limited by the “nearest neighbour exclusion principle”, which states that intercalators can bind to a maximum loading of one ligand per two base pairs[28]. Even though intercalators have been used extensively as anti-tumour, antineoplastic, antimalarial, antibiotics, and antifungal agents, not all intercalators are genotoxic (defined by the ability to alter a cell’s genetic material as a means of inducing a toxic effect). The presence of basic, cationic, or electrophilic functional groups is often necessary for genotoxicity[23].

2.2.1.1 CLASSICAL PLANAR FUSED-RING INTERCALATING AGENTS

The typical intercalators used as a common in cancer therapy have to fulfill several requirements concerning the properties of their structural compounds:

- polyaromatic (3-4 rings) and coplanar
- 3-4 Å wide and 6-8 Å long
- surface: approximately 28 Å
- charge-transfer-complexes, dipole-dipole-interactions, electro-static interactions
- para-conjugated chinonring and nitrogen (for a positive influence of intercalation)

In most cases, an intercalating substance is composed of 3 or 4 fused ring system, which is also acting as a chromophore, due to its ability to absorb light within an UV-visible spectrum[27]. Typical examples are acridinium salts including proflavine chloride (6) and the phenathridinium salts ethidium bromide (7) and propidium iodide (8) (Figure 7)[24].

Figure 7: Representative chemical structures of intercalating agents (L. Strekowski et al., 2007) [24].

2.2.1.1.1 ANTHRACYCLINES AND RELATED COMPOUNDS

Daunorubicin (DNR), Doxorubicin (DOX), Epirubicin (EPI), Idarubicin (IDA) – all known as anthracyclines originate from distinct species of *Streptomyces*[29].

Typically for the molecular structure of all anthracycline antibiotics is a planar ring system, originating from the anthrachinon structure, as well as the amino sugar, bound α-glycosidically on the saturated ring A[29].

Structural requirement for the anti-tumoural effect of anthracyclines provides a connection between a coplanar hydrophobic region (anthrachinon) with an angled hydrophilic structure (Figure 8). Thereby angulation occurs through the structure of the amino sugar component, which hydrophilic character is caused by OH groups and the (protonated) Amino group[29]. The X-ray structures of the two major drugs doxorubicin and daunorubicin involved in the interactions with oligonucleotide have shown that aglycon of both molecules intercalate between G-C pair bases[30].

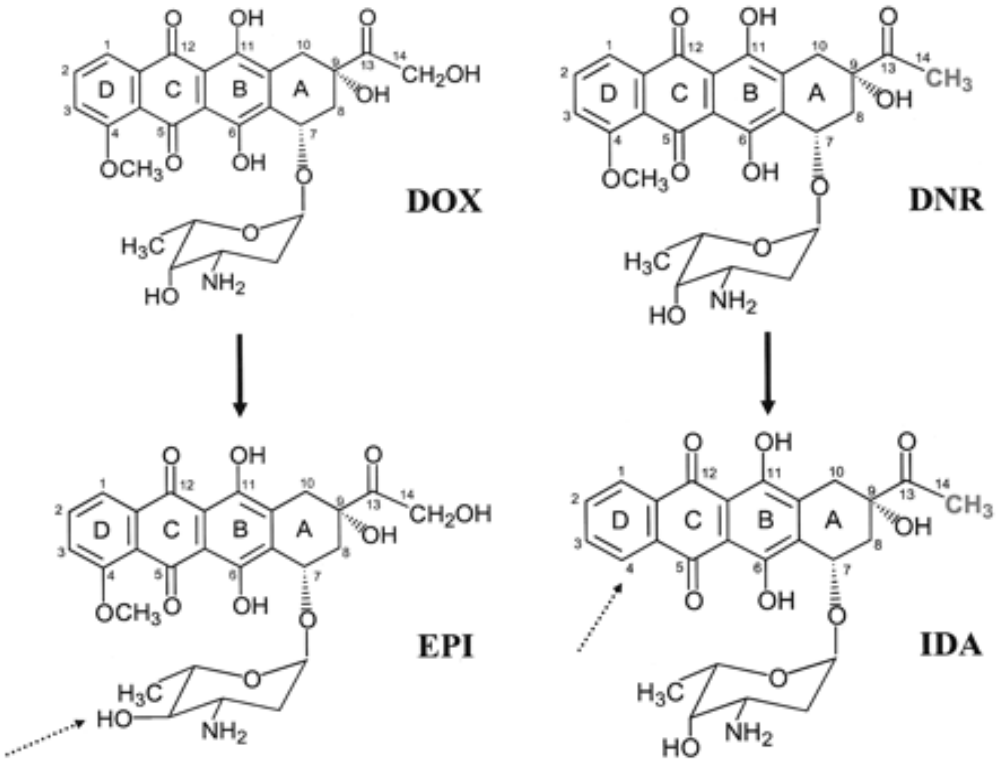


Figure 8: Structures of anthracyclines (G. Minotti et al., 2004)[20].

Whereas DOX is an essential component of treatment of breast cancer, childhood solid tumours, soft tissue sarcomas, and aggressive lymphomas, DNR shows activity in acute lymphoblastic or myeloblastic leukaemia. Like other anti-cancer agents, however, the clinical use of both DOX and DNR soon proved to be hampered by such serious problems as the development of resistance in rumour cells or toxicity in healthy tissues, most notably in the form of chronic cardiomyopathy and congestive heart failure (CHF)[20].

Cardio toxicity represents a very frequently occurring side effect of cytostatic drugs with an anthrachinon structure. Long-term use of such drugs might result in myocardial damage that is caused by peroxidation of lipids (Figure 9).

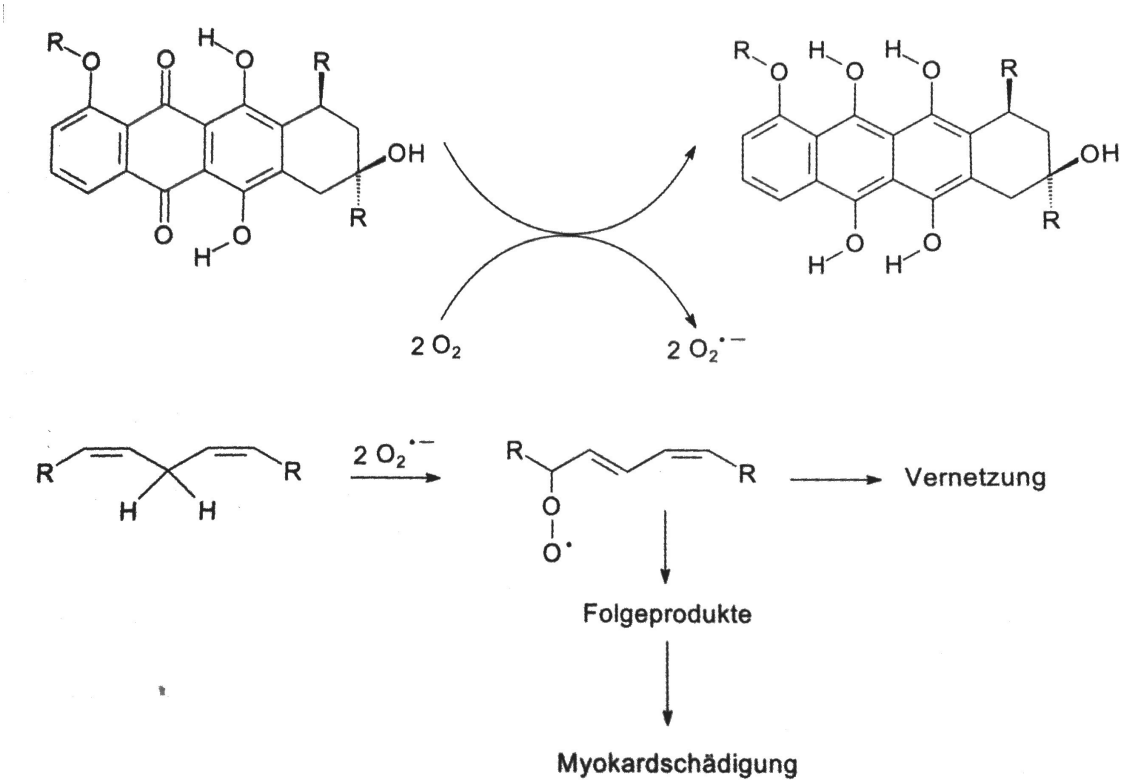


Figure 9: catalysis of lipid-peroidation , caused by anthracyclines (Steinhilber et al., 2005)[29].

Thereby reactive oxygen species (ROS) can arise and leading to cytotoxicity and damage of membranes. The redox system of anthrachinon/anthrahydrochinon develops O_2 radicals which again build hydro peroxide, which decays in turn cause a number of chain reactions. Due to the fact that the heart muscle doesn't possess any catalase enzymes, H_2O_2 can't be removed, which causes vast damage[29].

The last two decades have witnessed numerous attempts to identifying novel anthracyclines that proved superior to DOX or DNR concerning activity and/or cardiac tolerability. Yet only a few analogues have reached the stage of clinical development and approval; among them, EPI and IDA are popular as useful alternatives to DOX or DNR, respectively[20].

DNR and DOX can be refined due to their distinct substituent in the position 3 (Figure 8)[29]. EPI is a semisynthetic derivative of DOX[20]. They are acting as diastereomeres with a different configuration of position 4' of the amino sugar component[29]. This change has little effect on the binding mode or the activity of EPI, but increases the volume of distribution and enhanced total body clearance and thus a shorter terminal half-life. Thus, EPI was soon used at cumulative doses almost double those of DOX, resulting in equal activity. However, it was documented that replacing DOX with EPI does not eliminate the risk of developing chronic cardiotoxicity, although EPI's increased elimination[20].

IDA can be distinguished from DNR through the absence of an OCH_3 group in Ring D[29] and is active in acute myelogenous leukaemia, multiple myeloma, non-Hodgkin's lymphoma, and breast cancer. The broader spectrum of activity of IDA compared to DNR may be associated with increased lipophilicity and cellular uptake and improved stabilization of a ternary drug-Topo-II-DNA complex. IDA induces cardiotoxicity[31,32]. Other studies, however, demonstrate that IDA decreases left ventricular ejection fraction and causes CFH in patients with pre-existing CVDs or previous anthracycline treatment[20].

Despite extensive clinical utilization, the mechanisms of action of anthracyclines in cancer cells remain a matter of controversy. In a seminal commentary the following mechanisms were considered:

1) Intercalation into DNA, leading to inhibited synthesis of macromolecules DNA-synthesis-block, like interactions with RNA-polymerases[29], which are enzymes responsible for transcription by linking free complementary ribonucleotides along a single DNA strand, i.e. building a RNA strand as transcription-product for further translation into new proteins[22].

2) Initiation of DNA damage via inhibition of topoisomerase II[29];
   As already shortly mentioned in chapter 2.1, the double-helical structure of DNA, the two antiparallel strands are coiled around each other[33]. While transcription the replication fork has to rotate along its axis this is limited by DNA

supercoiling. Linking numbers are reduced behind the replication fork, while in front of it, i.e. in front of the RNA polymerase, coiling is accumulated[22]. The topological state of this closed-circular DNA molecules can be altered in case of replication via uncoiling performed by a class of enzymes called DNA topoisomerases[33].

> Topoisomerases are enzymes that control DNA winding and eliminated knots and tangles from DNA. So these enzymes have essential roles in replication and transcription, and allow for correct chromosomal configuration and partition. There are two principal types of topoisomerases, I and II, which cause single-stranded and double-stranded breaks in supercoiled DNA, respectively[23].

Anthracyclines react by stabilizing the formation and stability of the anthracycline-DNA-topoisomerase II ternary complex, which rely on specific structural determinants. The planar ring system is important for the intercalation into DNA (Figure 8), as rings B and C overlap with adjacent base pairs and ring D passes through the intercalation site. The external (non-intercalating) moieties of the anthracycline molecule (i.e. the sugar residue and the cyclohexane ring A) seem be important for the formation and stabilization of the ternary complex. In particular, the sugar moiety, located in the minor groove, is a critical determinant of the activity of anthracyclines as topoisomerase II poisons[34]. The nature of 3'-substituents highly influence the sequence selectivity of anthracycline-stimulated DNA cleavage[20].

DOX has also been found to inhibit topoisomerase I, an effect shared by IDA and investigational IDA analogues bearing a disaccharide moiety in which the second sugar retains an axial orientation relative to the first one.

The cell-killing property of anthracyclines weakly but significantly dependent on the cellular topoisomerase I content, suggesting that inhibition of topoisomerase I may shows an ancillary mode of action of anthracyclines[35]. Topoisomerase II-associated DNA damage leads to growth arrest in G1 and G2 and apoptosis. It follows that tumour cells may become resistant to anthracyclines due to altered topoisomerase II gene expression or activity[36].

3) Induction of apoptosis in response to topoisomerase II inhibition[20]

4) Generation of free radicals (ROS), leading to DNA damage, like doublestrand breaks or lipid peroxidation responsible for cardiotoxicity of anthracylines[29]

5) Direct membrane effects due to adhesion on cell membrane lipids, leading to intervention of cell functions, like for example an increase of membrane-fluidity and –permeability[20]

6) DNA binding and alkylation

7) DNA cross-linking

8) Interference with DNA unwinding or DNA strand separation and helicase activity[20], whereas the latter have been identified as proteins that catalyze the separation of duplex oligonucleotides into single strands in an ATP-dependent reaction[37].

Gewirtz et al.[38] pointed out, that several in vitro experiments reported in the literature had been performed at concentrations of DOX which were considered too high compared with peak or steady-state plasma concentrations (Cmax, Css) observed in patients after standard bolus infusions (approx. 5 µM and 25-250 nM, respectively). Therefore it was concluded that any study involving intact cells needed a cautionary re-evaluation.

In summary, it seems that the multiple mechanisms of action that have been associated to the anthracycline-functions may be related to the utilization of distinct drug concentrations under varied experimental conditions. If cells are exposed to drug concentrations in the sub-micro molar range, induction of cell differentiation and interference with DNA unwinding/DNA strand separation and DNA helicase may be evident. At drug concentrations that reflect Cmax after bolus application, the primary mechanism of drug action is likely to be through interaction with Topo

II. This conclusion is also echoed in a review by Cummings et al.[39]. It is feasible, however, that the genomic site of injury plays a crucial role in drug effectiveness. Interaction with the DNA–topoisomerase II complex is likely to be a primary triggering event for growth arrest and/or cell death due to a signalling pathway causing apoptosis, at least in leukemic cells. If drug concentrations exceeding approximately 2–4 μM, free radical associated toxicity and DNA cross-linking may become evident.

For more details the reader is encouraged to examine the single evaluation-results of Gewirtz et al.[38] and Minotti et al.[20].

2.2.1.1.2 MITOXANTRON

Mitoxantron is an alkaline substituted anthrachinon derivate (Figure 10). Structural it consists of a combination of the hydrophobic (tricyclic) coplanar system with a moveable cationoid centrum, bindable by phosphate residues. Thus, mitoxantron is able to cause intercalation. Following this strategy, active substances wouldn't be able to bind covalently (and thus reversibly) to the DNA without altering the primary structure of the same. Intercalation occurs between neighbouring base pairs and is "anchored" by the binding of the cationic central of the flexible side chains/amino sugar to free phosphate anions in the outer regions of the DNA helix. Following to this, alteration of the DNA conformation takes place, causing the loss of its template properties. Consequently DNA doubling is prevented and so the synthesis of DNA is blocked.

Compared with doxorubicine mitoxanthron causes less side effects like nausea and vomiting, stomatitis, alopezia[29]. Early reports indicated that mitoxantrone was less cardiotoxic than other anthracyclines but this conclusion has been challenged in more recent studies (Thomas et al., 2002)[40].

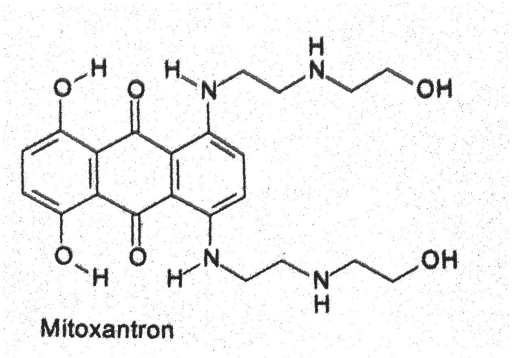


Figure 10: Synthetic trycyclic intercalating agents (Steinhilber et al., 2005)[29].

It is utilized in breast cancer, acute promyelocytic or myelogenous leucaemias, and androgen-independent prostate cancer[20].

2.2.1.1.3 Acridines and related Compounds

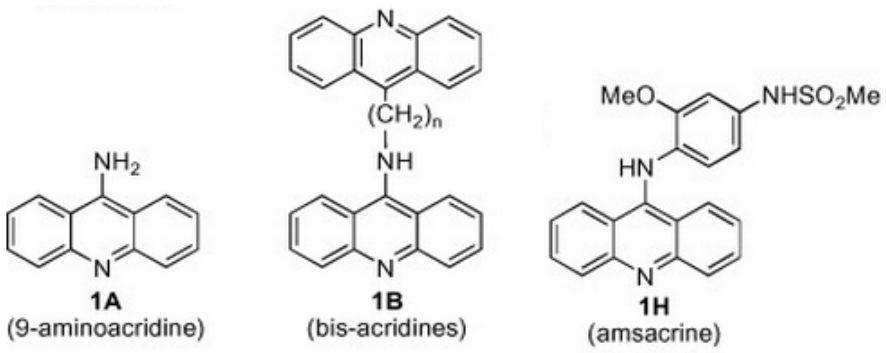


Figure 11: Acridine and its derivatives (Ferguson et al., 2007)[41].

Acridine derivatives are chemotherapeutic agents that were first used as antibacterial and anti-parasite agents. The biological activity of acridines is mainly attributed to the planarity of these aromatic structures[41], which bind reversibly but non-covalently[42] to DNA, intercalate within the double-stranded DNA structure, and thus interfering with the cellular machinery. Acridines are anthracene derivatives, which differ only in a C to N exchange in ring B. The archetypal agents 9-aminoacridine (Figure 11) and its derivatives show high mutagenic activity[29].

One common compound of such acridines is the substance amsacrin (Figure 11, 1H). Considering its planar structure, amsacrin owns intercalative functions[29]. Moreover amsacrine is able to inhibit the activity of topoisomerase II enzymes[41]. The main indication for this agents is acute myeloid leukaemia[16].

Derivatives of acridines that have shown interesting anti-tumour properties are acridone-4-carboxamide and the bis-functionalized acridone/acridine-4-carboxamides. Although, good correlation between high affinity for DNA and cytotoxicity was observed, the intercalating property was found not to be sufficient for anti-tumour activities. It was therefore proposed that the anti-tumour properties of acridines and acridones were not only due to their mode of binding to DNA, but also due to their specific interaction with other nuclear receptors[43].

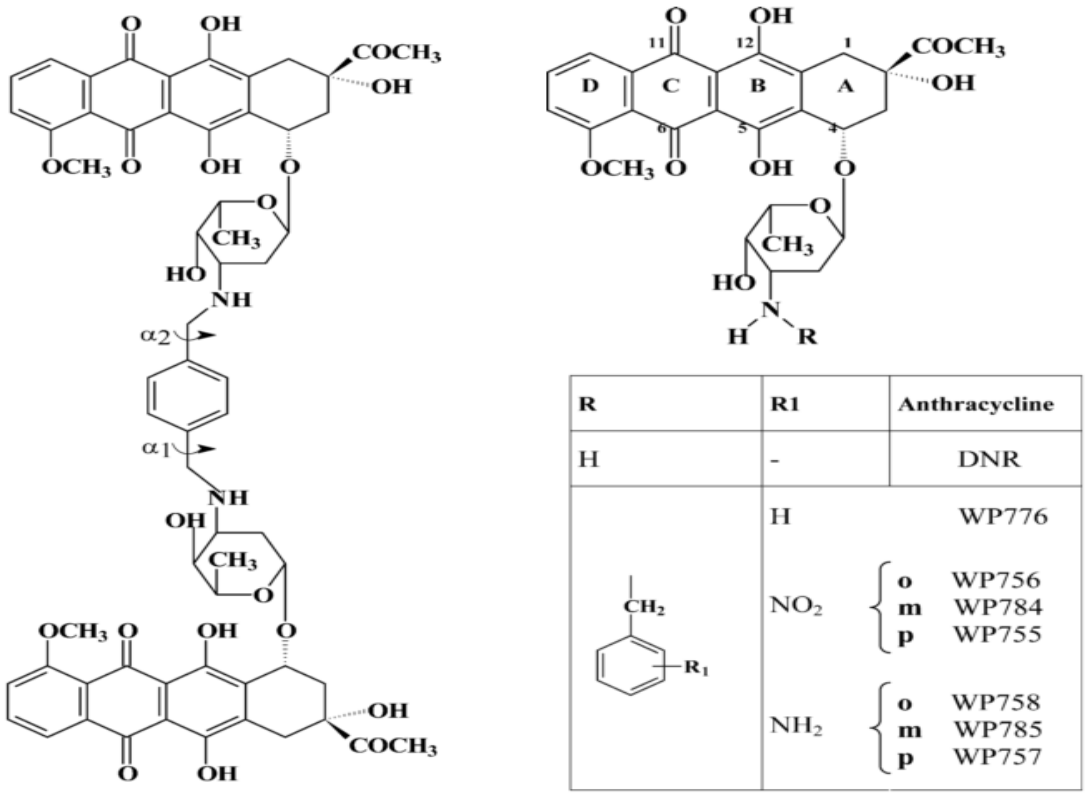
2.2.1.1.4 BIS-INTERCALATORS

Bisintercalators are often stronger binders than a single intercalating group[25] as a result of synergistic contributions from the constitutive intercalating moieties.
Linkage of two intercalators to form a bisintercalator may also increase specificity, through the recognition of consecutive motifs within short sequences [42]. The greatest requirement of a bisintercalating agent for binding every potential intercalation site is that the linker between the its two intercalating moieties is flexible enough to allow for intercalation between base pairs because binding of a ligand excludes the binding of the other ligand to neighbouring sites[24].

BISDAUNORUBICIN

In a recent study the compared daunorubicin (DNR), and new anthracycline derivatives - bisdaunorubicin (WP631) and its monomeric analogues (WP700 series) , were investigated (Figure 12). The most interesting difference between bisdaunorubicin and its monomers with attached benzyl linker is much more effective binding of the latter molecules (WP700 series) to DNA than DNR or WP631. The grate of self-association is also higher compared to parent DNR molecules. The benzyl linker being the only dif-

ference between WP series and DNR thus is the factor inducing the additional interactions favouring the self-association between anthracycline moieties and simultaneously the interaction between the WP700 series and DNA. Thus, the stabilising effect of small benzyl linker is very effective in making WP700 molecules stronger intercalators than parent DNR and WP631. In cell accumulation WP700 agents reach also best rates (60-70%) compared to DNR (40-45%) and WP631 (20%). Concerning cytotoxicity WP700 agents, which are very similar to each other in their DNA binding mode, self-association and cell accumulation, differ very distinctly in their cytotoxicity power. Most effective compounds are amino-benzyl derivatives of WP700 series with WP757 and WP785 being the most effective ones. The nitro-benzyl compounds have very low toxicity, even if they bind to DNA with similar power compared to their amino-benzyl relatives. The IC_{50} of WP631 is a little lower compared to DNR and two to three times lower than that of amino-benzyl derivatives. Thus, there is no relation between cyto-



R	R1	Anthracycline
H	-	DNR
	H	WP776
	NO_2	o WP756 m WP784 p WP755
	NH_2	o WP758 m WP785 p WP757

Figure 12: Bisdaunorubicin (WP631) and its monomeric derivatives (WP700 series) (H.T. Haj et al., 2003)[30]

toxicity and the ability to intercalate DNA. Due to the fact that anthracyclines are very effective poisons for topoisomerases it is likely that the very large differences in cyto-

toxicity of DNR and its derivatives derived from different levels of topoisomerase poisoning[30].

ACRIDINE BISINTERCALATORS

Di-acridines or di-quinolones may be either mono- or bis-intercalators, depending upon the length of the alkyl chain separating the chromophores[42] (Figure 11; 1B). It was reported that the minimum length require for effective bis-intercalation into DNA the length of two base pairs along the DNA axis[44]. Diacridines with a short chain length were monointercalators, but those with an alkyl linker chain longer than C6 were bisintercalators. Although bisintercalation greatly increased the molecule's DNA binding activity, none of the bisintercalators was as effective frameshift mutagen as the 9-aminoacridine chromophore. Although this showed no ability for "petite" mutagenesis, the diacridines of longer chain length were very effective their binding affinity[42].

2.2.1.1.5 ANTIBIOTICS WITH INTERCALATIVE FUNCTION

Additionally to anthracyclines, which also act as intercalative antibiotics, dactinomycin (actimoycin D) has intercalative potential as an antibiotic substance. It provides a planar chromophore system, consisting of two identically cyclically pentapeptides as substituents.

For application as an antibacterial drug, the chromopeptide turned out to be too toxic. At a later date it was established, that dactinomycin owns cytostatic effects. It acts via binding of DNA and blocking of RNA-synthesis. Mechanisms for this procedure are listed below:

- complete intercalation of the chromophore between two adjacent guanine-cytosin/cytosine-guanin base pairs
- reversible addition to a guanosin-parital-structure within linkage of hydrogen bridges to a so called outside-complex

- Block of topoisomerase II
- ROS-production[29]

Another antibiotic substance group produced by *Streptomyces verticillus* are bleiomycines. As molecules, which are very complicated in their structural design, they intervene the functions of the DNA. Moreover they are glycopeptides, consisting of 7 amino acids and two monosaccharides (L-glucose and D-mannose). Bleiomycin binds DNA and causes single and double strand breaks through establishing an iron(II) chelate via binding of oxygen and its activation to ROS.

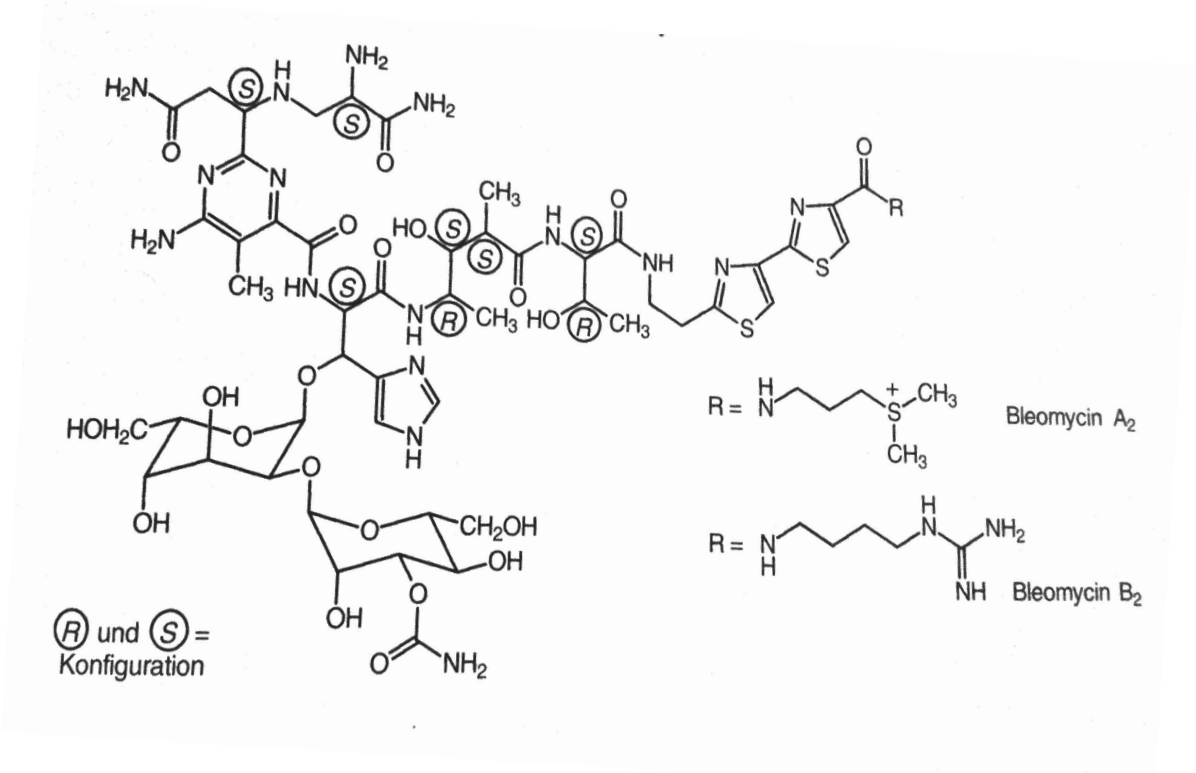


Figure 13: Bleomycin and its derivatives (Steinhilber et al., 2005)[29]

For clinical application a mixture of bleiomycin A_2 and B_2 is used. The antineoplastic effect of those drugs is based on the incorporation into DNA. Repeated redox reactions once more lead to the production of ROS and elimination of pyrimidine and purine bases from DNA[29].

2.2.2 GROOVE-BINDING COMPOUNDS

The double helix of DNA is of great length and short diameter. The minor groove is narrow and shallow, only about 10 Å wide. The major groove is deeper and wider, approximately 24 Å in width. These distinct grooves are caused by hydrogen bonds between the complementary bases of DNA[45].

Groove binding, unlike intercalation, does not induce large conformational changes in DNA and may be considered similar to standard lock-and-key models for ligand-macromolecular binding. They are stabilized by intermolecular interactions and have typically larger association constants than intercalators, as a cost in free energy is not required for creation of the binding site[23].

Essential DNA-protein interactions, occur when proteins bind at the floor of the DNA grooves through hydrogen bonds and nonspecific binding. Thereby proteins recognize hydrogen bond donors and acceptors as well as hydrophobic methyl groups. In nature, proteins can bind to the major groove, some to the minor groove, and some require binding to both. The majority of DNA interacting agents bind to the major groove, most small molecules less than 1000 Da, however, target the minor groove[45].

2.2.2.1 READING THE MINOR GROOVE

In addition to their anti-microbial properties agents, which bind thymine-adenine (TA)-rich sequences of the minor groove have shown anti-cancer activity. Due to their TA-region specificity, these minor groove binders are distinct than more typical DNA-reactive anti-tumour drugs, which often cause vast damages in healthy tissues.
Minor groove binders can be divided into two classes: reversible inhibitors of DNA-depended functions, and such causing permanent damage through direct or indirect irreversible interaction with DNA nucleotides, such as alkylating the N3 of adenines lying in the minor groove. Molecules such as synthetic polyamides or diamidines that bind in the DNA minor groove can be used as highly selective agents capable of inter-

fering with specific protein and DNA interactions that occur in AT-rich domains. Thus, they act as competitive inhibitors with normal proteins such as Topo II[45].

Much of what we know about the way in which minor groove binding compounds interact with DNA is based on the studies of the polypyrrole antibiotics distamycin and netropsin[46], which read DNA directly through donor-acceptor interactions and shape complementary[47]. Repeating pyrrole units connected by amide bonds and ending with one or more positively charged nitrogen atoms characterize them. In general, the deeper and narrower minor groove of AT-rich sequences, and the absence of the protruding 2-amino group of guanine, is optimal for accommodating the shape of the compound and for maximizing stabilizing van der Waal's contacts. Hydrogen bonding between the groove floor base pairs and the linking amides as well as electrostatic stabilizing interactions with the protonated amines are primary contributors to the overall ligand/DNA stability[46].

Though devoid of anti-tumour activity in its unmodified state, the antibiotic distamycin A (Figure 14) has been used as a backbone for the development of new minor groove binding drugs since its isolation from *Streptomyces distallicus* in 1964. In its natural state distamycin A binds reversibly to DNA, with a preference for regions containing four or more AT base pairs[45].

Analogues of distamycin A that contain more pyrrole units have greater specificity for longer AT tracts due to an increase in hydrogen bonding and van der Waal surface contacts[46].

One of the first derivatives of distamycin to show potent anti-tumour activity was the derivative tallimustine (Figure 15)[45]. In the case of tallimustine the formyl group of distamycin has been substituted with benzoyl nitrogen mustard, chlorambucil, and epoxycarbonyl moieties[46]. Tallimustine combined the DNA damaging effects of an alkylating agent with the DNA specificity of the distamycin frame. A comparison between cell-cycle effects of the major groove alkylator melphalan and tallimustine showed that when cells are alkylated in their major groove a S-phase delay occurs, which is followed by a G_2 arrest. When the minor groove is alkylated, cells passed through a complete cell cycle before becoming blocked in G_2. As determined in diverse phase I trials the toxicity of tallimustine was significant. Nevertheless the drug proceeded into phase II trials completed in colorectal and small cell lung cancer failing first line treatment: Despite several advances of tallimustine, the development was halted due to its low therapeutic index in patients with advanced solid tumours. Tallimustine mostly caused side effects such as neutropenia and thrombocytopenia. Concerning acute myeloid leukaemia (AML) it is feasible that tallimustine or some derivatives of it might still have clinical efficacy in the treatment of a subset of patients with AML[45].

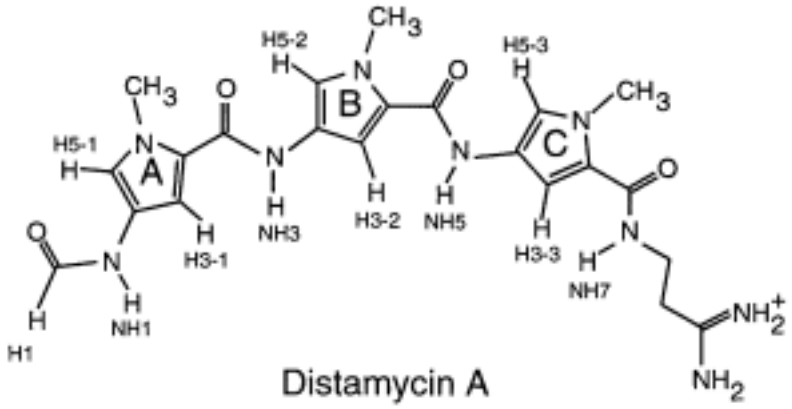


Figure 14: Distamycin A (H. Sugiyama et al., 1996)[151]

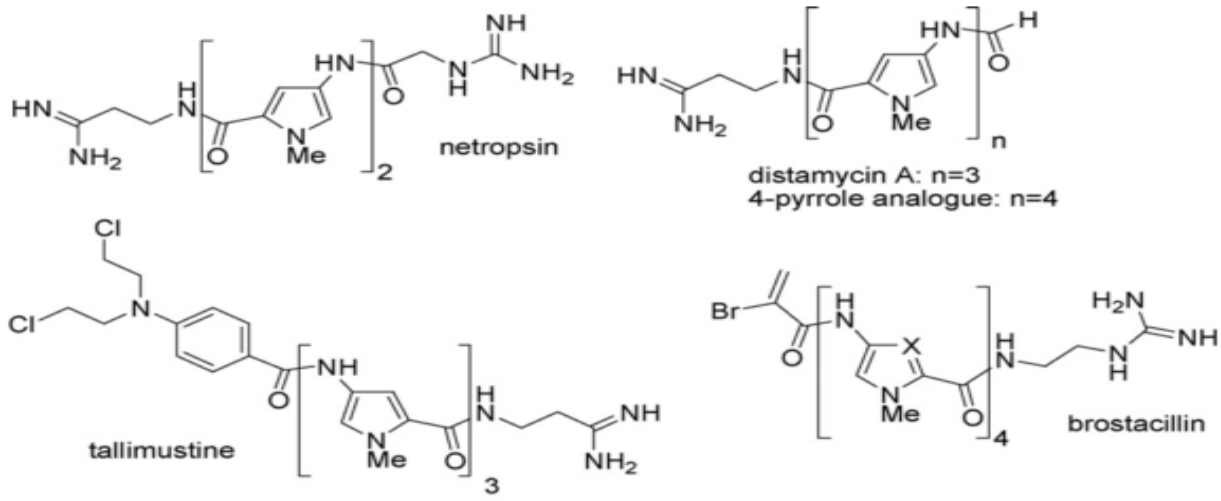


Figure 15: examples of poypyrroles (S. M. Nelson et al., 2007)[46].

2.2.2.2 Reading the Major Groove

Most often proteins can recognize and bind to DNA via the major groove. It would be desirable to have major-groove-binding molecules that block the access to proteins that recognize the same groove. This occurs only in order to sufficient affinity and sequence selectivity[47]. Details about compounds are described within chapter 3.2.

2.2.3 Covalent DNA Cross Linking

Nowadays some of the most useful anti-cancer drugs still include DNA cross-linking agents, such as cyclophosphamide and cisplatin, and topoisomerase blockers, such as anthracyclines.

The concept of cross-link formation requires the presence of at least two alkylating groups in the molecule which permit the molecule to react at two distinct points of the DNA and cause DNA interstrand cross-linking[48].

A huge variety of intercalators, groove binder and cross linkers have been documented as well as topoisomerase I or II targeting agents. Moreover the majority of DNA binding agents takes advantage of every mode of binding as they possess an intercalative unit as well as a groove-binding side chain[23].

The physiological responses of cells to a variety of such DNA-damaging drugs are that treated cells tend to arrest at certain points in the cell cycle (mostly G_2) and in some cases undergo apoptotic death[48].

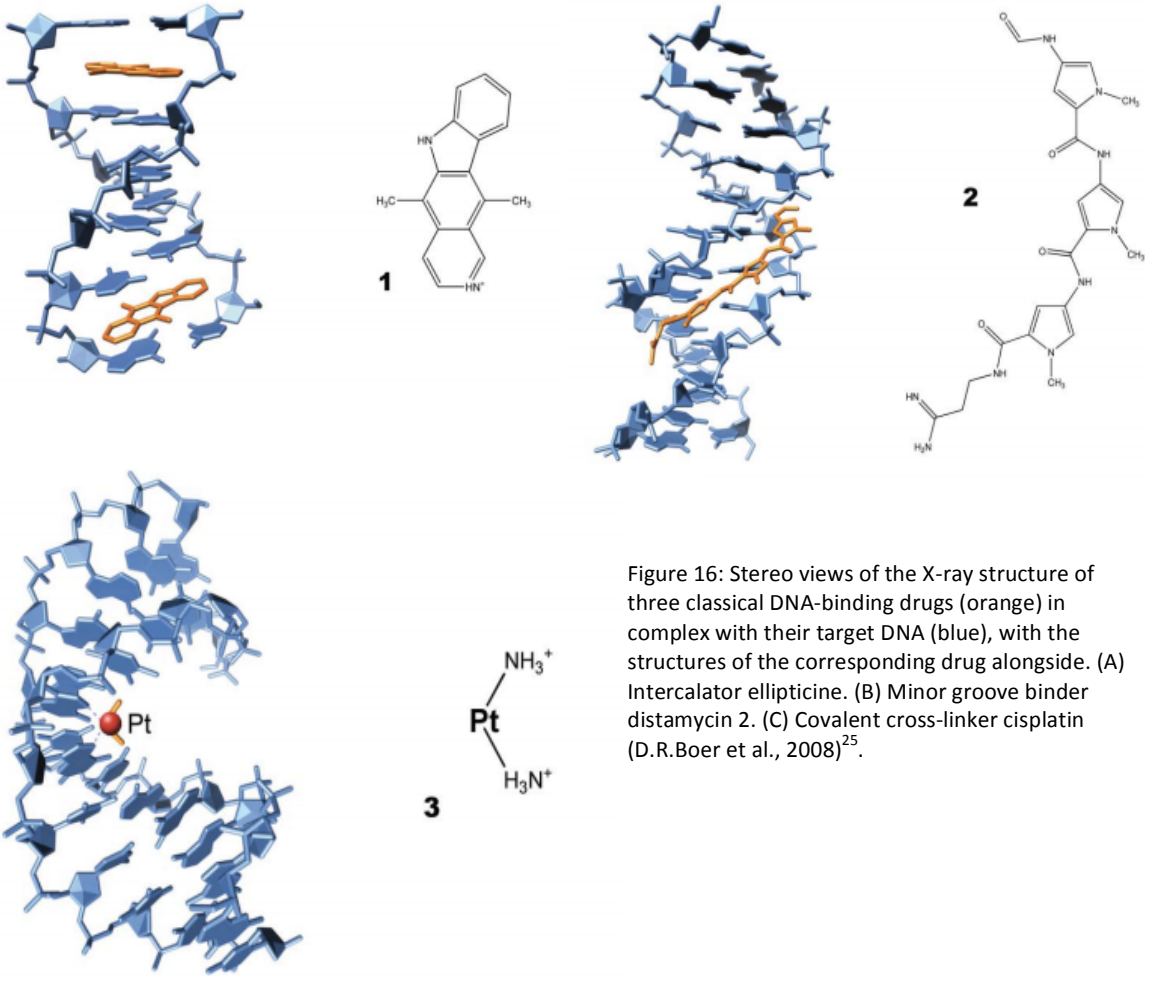


Figure 16: Stereo views of the X-ray structure of three classical DNA-binding drugs (orange) in complex with their target DNA (blue), with the structures of the corresponding drug alongside. (A) Intercalator ellipticine. (B) Minor groove binder distamycin 2. (C) Covalent cross-linker cisplatin (D.R.Boer et al., 2008)[25].

3. NEW PERSPECTIVES

Overall explained in chapter 2, our understanding of the relationships between DNA interaction, chemical structure, and biological activity, including genotoxicity, remains still incomplete[26]. The development of new and improved DNA-binding drugs may come from slight variations of the classical binders. Also altered DNA conformations or new binding modes are used to design alternative and trailblazing compounds. Devastating side effects often arise from poor specificity. Thus, it is expected that sequence specificity will be achieved more straightforwardly, since the opening of the DNA duplex to increased exposure to new interactions of the otherwise buried bases[25].

3.1 New Structures – binding the classical Way

Commercially available acridine and anthracycline derivatives have been widely studied from a variety of viewpoints, such as physicochemical properties, structural requirements, synthesis and biological activity. However, the clinical application of these and other compounds of the same class have encountered problems such as multidrug resistance (MRD), and secondary and/or collateral effects. These shortcomings have motivated the search for new compounds to be used either in place of, or in conjunction with the existing compounds[49]. In this section, recent advances in the structural characterization of compounds that bind DNA in a "classical" way will be discussed. These includes the purely minor and major groove binders, intercalators and molecules that form covalent adducts with DNA[25].

3.1.1 Minor Groove Binding

Multiple minor groove binders have been in clinical trails for many years. Evidence is just now emerging that these class of agents indeed have significant clinical activity and there still remain a great number of compounds with potential clinical activity[45].

3.1.1.1 Distamycin & Netropsin Derivatives

3.1.1.1.1 PNU-145156E

A new clinically relevant derivative of distamycin (discussed within the chapter before) to enter development has been the drug PNU-142156E, previously renamed FCE-26644. Though derived from the DNA binding distamycin backbone, initial experiments revealed that the primary action method of this drug and similar agents is to bind the basic fibroblast growth factor, a cytokine important in angiogenesis. Despite apparent lack of cytotoxicity on its own, PNU-145156E showed combinatorial efficacy with other cytotoxic drugs and entered phase I trials, where some disease stabilization was observed in patients with solid tumours. Dose-limiting toxicities were thrombophlebitis, dyspnoea and pulmonary embolism. Due to the relative difficulty in detecting changes in its surrogate endpoint and its unique toxicity profile, development of PNU-145156E has currently been halted.

Though this drug failed as a general anti-tumour agent it may yet have applications in such tumour types, which are total dependent on its major molecular target: The efficacy of this drug in treating bFGF -dependent sarcomas in mice was initially shown and has yet to be investigated further[45].

3.1.1.1.2 Brostallicin

Perhaps the one single distamycin derivative generating significant interest in the clinic is the bromoacrylol derivative brostallicin (Figure 15). It is derived from the lead compound PN-151807, which was originally shown to have activity against cyclin-dependent kinases in addition to its DNA binding activity. Because of its favourable myelotoxicity/cytotoxicity ratio it was selected for clinical development. Studies revealed that brostallicin is only an effective DNA alkylator in the presence of high levels of cellular thiols (such as glutathione). Phase II trials had been completed showing partial response for patients with soft tissue sarcomas who had failed in first line therapy. These results were encouraging and seem to indicate that brostallicin functions primarily as a cytostatic agent rather than a cytotoxic one[45].

3.1.1.1.3 LEXITROPSINS

The lexitropsins are analogues o distamycin (Figure 17; 1) and netropsin (2) both of which bind selectively and with high affinity to adenine/thymine sequences of DNA, in the minor groove. A substantial effort has been invested in finding ways of controlling the selectivity of binding for other DNA sequences based on the preference of distamycin for binding two molecules side-by-side, which allows for the pairing of different heterocycles and the development of the "pairing rules". Thus, pyrrole/pyrrole pairs confer A/T selectivity, while analogues with pyrrole/imidazole, imidazole/pyrrole, or pyrrole/hydroxypyrrole pairs can be designed to read C/G or G/C and distinguish A/T from T/A[50].

Lexitropsin conjugates with anti-cancer drugs are synthesized to preferentially generate N3-methyladenine (3-MeA) adducts with the DNA. The presence of these 3-MeA lesion in DNA is thought to halt DNA polymerase, and this initiates cell death. The challenge is thus to produce drug-like molecules with high affinity for DNA while maintaining sufficient sequence selectivity to have useful therapeutic effects. Thus, new anti-cancer drugs with novel modes of action can be developed[51].

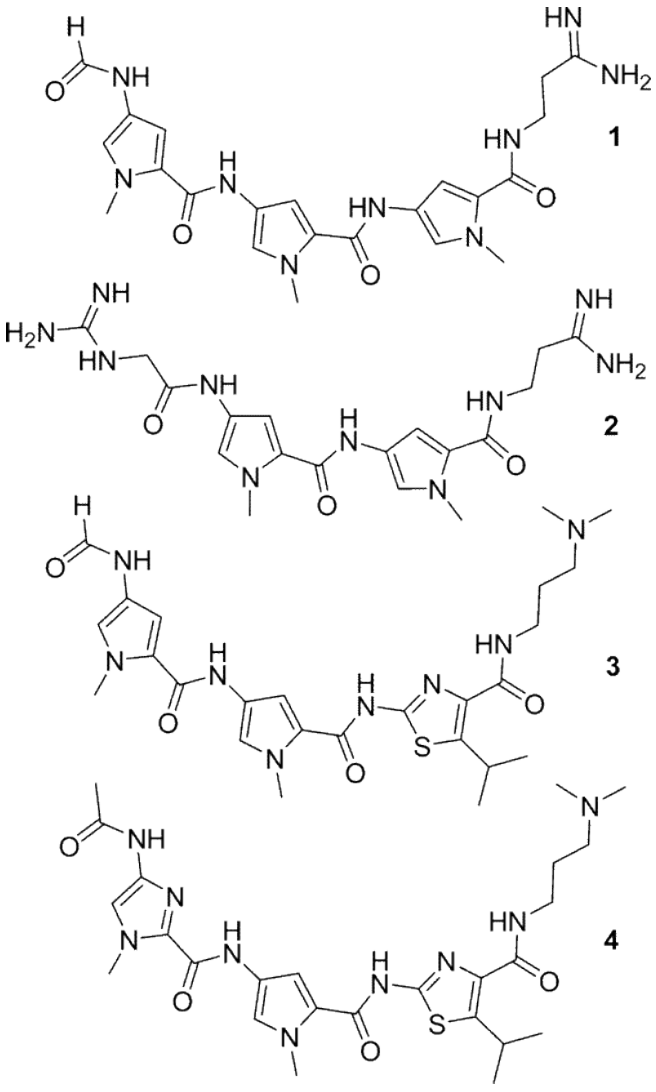


Figure 17: Lexitropsins: (1) Dystamycin. (2) Netropsin. (3) Thiazotropsin A. (4) Thiazotropsin B (Anthony et al., 2007)[50].

3.1.1.1.4 HAIRPIN POLYAMIDES

Distamycin and netropsin have also served as base for the development of "synthetic programmable oligomers" - molecules made up of functional groups capable of reading individual base pairs in the order by which these functional groups are joined together. Simple compounds, such as the polypyrrole antibiotics provide compounds of enormous selectivity and versatility and thus, have been used as carriers for alkylating agents. This leads to directing alkylation to specific DNA sequences. These compounds are able to compete with natural substrates, such as specific transcription factors, and alter gene expression via both affecting gene repression (e.g., GFs) and activation (e.g., tumour suppressor genes).

In one scenario the polyamide selectively binds to a DNA sequence that is normally occupied by a transcription factor. Thereby it prevents this protein from its interactions, which would support gene transcription. Thus, hairpin polyamides provide a high degree of control of gene expression and this suggests enormous promise for their clinical utility.

Hairpin polyamides (HP) that are capable of precise sequence selective binding have been successfully developed (Figure 18). Pairs of pyrrole (Py), imidazole (Im), and hydroxypyrrole (Hpy) rings are capable of distinguishing the four Watson-Crick base pairs with the following base pairing rules:

- Py/Im targets C-G
- Im/Py targets G-C
- Py/Hpy targets A-T and
- Hpy/Py targets T-A.

Thereby affinity and specificity of the polyamides were increased via linking antiparallel polyamide dimers and forming a hairpin structure as seen in figure 18 below.

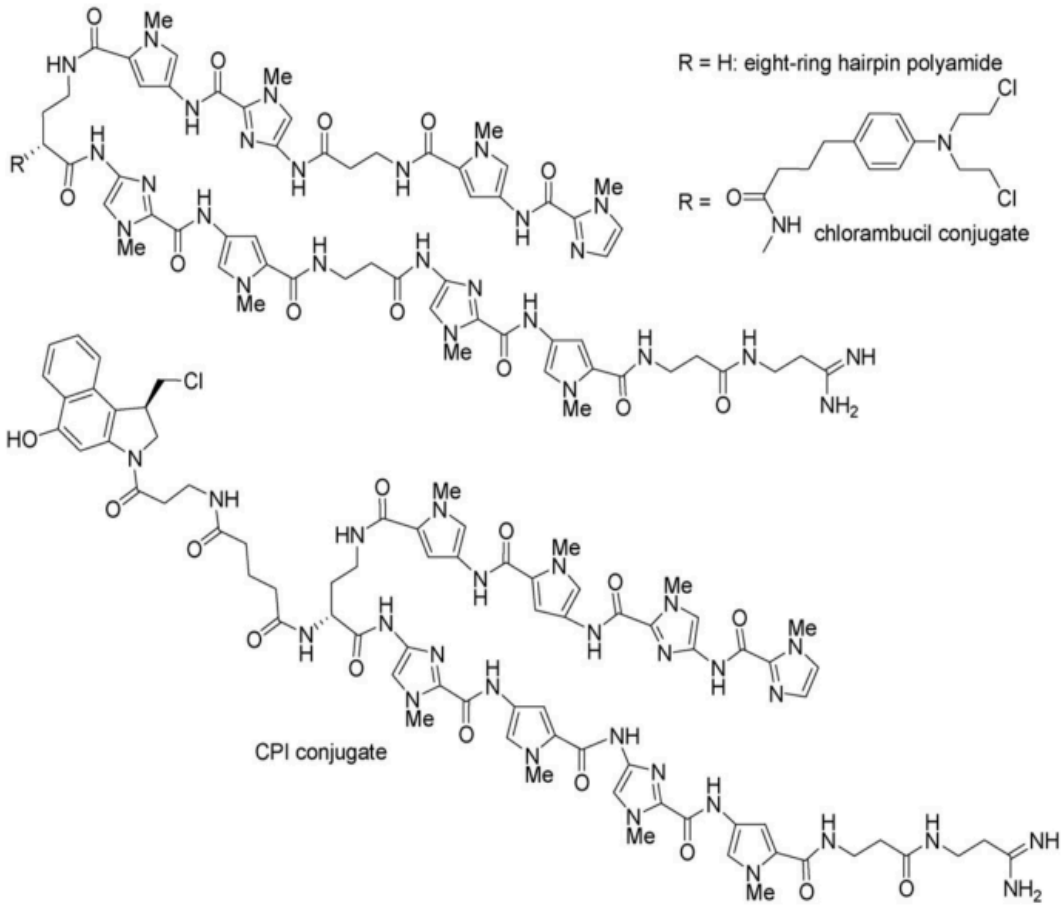


Figure 18: Examples of hairpin polyamides (Nelson et al., 2007)[46].

The hairpin is maintained by acetylation on the chiral amine, which prevents the linear oligomer from binding to the minor groove. The hairpin turn and the ß-dimethylaminopropylamine tail are able to recognize A-T bases. The size of the DNA binding site can be increased by changing the numer of included rings, proportional the capable recognition site (11-16 pairs)[46]. Several recent studies about this subject are reviewed in the following paragraphs:

Lai et al.[52] developed a hairpin polyamide that recognizes specific sequences of TGF-$ß_1$ promotor. It was shown that and this HP was able to significantly decrease TGF-$ß_1$ transcription and protein expression in human vascular smooth muscle cells. Given the stability and function of cell permeability of HPs, this compound is indeed feasible to be an useful gene therapy agent. Suggested indication has been cardiovascular and renal diseases.

Another study demonstrated that minor groove binding HPs also disrupt protein/DNA interactions, which occur in the major groove. Human oestrogen-related receptors

belong to a class of nuclear receptors that bind to DNA via an oestrogen response element (ERE). It was demonstrated that human oestrogen receptor alpha of the ERE, which binds in the major groove of the ERE, can be inhibited by a polyamide- bound in the opposing minor groove of the ERE. Thus, two mechanisms are involved: direct steric blockage of minor groove DNA, and change of the helical geometry of DNA resulting in decreased major groove interactions[53].

Another study by Buchmueller et al.[54] described a new HP, designed to target the inverted CCAAT box in the multidrug resistance (MDR) gene that encodes the P-glycoprotein (Pgp). Pgp-activation leads to chemotherapy resistance via active transport of small, foreign molecules out of the cancer cell.

Furthermore distinct HP-chlorambucil conjugates (Figure 18) were synthesised and screened for activity in human colon cancer cells. Each compound was designed to recognise certain DNA sequences via its polyamide component. One out of five examined compounds has shown significant changes in cellular morphology and growth arrest of cancer cells *in vitro*. An additional polyamide chlorambucil conjugate was designed to bind to certain sites along the HIV-1 promoter and was indeed found to block HIV viral replication. HP-cyclopropylpyrrolindole (HP-CPI) conjugates (Figure 18) showed cell growth inhibition activity in mammalian cell. Compared to its parent CPI (adozelesin), however, it showed decreased growth inhibiting activity.
Another area of research using the HP compounds has been the selective targeting of AT-rich sequences. These sequences are structural components of chromatin, which is essential for cell proliferation. The localization of proteins that bind to these regions can be disrupted by the presence of HP compounds and thus, affects the activating function of such proteins[46].
Due to the fact that polyamide-conjugates are less toxic than their parent agents, it is proposed to use such compounds for prospective clinical applications[46].

One problem provides the size of these agents. They are too large and have too many hydrogen-bonding groups. However, there is good evidence that this principle can be extended to much shorter molecules, such as thiazotropsins where 2:1 binding in the

minor groove occurs with limited overlap between the minor groove binders[50] (Figure 17; 3 and 4).

3.1.1.2 Bis-Benzimidazoles

The bis-benzimidazole compounds, Hoechst 33342 and 33258 (Figure 19) show AT sequence selectivity and also bind in the minor groove of DNA. Hoechst 33258 (pibenzimol) has undergone Phase I clinical evaluation as anti-cancer agent; the mechanism of cytotoxicity has been suggested as inhibition of topoisomerase and DNA helicase. It have been developed a wide range of conjugate molecules of such agents:

Hairpin polyamides have been linked to Hoechst 33258 analogues to acquire longer sequences that are able to track along the double helix. In addition these conjugates were proposed as one way to increase cellular and nuclear uptake[46].

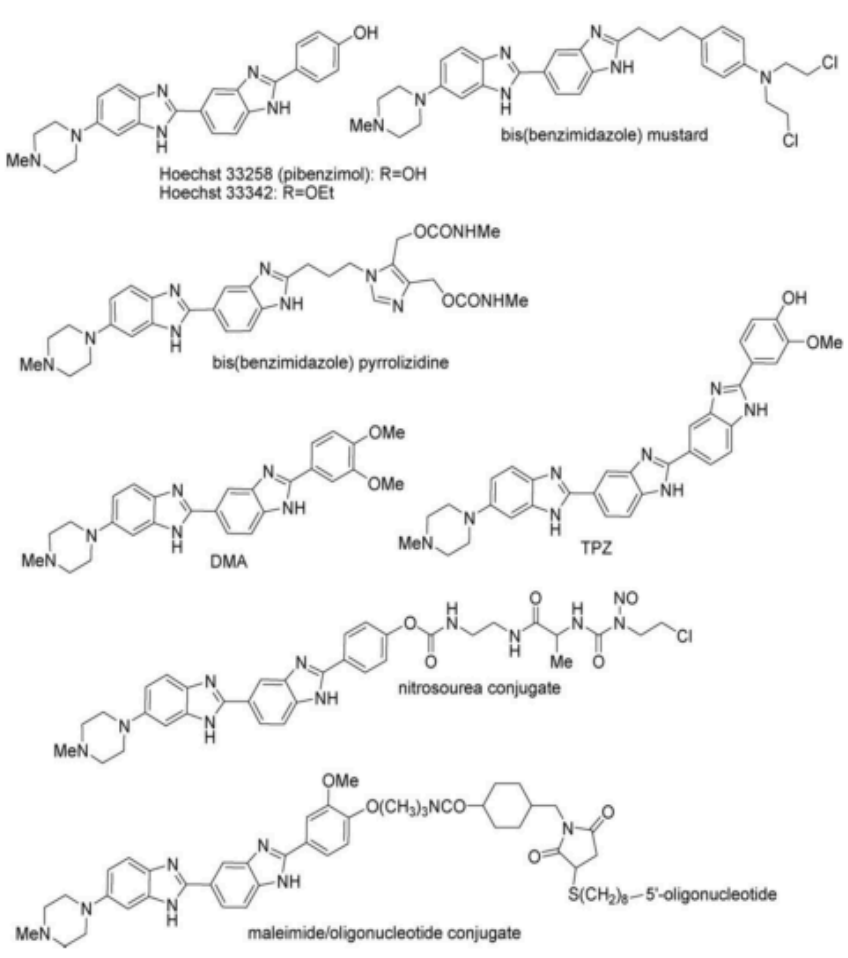


Figure 19: Examples of bis-benzimidazole conjugates (Nelson et al., 2007)[46].

A Hoechst analogue conjugated with a triplex forming oligonucleotide is further explained in chapter 3.4.1.

Mustard analogues of bis-benzimidazoles where the mustard moiety was linked via a variable-length polymethylene chain (Figure 19) were found to have great cyto-

toxic potential (up to 85-fold more than chlorambucil). Thereby the region of DNA, which is desired to be alkylated can be varied via modifications of chain length: analogues with short linker chains alkylated strongly at adenine sites in poly-AT regions, while those with longer chains showed substantial N7 alkylation at guanine sites.

Carbamate and 2-chlorethylnitrosourea conjugates (Figure 19) of Hoechst 33258 are also more cytotoxic than the parental compound Hoechst 33258. The increase in cytotoxicity correlates with increased DNA affinity and may be due to inhibition of topoisomerase enzymes.

Hoechst 33258 has been also shown to bind to G-quadruplex DNA (detailed within chapter 3.3.3)[46].

Summing up, bis-benzimidazole groove binders can be highly expected to be used in clinical applications of the future.

3.1.1.3 IROFULVEN

One of the most exciting agents may entering the field of application in recent years is irofulven; a semisynthetic derivative of the mushroom-derived compound Illudin S. Irofulven was immediately noted to retain its anti-tumour activity in solid tumour cell systems known to be highly resistant to nearly all rested compounds. Moreover Irofulven also demonstrated synergistic activity with various anti-cancer treatment, most notably topoisomerase inhibiting agents as well as gamma radiation. Due to its excellent preclinical activity irofulven moved into clinical trials quickly. Numerous phase I and II trials of irofulven are summarized by Cai et al.[45].
While irofulven is capable of inducing apoptosis in a wide variety of tumour types but also demonstrates unique retinal toxicity, which can limit its use[45].

3.1.1.4 TRABECTEDIN (ECTEINASCIDIN-743)

Trabectedin, also termed ET-743 is member of the ecteinascidin family and derived from the Caribbean tunicate *Ecteinascidia turbinata* (Figure 20). It is a tetrahydroisoquinoline alkaloid composed of three subunits[45].

ET-743 binds tightly in the DNA minor groove where it alkylates guanine-N2 positions in a sequence-selective manner. The bond between ET-743 and DNA is reversed upon DNA denaturation, which sets ET-743 apart from the DNA alkylating agents presently used in cancer chemotherapy[55]. The binding of Trabectedin causes DNA helix distortion with the DNA bending towards the major groove rather than the site of the drug interaction. Additionally it blocks the G_2/M phase of the cell cycle and is a selective inhibitor of activated gene transcription important for maintaining tumour cell viability[45]. Although DNA alkylation by ET7-43 can trap topoisomerase I, topoisomerase I inhibition is only detectable at micro molar concentrations that exceed pharmacologically active concentrations by a factor of 100 or more[55]. It is believed that DNA breakage, formed by the drug, is caused by its activation of the nucleotide excision repair (NER) pathway. NER enzymes recognize bulks in DNA produced via alkylation of ET-743 and remove them by breaking the strands[45]. Figure 20 shows this unique mechanism of the agent[55].

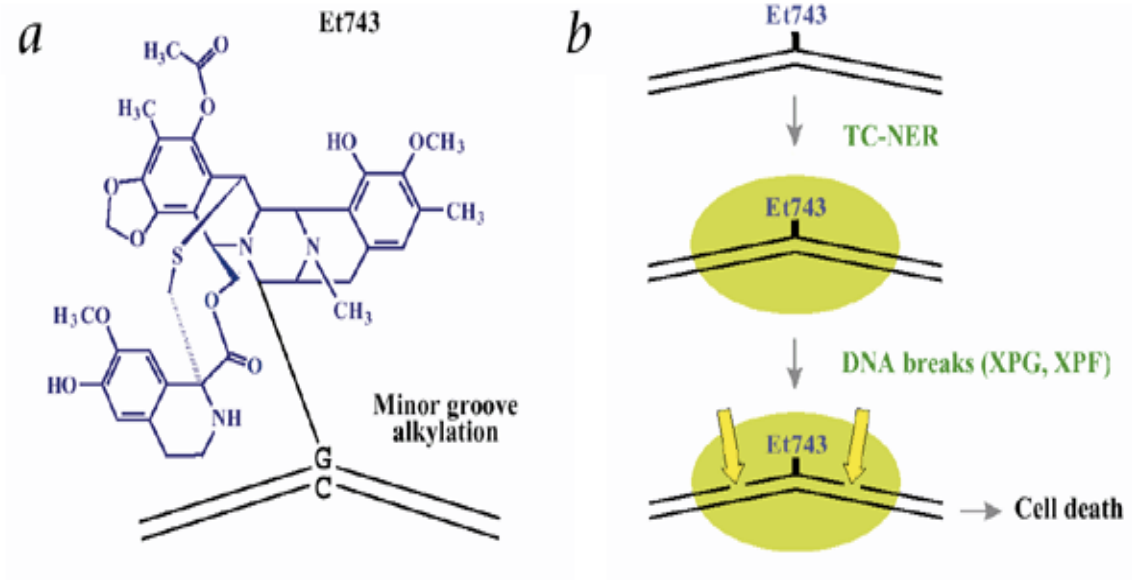


Figure 20: Model for TC-NER–mediated cytotoxicity of Et743.
a, Structure of Et743 alkylated to N2 of guanine in the DNA minor groove. Alkylation produces a bend toward the DNA major groove.b, Schematic representation of the trapping of the TC-NER complex stalled after DNA cleavage. Arrows represent DNA cleavage by the NER endonucleases, XPF and XPG. (Takebayashi et al., 2001)[55].

ET-743 seems to work by trapping this DNA-binding protein[47]. Trabectedin might also be analogous to topoisomerase poisons because as recently reported, it can cause protein-associated strand breaks, probably as a result of a halt of NER.

Another mechanism of action involves reversal of multidrug resistance. MDR1 (multidrug resistance 1) is a broad specificity pump that exports drugs from cancer cells. ET-743 is able to prevent such efflux. However, the underlying mechanism remains unclear.

In any event, the unique features of the drug-DNA complex (major-groove bend associated with minor-groove occupancy, extrahelical protrusion and hydrogen-bonded stabilization of the helix) provide ET-743 with structural and biological properties that set it apart from other known agents[55].

In April 2005, the FDA granted Trabectedin orphan drug status for ovarian cancer. In 2007 trabectedin received European approval for its use in patients with advanced or metastatic soft tissue sarcomas who have failed or are not suitable for first line therapy with anthracyclines and ifosfamide. A combination therapy of trabectedin with doxorubicin is suggested[45].

3.1.1.5 AUREOLIC ACID ANTIBIOTICS

3.1.1.5.1 MITHRAMYCIN

Mithramycin (MTR) binds to GC-rich DNA sequences in the minor groove and belongs to the aureolic acid group of anti-cancer drugs. Binding to GC-rich sequences in eukaryotic promotors may be the mechanism by which MTR inhibits gene expression. For example, MTR inhibits expression of the c-MYC proto-oncogene in several different cell types. MTR forms complexes with several different metal ions such as Mg^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} [46]. For explaining the binding of those ions, the interaction of Mg^{2+} with a further compound of this group of anti-cancer drugs - chromomycin A_3 is used.

3.1.1.5.2 CHROMOMYCIN A_3

The figure below shows the X-ray structure of chromomycin A_3 in complex with a 5-d(TTGGCCAAA)-

3 duplex DNA. This structure establishes the exact location of a Mg^{2+} ion, which coordinates two chromomycin A_3 molecules (Figure 21; 13) and thus, may have great impact on the pharmacology. The two chromomycin A_3 moieties bind to the same minor groove of the DNA duplex. The chromophore and C-D-E trisaccharide moieties interact directly with residues in the minor groove, whereas the A-B disaccharide and part of the chromophore interact with the phosphate backbone of DNA[25].

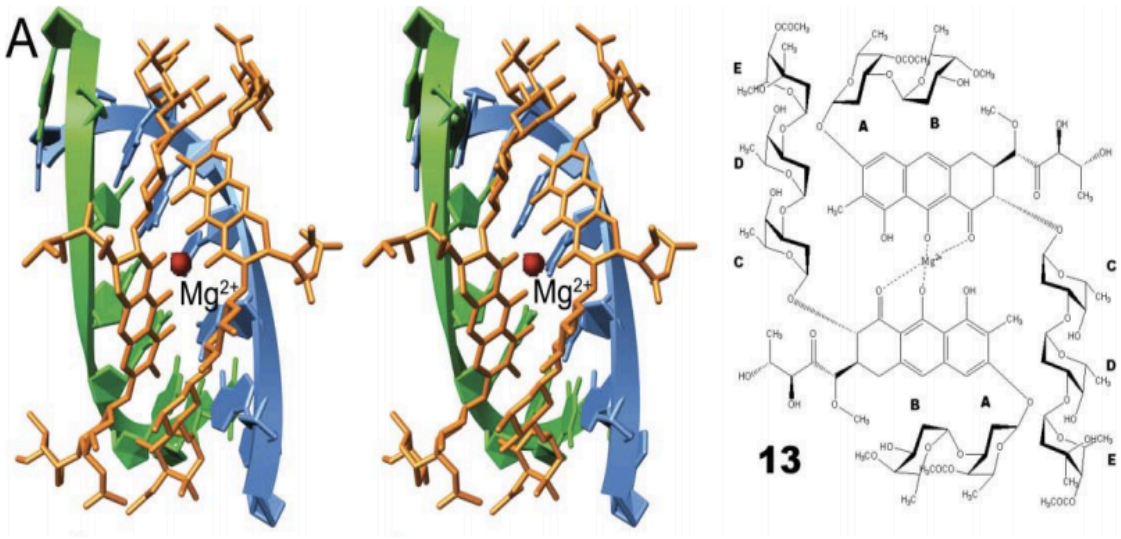


Figure 21: Stereo views of recent structures of compounds that interact through and combine classical binding modes. (A) Minor groove binding of the antibiotic chromomycin A3 13. (D.R. Boer et al., 2008)[25].

3.1.1.6 BISQUATERNARY AMMONIUM HETEROCYCLES

Bisquaternary ammonium heterocycles (BQAH) are a class of synthetic DNA minor groove binding agents related to the polyamidines, with quaternary ammonium heterocycles as charged centres. The terephtalanilide NSC-66761 (Figure 22) was an early example that was clinically evaluated for anti-cancer activity. Different compounds of BQAHs have been investigated for possible sequence-selective binding *in vitro*. It was indicated that several of them prefer binding to AT runs, consisting of at least 4 bps.

Shortening of one or two spacer groups between the aromatic rings emerged as a prercondition for sequence-specific binding. Polymerase-catalysed synthesis of DNA and RNA in vitro were inhibited by all derivatives while inhibition ability correlates with sequence-specificity. SN 6999 and its amino analogue SN 7167 show a significant distortion in the DNA structure[46].

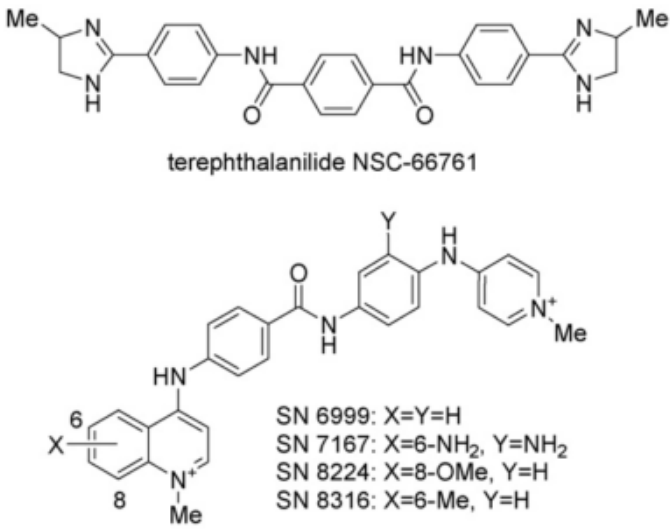


Figure 22: Examples of bisquaternary ammonium heterocycles (Nelson et al., 2007)[46]

3.1.1.7 The "Amplifier Effect"

Another novel way in which minor groove binding agents have been used, is to amplify the cytotoxic effects of DNA alkylators and DNA cleaving compounds in an attempt to enable the use of lower cytotoxic drug doses resulting in fewer side effects. For example, distamycin A has been used in combination with bleiomycin, C1027 and duocarmycin A (alkylating agents), resulting in increased DNA cleavage and apoptosis in experimental model systems. Increased activity results from altered DNA conformation

via distamycin binding to the minor groove, which enables greater access of the cytotoxic compound to its targeted DNA sequences[46].

3.2 New mixed Major/Minor Groove Binders

3.2.1 Polyethylenglycol

One recent study reveals specific interactions between polyethylene glycol (PEG) molecules and the major groove of a promotor DNA recognition site. In the reported structure, the DNA adopts A conformation and contains three curved PEG molecules per duplex. The presence of the PEG molecules opens the major groove by approximately 1 Angstrom[25]. Many chemotherapeutic agents have limited penetration into the brain through the Blood brain barrier. This is mainly caused by drug efflux systems in the brain capillary endothelial cells. To benefit from the PEG mechanisms several multifunctional peptide-PEG intercalating conjugates have been designed, to use PEG as a delivery systhem[56,57].

3.2.2 Polyamines

Polyamines bind to the major and/or minor groove of the DNA double helix, interacting with nucleic acid bases and neutralizing the charges of phosphate groups. Polyamines can affect the local conformation of DNA, and due to their ability to stabilize highly charged structures they promote the conversion from right-handed B-DNA to either left-handed Z-DNA or A-DNA and also promote DNA binding[58]. Polyamines are small, naturally occurring, polycationic alkylamines, which are essential for normal cell

growth and development in eukaryotes. Their roles in physiologically cells involves the maintenance of chromatin structure, regulation of ion-channels and control of membrane stability[59]. The ability of polyamines to condense DNA decreases in the order: pentamine > tetraamine > triamine. Moreover spermine is known to protect DNA from the ROS-induced strandbreakage by either altering the DNA structure and making it less susceptible to attack, or by directly scavenging free radicals and inactivating ROS[58].

Ornithine decarboxylase (ODC) converts the amino acid ornithine into the diamine putrescine. The expression is regulated through mechanisms including post-transcriptional processing, alteration of protein stability, and transcription where growth factors, hormones, and tumour-promoting agents control ODC. A second catalysator of the PA biosynthesis is the S-adenosylmethionine decarboxylase (AdoMetDC), which decarboxylates S-adenosylmethionine (SAM) creating a product, which donates its propyl amines for the conversion of putrescine to spermidine and spermine.

The intracellular polyamine pools are mediated by spermidine/spermine N^1-acetyltransferase (SSAT) which catalyses the formation of N^1-acetylspermine/spermidine by transferring the acetyl group from acetyl-coenzyme A to the N^1 position of spermine or spermidine, respectively. These acetylated polyamines serve as substrates for the flavin-dependent polyamine oxidase (APAO), which again produces spermidine or putrescine, as well as 3-aceto-aminopropanal and hydrogen peroxide (H_2O_2). Another enzyme is the spermine oxidase (SMO), which oxidized unsubstituted spermine to spermidine, 3-aminopropanal, and H_2O_2[59].

A key regulator of these enzyme activities is antizyme (AZ), a protein that controls the activity of ODC. AZ is synthesized in response to elevated cellular polyamine levels, binds ODC and promotes its degradation. AZ also plays a role in the feedback control of the polyamine transport system (PTS) activity by a still unknown mechanism.
Although polyamines are highly charged molecules they are transported across the lipophilic cell membrane via PTS. This is possible due to interaction of the charged polyamines with a negatively charged structure present on the cell membrane - heparan

sulphate. The membrane-bound polyamine is then taken into cells through endocytosis[60].

An increase in intracellular polyamine concentration, e.g., through an upregulation of ODC, correlates with increased cell proliferation as well as tumourigenesis. Enhanced levels of polyamines have been associated with breast, colon, prostate, and skin cancers. Moreover the polyamine pathway is a downstream target of numerous oncogenes, such as Myc and Ras[59]. Everybody consumes large quantities of polyamines every day: Broccoli and cauliflower are high in spermidine, and citrus fruits are rich in putrescine, whilst all three polyamines are found in high concentrations in meat. Studies with refractory prostate cancer have shown that even a simple low polyamine diet can result in improved survival, a better quality of life and a significant decrease in pain scores[61]. A significant reduction in metastases in has been obtained in treated animals[62]. An increase of polyamines has also been associated with breast cancer[63]. Moreover a SMO induction was demonstrated in inflammation-associated cancers of the colon and lung, mediated by TNFα or other cytokines, resulting in ROS production and thus, DNA damage[59]. SMO levels as well were shown to be increased in ulcerative colitis, another inflammatory condition leading to a high incidence of colon cancer. Xu et al. demonstrated that helicobacter pylori, which is able to cause such type of cancer, upregulates SMO expression for increasing DNA damage and H_2O_2 production[64]. These studies established SMO as a downstream target of H. Pylori and thus, a contributor to gastric cancer development.

Based on these observations and the absolute requirement for polyamines in tumour growth, the polyamine pathway is a rational target for chemoprevention and chemotherapeutics. There are two ways of polyamine cancer treatment: Targeting the polyamine pathway via enzyme inhibitors and polyamine analogues[59]. The following paragraphs describe the recent advances made in the polyamine field.

3.2.2.1 Enzyme Inhibitors

Since the beginning of targeting the polyamine biosynthetic pathway numerous types of agents have been developed (Figure 23). The common therapies affect both metabolic enzymes ODC and AdoMetDC.

3.2.2.1.1 DFMO

2-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, was first shown in the 1970s to have anti-cancer properties (Figure 23; a). *In vitro*, DFMO depletes putrescine and spermidine. This depletion is associated with cytostatic growth response. Its cytotoxic effects on human small-cell lung cancer *in vivo* and *in vitro* prompted clinical trials for DFMO. However, as single agent or in combination it was largely ineffective. It is generally well tolerated with mostly reversible toxicities including thrombocytopenia, gastrointestinal anomalies, and hearing loss. This lack of effectiveness is likely due to its low transport into the cell, the fact that it is typically cytostatic and not cytotoxic, and finally due to compensatory mechanisms (increased polyamine transport or upregulation of

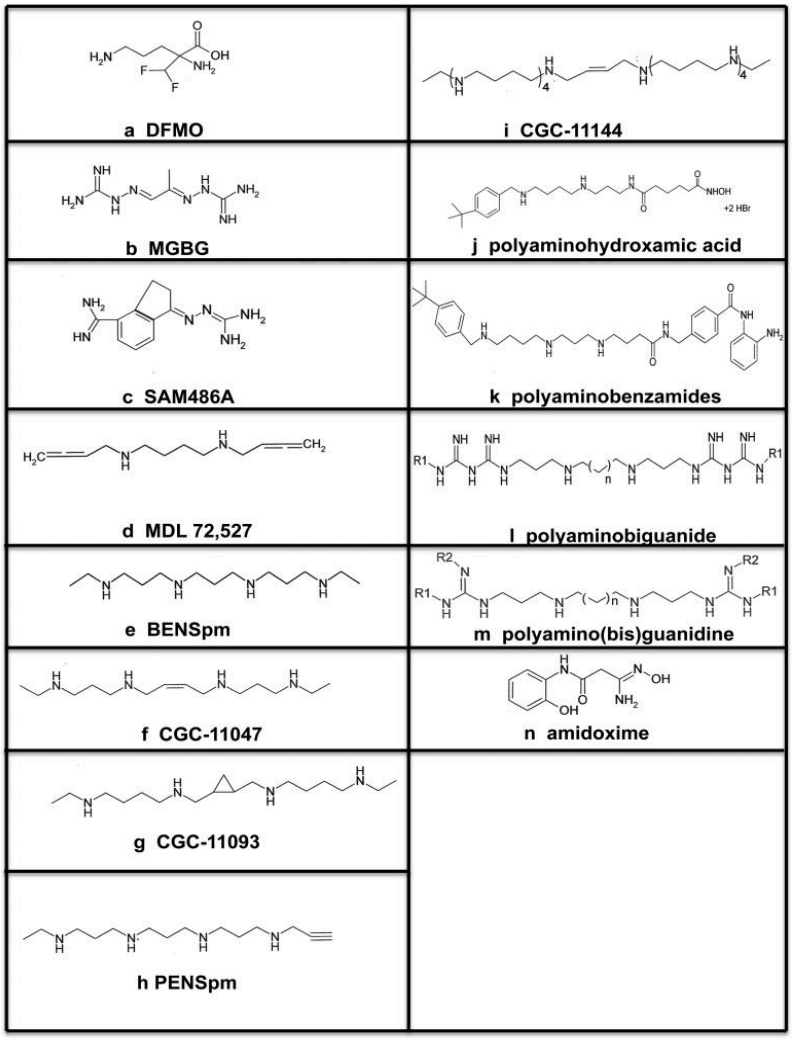


Figure 23: Representative agents targeting the polyamines, polyamine metabolism, and polyamine catabolism silenced genes. (Nowotarski et al., 2013)[59].

AdoMetDC) that occur as a result of the depleted polyamine pools. Thus, tumour cells are able to overcome the effects of ODC inhibition[59].

3.2.2.1.2 MGBG

Methylglyoxal bis(guanylhydrazone) (MGBG) was already used in treatment for leukaemia before it was demonstrated to be a competitive inhibitor of AdoMetDC. In vitro, MGBG reduces the levels of spermidine and spermine while increasing putrescine and inhibits cellular growth. Its use as a chemotherapeutic agent was limited due to its mitochondrial toxicities.

Subsequently, a number of nucleoside-based inhibitors of the enzyme were developed, most based on the structure of S-adenosylmethionine[59,65].

3.2.2.1.3 SAM486A AND ITS DERIVATIVES

More recently, 4-amidinoindan-1-one-2'-amidinhydrazone (SAM486A/CGP48664), a competitive inhibitor of AdoMetDC, was found to produce low mitochondrial toxicity and has been tested in phase I and phase II clinical trials for multiple cancers[66]. SAM486A/CGP48664 decreases the levels of both spermidine and spermine while increasing the levels of putrescine.

Further inhibitors have also been developed, which target aminopropyl transferases spermidine and spermine synthase: S-adenosyl-3-thio-1,8-diaminooctane (AdoDATO), a spermidine synthase inhibitor, caused little growth inhibition in cancer cell lines[59].

3.2.2.2 SYMMETRICALLY SUBSTITUTED POLYAMINE ANALOGUES

As indicated before, the initial focus of polyamine-targeted therapy was directed at inhibiting its biosynthetic enzymes. This, however, showed minimal success. Thus, current research emphasises on exploiting the self-regulating nature of the polyamine pathway using polyamine analogues.

Polyamine analogues were able to utilize the PTS without evoking compensatory mechanisms or functionally substituting natural polyamines. This results in the down-regulation of the polyamine biosynthetic pathway and an increase of the catabolic pathway, i.e., a significant depletion of all 3 polyamines. Thus, growth inhibition and, in specific cases, cell death are the consequences[59].

The first polyamine analogues were the bis(alkyl) polyamines, which are similar to spermidine and spermine. Their primary amino termini are protected by bis(alkyl) groups, preventing oxidation via multiple amine oxidases. This generation of analogues downregulates both ODC and AdoMetDC through a combination of multiple mechanisms, involving product inhibition and changes in mRNA translation[67]. Moreover, several of these analogues induce polyamine catabolic enzyme activity, which has been linked to cytotoxicity[68].

3.2.2.2.1 BENSPM

BENSpm, an N^1, N^{11}-bis(ethyl)norspermine uses the polyamine transport system to enter the cell and downregulates both ODC and AdoMetDC. It induces the catabolic enzymes SMO and SSAT[59], which production of H_2O_2 implicates a cytotoxic response for many tumour types[69]. There is a huge amount of bis(alkyl) polyamine derivatives, e.g., spermine (BESpm) and homospermine (BHSpm) compounds. Among them especially BENSpm displays significant anti-tumour activity in vitro. Phases I and II trials, however, were disappointing. The initial phase I dosage-trials suggest that although BENSpm was well tolerated with the once daily dosing, the compound failed to show

significant anti-tumour effects against breast or lung cancer. Studies that combine BENSpm with standard chemotherapeutic agents appear promising and are likely to serve as catalysts for future research[59].

3.2.2.3 CONFORMATIONAL RESTRICTED ANALOGUES

Another type of bis(alkyl) polyamine analogues is characterized by a restriction at the central carbons of the polyamine chain. From this subset of compounds, CGC-11047 (PG-11047)[70] and CGC-11093 (PG-11093)[71] have been the most encouraging therapeutic agents.

CGC-11047 is a second-generation analogue of BESpm that is also symmetrically substituted, but conformationally restricted with a cis double bond between the central carbons (Figure 23; f). Treatment with CGC-11047 has demonstrated significant growth inhibition in numerous tumour cell lines[70]. CGC-11093 is also a BEHSpm analogue that contains a cyclopropyl ring at the central 2 carbons of the 4-carbon methylene bridge. These alterations increased the anti-proliferative activity of these compounds as well as reduced the toxicity. CGC-11047 inhibited growth in both small cell and non-small cell lung cancer cell lines as well inhibits the growth of breast cancers and colon cancers *in vitro*. Phase I studies of both compounds has been initiated[59].

3.2.2.4 OLIGOAMINES

It was hypothesized that an increase of the number of protonatable nitrogens in the polyamine analogues would increase the affinity of these molecules to DNA. Oligoamines contain 8-14 amines and have saturated, unsaturated, and conformationally restricted forms.

The oligoamine CGC-11144 (Figure 23; i) shows growth inhibition of prostate cancer cell lines and breast cancer, correlated with the ability of the analogue to aggregate DNA. Moreover CGC-11144 has epigenetic effects, leading to changes in gene expres-

sion of specific tumour cell types. However, at present there have been no clinical trials for CGC-11144[59].

3.2.2.5 UNSYMMETRICALLY SUBSTITUTED ANALOGUES

The second generation of polyamine analogues contains unsymmetrical substitutions to the spermine and norspermine backbone. These compounds included: N^1-propargyl-N^{11}-ethylnorspermine (PENSpm), N^1-cyclopropyl-methyl-N^{11}-ethylnorspermine (CPENSpm), and N^1-cycloheptylmethyl-N^{11}-ethylnorspermine (CHENSpm). These compounds are cytotoxic to representative small-cell-lung carcinoma line, while cytotoxicity is accompanied by a significant increase of the expression of SSAT. Thus, those low structural modifications can result in significantly altered biological responses to the individual polyamine analogues[59]. It was also found that reactive functional groups can be added to the polyamine backbone to enable the entry of such conjugates into the cell through the polyamine transport system, leading to the synthesis of multiple compounds, utilizing this strategy. Several examples are given within the next chapter.[59]

3.2.2.6 PA-ANALOGUES TARGETING EPIGENETIC MODIFICATIONS

Epigenetic information is regulated by chromatin modifications that involve both histones and DNA. Histones can undergo different types of modifications, such as phosphorylation, acetylation, methylation of its lysine and arginine residues[72]. It provides a mechanism for regulating gene expression. The acetylation of the cationic lysine tails of histones neutralizes the charge and promotes a relaxed chromatin structure, thereby enhancing chromatin accessibility and potentiating gene transcription. On the opposite, desacetylation causes condensed chromatin, reduced accessibility, and transcriptional repression. In some cancer types, a hypoacetylation of histones is observed, which leads to underexpression of important transcripts, such as the tumour suppress-

ing cell cycle regulator p21, contributing to tumourigenesis. Additionally promoter-region DNA hypermethylation, including histone methylation, is associated with this tumour suppressor gene silencing[59].

3.2.2.6.1 HDAC-INHIBITORS

HDAC regulates the level of acetylation of specific histone lysine residues in chromatin. Analogues contain a polyamine chain to increase its affinity for chromatin and facilitate cellular import[73].

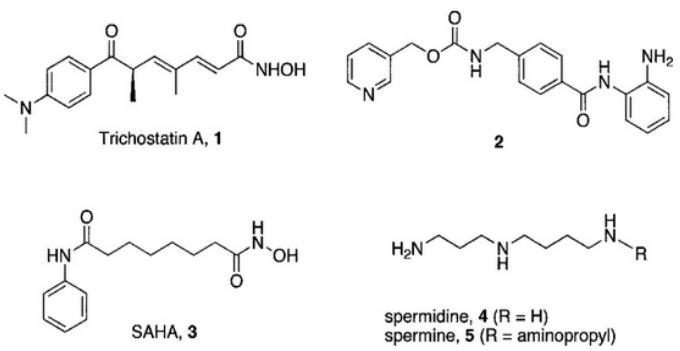
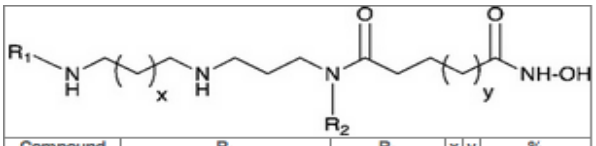


Figure 24: Structures of 1 (trichostatin A), 2, 3 (SAHA), 4 (spermidine), and 5 (spermine) (Varghese et al., 2008)[73]

Histone hyperacetylation caused by HDAC inhibitors such as trichostatin A (Figure 24; 1), MS-275 (2), and SAHA (3) can cause growth arrest in a wide range of human tumour xenografts. It is desirable to identify potent HDAC inhibitors that restore the expression of normal tumour suppressor genes without producing significant dose-limiting toxicity.

Polyamine hydroxamic acid (PAHA) derivatives incorporate structural features of spermidine and spermine (Figure 24, 4 and 5) whereas their linker and hydroxamic acid moieties are found in 1-3**.** Studies of Varghese et al[73] found that the length of the linker chain adjacent to the hydroxamic acid binding group appears to have a dramatic effect on HDAC inhibitory activity. For example, HDAC inhibition increases for compounds 7-9 (Figure 25) as the length of the chain increases from five to eight carbons. Compound 18 is the most effective inhibitor in this series and has a seven-carbon linker chain. The inhibitory activity of 13, however, cannot be explained on the basis of linker length, suggesting that other factors can contribute to the activity of PAHAs as well. Those three latter compounds are significantly more active compared with compounds 19 and 20, which contain a traditional “cap” group that is thought to bind to a certain surface region in the HDAC active site[73].



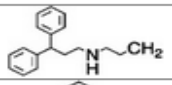
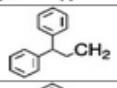
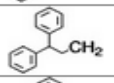
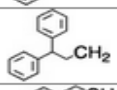
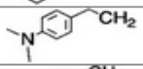
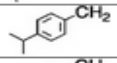
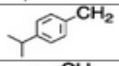
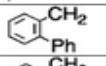
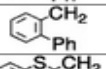
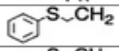
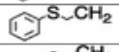
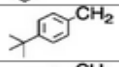
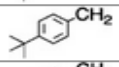
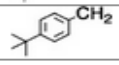
Compound	R_1	R_2	x	y	% inhibition (1.0 μM)
6		H	1	4	55.2
7		H	2	3	46.3
8		H	2	5	54.6
9		H	2	6	73.9
10		H	2	4	46.7
11		H	2	2	15.9
12		H	2	4	41.4
13		H	2	2	71.3
14		H	2	4	38.6
15		H	2	2	6.7
16		H	2	4	16.5
17		H	2	3	51.5
18		H	2	5	88.7
19			2	2	2.0
20	H		2	4	58.6

Figure 25: Structures of PAHA Analogues 6–20 and their inhibitory activity against HeLa CellExtract Histone Deacetylase (Varghese et al., 2008)[73].

3.2.2.6.2 DNMT1 INHIBITORS

As discussed above, tumour suppressor genes hypermethylation often occurs in cancer cells; therefore their demethylation provides an interesting therapeutic strategy. Many DNA methyltransferase (DNMT) inhibitors have been described and are divided into two families: the nucleoside analogues (Figure 26) and non-nucleoside inhibitors, which structure varies according to their inhibitory mechanism[72].

NUCLEOSIDE-LIKE INHIBITORS

The first molecules that have been characterized as DNMT inhibitors were initially used as antimetabolites and cytotoxic agents in leukaemia chemotherapies. Azacitidine and decitabine are 5-aza- and 5-aza-2'-deoxy-analogoues of cytidine, respectively (decitabine), i.e., the carbon atom in position 5 of cytidine is replaced by a nitrogen atom and linked to a ribose or a desoxyribose, respectively (Figure 26). They are used at low doses in order to achieve only their demethylation effect with little cytotoxicity. DNA incorporation of ribose analogues is decreasing. However, they also bind to RNA and thus, disrupt protein synthesis. This explains why decitabine is more active than azacitidine, but also why it has also less sideeffects, given that the latter can be incorpo-

rated not only in dividing cells, but also in quiescent cells. Both have been approved by the FDA for the treatment of MDS and AML. At present Phase II is conducted for a use in solid tumours including melanoma, ovarian and prostate cancer – also in combination with HDAC inhibitors[72].

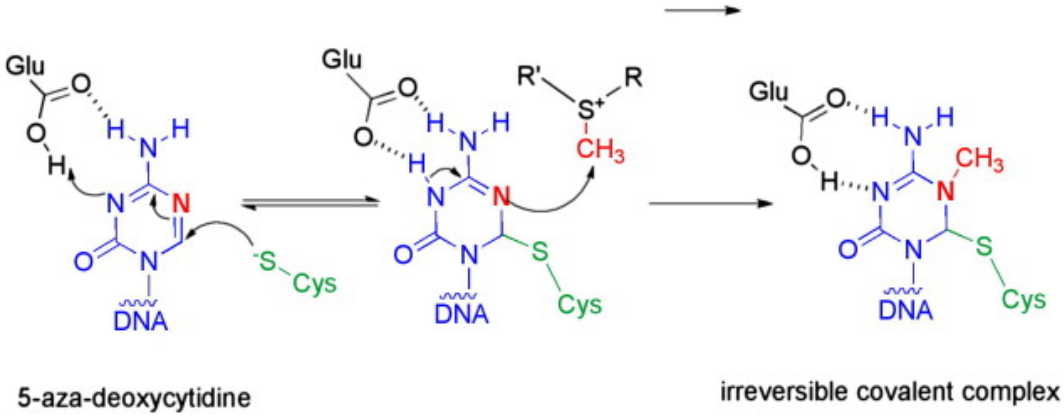


Figure 26: Nucleoside analogue inhibitors of DNMTs. (Gros et al., 2012)[72].

More recent developed derivatives shows improvement of the drug cellular uptake, better therapeutic efficacy (CP-4200 – azacitidine prodrug)[74], better stability or lower in vivo toxicity (SGI-110 – decitabine pro-drug)[75].

NON-NUCLEOSIDE INHIBITORS

Several DNMT inhibitors of various origins and structures have been described during the last years, showing affinity via a DNA incorporation independent mechanism. DNTM inhibition activity has been found among others in following structure families: Flavonoides[76,77,78], hydralazines[79], procainamides and procaines[80,81], oxazolines and isoxazolines[82], psammaplin A[83], nanaomycin A[84], curcumin[85], etc.[72].

For more detailed information feel free to consider the single references.

3.2.2.6.3 LSD1 INHIBITORS

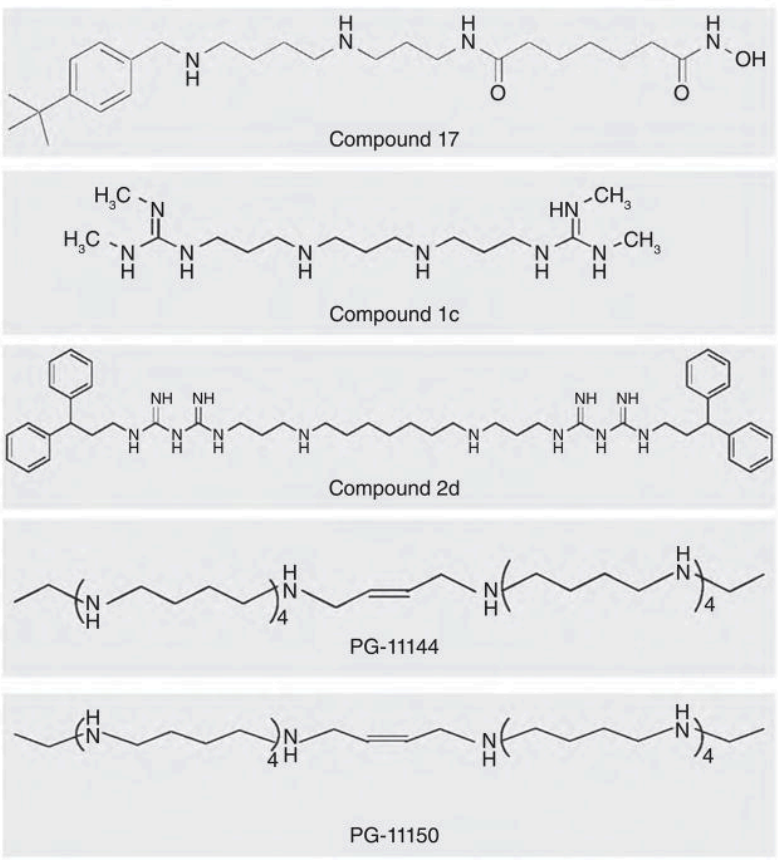


Figure 27: 1c and 2d are potent inhibitors of LSD1 activity and re-activate aberrantly silenced genes in tumour cells. PG-11144 and PG-11150 polyamine analogues contain ten amines and are a cis/trans pair with double bonds in the centre of their structure. Oligoamines competitively inhibit LSD1 activity and re-activate aberrantly silenced genes in colorectal cancer cells (Huang et al., 2009)[86].

With the discovery of the transcriptionally repressive lysine-specific demethylase LSD1, it was hypothesized that inhibition of this enzyme could lead to the re-expression of some aberrantly silenced tumour-suppressor genes in the progression of cancer cells. As already noted, changes in chromatin, including histone methylation, acetylation and phosphorylation are very important to mediate gene expression. LSD1 is structurally similar to, and functions in a mechanistically similar manner as, the polyamine oxidase SMO). Through this homology specific bisguanidine, biguanide polyamine analogues and long-chain polyamine analogues (Figure 27), respectively, can effectively inhibit LSD1 activity, leading to re-expression of previously silenced genes in tumour cells[86].

Studies demonstrate the pre-treatment of human colon cancer cells with ODC inhibitor DFMO (described in chapter 3.2.2.1.1), followed treatment with LSD1 inhibitors coupled with co-treatment by DFMO again. DFMO can be used to reduce intracellular polyamine concentrations. Thus, it was hypothesized that the reduction of the natural polyamines in cancer cells by pre-treatment with DFMO would enhance the epigenetic effects of oligoamine treatment through two mechanisms: The reduction of the natu-

ral polyamines would allow analogues to have greater access to their targets; and the reduction of natural polyamines would result in increased uptake of the oligoamines, thus, rapidly increasing the effective intracellular analogue concentration.
The results of the study had been an intracellular polyamine depletion, an increased accumulation of analogue, synergistic re-expression of an aberrantly silenced tumour suppressor genes and increased cell death through apoptotic mechanism[87].

It has also been demonstrated that the combination of DNA methyltransferase and HDAC inhibitors provides synergistic effects in. re-expressing epigenetically silenced genes in cultured cancer cells[88], and provides clinical responses in patients with leukaemias[89].

3.2.2.7 Polyamine/Anti-cancer Drug Conjugates

3.2.2.7.1 The Polyamine Transport System

Many tumour types have been shown to contain not only elevated polyamine levels, but also an activated polyamine transport system (PTS) for importing exogenous polyamines[60]. Thereby it is known that polyamine transport is carrier mediated, time-, temperature- and concentration-dependent, energy requiring, and saturable. A number of cell types have a single transport system for uptake of all three polyamines. However, in most cell types, two transport systems have been recognised. The first has preference for putrescine, although it is capable of transporting all three polyamines. The second is capable of transporting spermidine and spermine (being more selective to spermine), but not putrescine[90].

A Intracellular concentrations of free polyamines control the activity of such uptake system as well as the anabolic pathways [91]. Exploiting the transport mechanism, the presumed high affinity of polyamonium cations for DNA[92], the enhanced cellular need for these amine growth factors and an activated transport system for their import[60] it has been attempted to develop ways for specific cancer cell DNA targeting. Polyamine-

conjugates with different types of anti-cancer drugs has been in great interest of current research[92].

Moreover the vectorization of chemotherapeutic drugs by polyamines has been expected to enhance cytotoxicity to tumour cells and diminish secondary effects on normal cells. This is possible due to particular structural tolerances of the PTS, which allows for import of non-native polyamine constructions[93].

3.2.2.7.2 Approaches in Anti-cancer Conjugates for Clinical Use

It was found that presence of a polyamide side chains on intercalating agents such as anthracene or acridine derivatives does not disturb their intercalating capability as well as their topoisomerase inhibiting properties[91].

One of the first polyamine-anti-cancer drug conjugates investigated was the combination with chlorambucil. Chlorambucil, believed to elicit its cytotoxicity through the formation of DNA interstrand crosslinks that eventually lead to apoptosis[94], showed in conjugation with spermidine a 10^4 -fold increase of acylation and cross-linking of DNA while it retained its cytotoxicity and anti-tumour activity[92]. However, the activity of these conjugates greatly depends on their architecture: for example, the attachment of a chlorambucil moiety to spermidine improved its in vitro cytotoxicity when the nitrogen mustard moiety was bound to the secondary amine; in contrast, no improvement resulted from the linkage of chlorambucil to the C-5 of spermidine[95]. Cohen et al., also examined the attachment of nitroimidazoles to polyamines and again used the subsequent increase in toxicity induced by DFMO via increased transport into the cell[96].

The following paragraphs provide a short insight into the concern of current research:

The natural enediyene antibiotics are one of the most potent anti-cancer agents ever discovered. However, a major obstacle to successful clinical use is their lack of tumour selectivity. The partial success of gemtuzumab ozogamicin, an anti-CD33 monoclonal antibody conjugated to an enediyene antibiotic (calicheamicin) has shown great potential in acute myeloid leukaemia[97]. Thus, enediyenes has been equipped with all three polyamines and showed potent DNA damaging abilities[98].

Also the attachment of a polyamine vector to camptothecin, another enediyene derivative, and its related compounds have shown great potential (90 times more than topotecan – a camptothecin agent) in human non-small-cell lung cancer cell lines[99]. Tetracyclic amidines conjugated to putrescine have shown inhibition of tumour cell growth in lung cancer cell lines. Like the previously mentioned studies, these compounds successfully competed with their native polyamines for entry into the cell[100].

An amazing approach of Eiseman et al.[101] was to incorporate two small moieties of aziridine onto spermine. Aziridines are electrophiles, and are attracted to endogenous nucleophiles, like nitrogenous bases within DNA. The addition of spermine provides it with a targeted DNA cross-linking ability, making it an ideal alkylating agent, which shows potent cytotoxicity via the induction of apoptosis in distinct human prostate cell lines. Moreover, treatment with the conjugate prior to radiotherapy sensitised the cells, resulting in the decrease of apoptotic threshold to radiation.

A further anti-cancer agent connected with a polyamine tail has been F14512, a novel spermine-etoposide conjugate investigated by Barret et al.[102] (Figure 28). Etoposide, discovered in 1961, is still one of the first line agents in the treatment of various tumours. It possesses potent anti-cancer activity and is still one of the best inhibitors of topoisomerase II, whereas those effects are limited in vivo by its low cancer cell specificity[62]. It was evidenced that F 14512 uses the PTS, being eight times more potent than etoposide concerning cytotoxicity. The use of human breast tumour xenografts resulted in partial or complete regression of the tumour in a dose of 1,25mg/kg/injection. In comparison, etoposide needed to be dosed at 30mg/kg/injection to achieve the same results. However, even this reduced dose also

caused toxicity. This was the first report of a polyamine drug conjugate being used successfully[102].

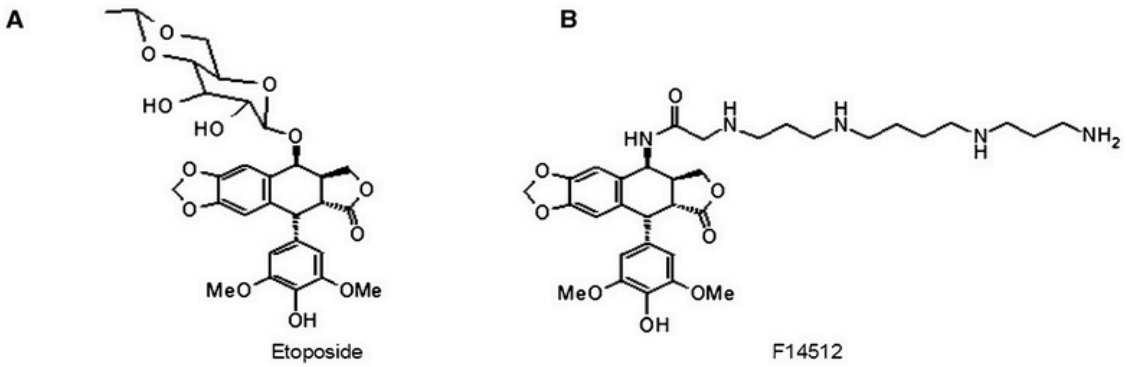


Figure 28: Etoposide and F14512 (Barret et al., 2008)[102].

3.2.2.7.3 Advances in creating optimal PTS-Ligands

In present research the challenge to design models that could help to identify the molecular recognition elements involved in polyamines transport and to delineate the structural tolerance accommodated by PTS is of great interest.

Cullis et al. established first generalizations for structural requirements:

- The principal determinant of the binding to the polyamine receptor appears to be the number of charges
- The spacing between charges alters the binding affinity - homospermidine derivatives bind tighter than norspermidine derivatives
- "Non-branched" conjugates are significantly more tightly bound than the corresponding "Branched" conjugates (compare figure 29; 1 and 7; 6 and 8)
- There is no absolute requirement for primary amino groups (compare 6 and 9; spermine and 10)[92].

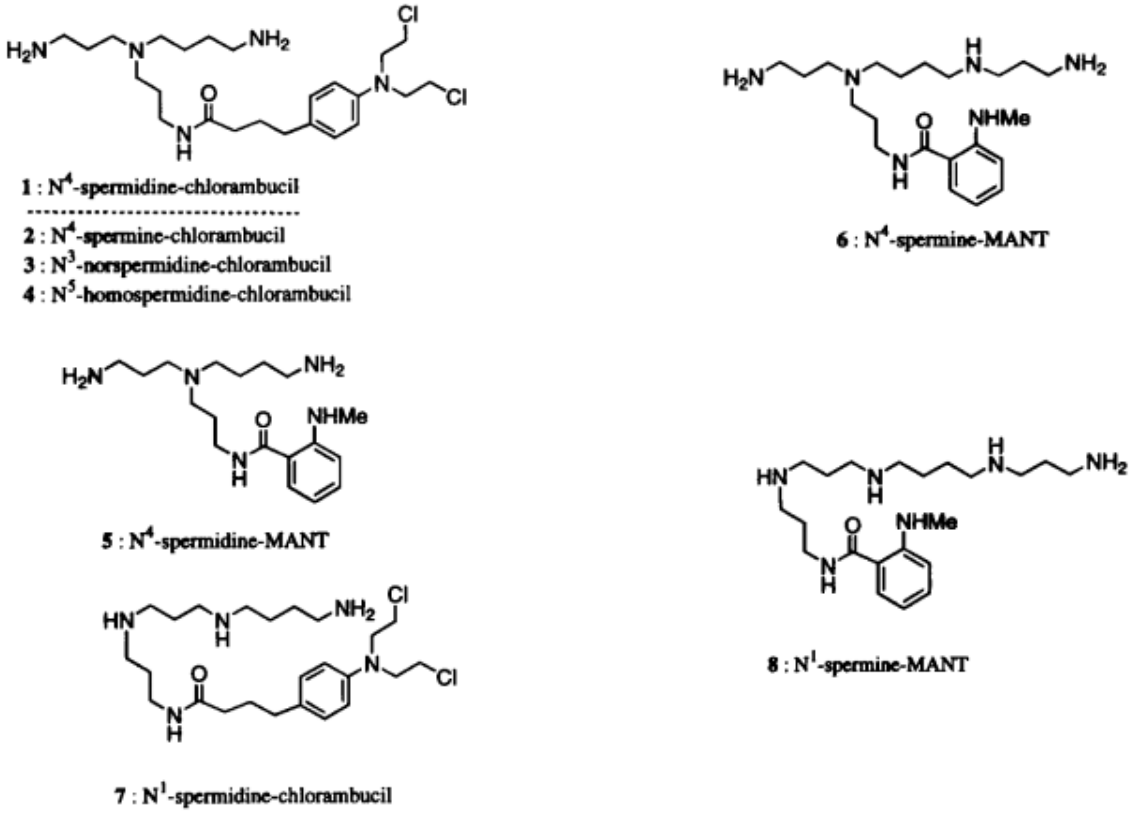


Figure 29: Some of the polyamine conjugates synthesized to probe the structural tolerance of the polyamine receptor (Cullis et al., 1998)[92].

Furthermore, Cullis and his group found that once inside the cell, these conjugates were not evenly distributed within the cytoplasm, but stayed within vesicle-like structures and do not readily associate with the nucleus. Given that the increased cytotoxicity of this conjugate is believed to be due to its increased DNA-binding ability, this was an unexpected finding. According to this it is interesting that internalisation by endocytic processes is the preferred delivery strategy for anti-cancer drugs. This avoids degradation by cellular components. Thus, it is possible that transport via PTS is providing another benefit offering the conjugate a degree of protection[103].

Phanstiel and collaborators[104] raised the question if polyamine conjugation with bulky DNA binding agents such as linear tricyclic systems (Figure 30; 5 and 6) may improve their selectivity by targeting the PTS. It was shown that the monoalkylated SPM motif present in 6 seems to be a better vector than its SPD homologue 5. The same issue was investigated by Delcros et al., 2002[91] with novel analogues consisting of SPM covalent-

ly bound to an acridine via an aminoaliphatic linker. The linker was placed to the 9-position of acridine, through either an amide bond (Figure 30; 8-10) or directly as the aniline (11-13). The architecture was based on the following observations: (1) N1 derivatives of polyamines show a higher affinity for the PTS than their N4; (2) The relationship between the number of protonated nitrogens along polyamines and their affinity for the PTS and for DNA.

By studying conjugates 8-13 it was found that selectivity will be reached if affinity of the drug for the PTS is higher than for any other transport system(s). It is, however, expected that the tumour selectivity of drugs delivered by polyamines will again need to be co-treated by DFMO to up-regulate the PTS activity of the tumour cells.
However, expected benefits expected were diminished by the ability of this conjugates to repress the activity of the PTS and thus, to inhibit their own transport[91].

In later studies two collections of polyamine conjugates were synthesized and screened for affinity for the PTS. The first collection consisted of several N-substituted linear or branched di-, tri-, or tetraamine scaffolds with different tether lengths between nitrogen centres. The N-substituents were N1-alkylarenes, with the alkyl spacer ranging from methyl to propyl and the aromatic nucleus from benzene to pyrene (Figure 31)[105].

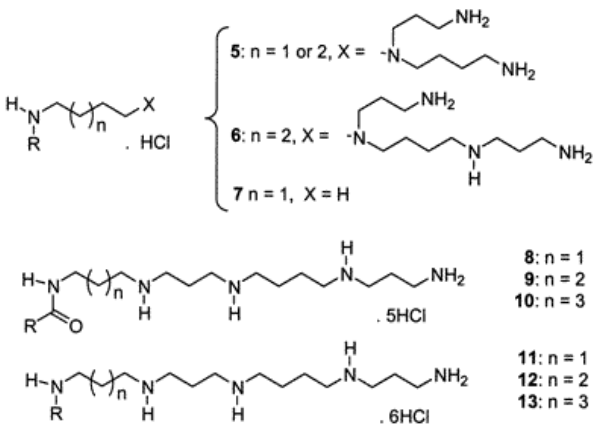


Figure 30: Phanstiel's conjugates (5−7), and Delcro's acridine−spermine conjugates (8−13) (Delcros et al., 2002)[91].

These conjugates helped to define key characteristics required for the selective delivery of polyamine−drug conjugates into cells via PTS: Triamine homospermidine appeared to be a highly selective vector motif to ferry an arenyl group into cells via the PTS.

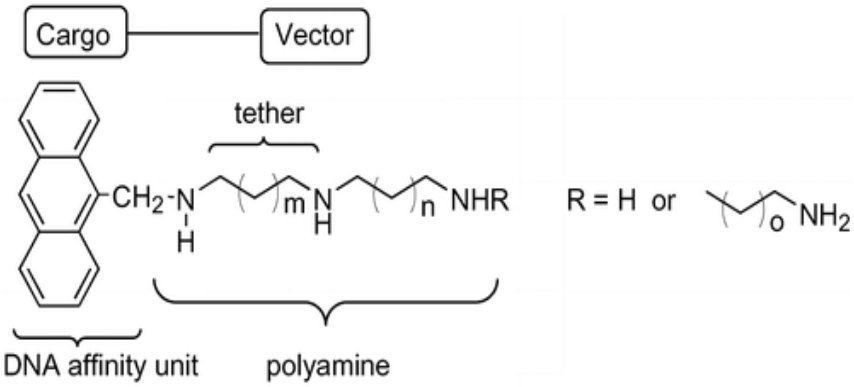


Figure 31: (Wang et al., 2003)[105]

Tetraamines conferred high PTS affinity for conjugates the drug wasn't more efficiant[95]. Thereby length of the tether connecting the polyamine chain to the arenyl group is crucial. N1-tethers longer than ethylene showed dramatic loss of selectivity for PTS. There are also clear limits to the size of N1-substituents, which can be accommodated by the polyamine transporter. A direct correlation was observed between polyamine-conjugate uptake and cytotoxicity. In this regard, a cytotoxicity model was proposed, which describes a hydrophobic pocket of set dimensions, adjacent to the putative PTS polyamine-binding site[93].

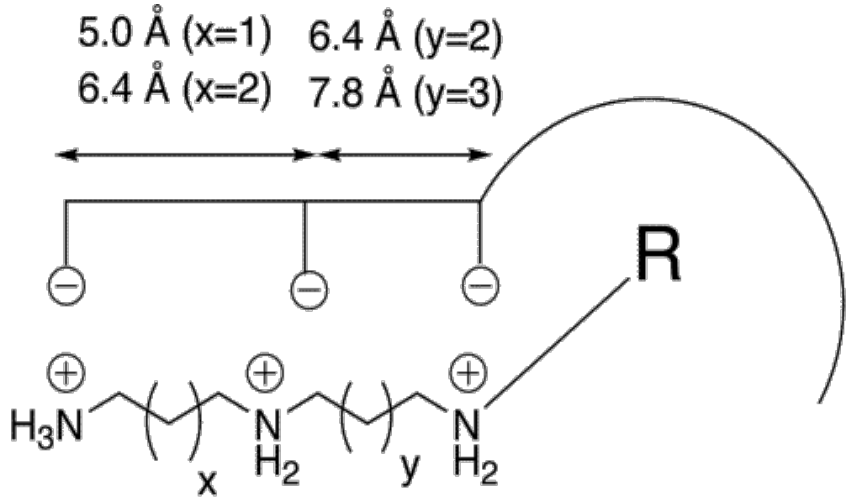


Figure 32: (Gardner et al., 2004)[93].

The model will be discussed from left to right. For the value of x either a propylene (x= 1) or a butylene fragment (x= 2) should be chosen for an excellent PST selectivity. Earlier studies provide optimal values for y (y= 2 or 3)[93]. In figure 32 the size of R defined by the fact that selective targeting of cells with active polyamine transporters can be further enhanced via a smaller, less hydrophobic naphthylmethyl unit[106]. Considering R would be a smaller group (e.g., naphthyl), one can lengthen the N1-tether to ethyl and still maintain high PTS selectivity. Considering a significant decrease in uptake and cytotoxicity associated

in lengthening the methyl tether to an ethyl tether, one can estimate the hydrophobic pocket of the PTS (which accommodates R). It is supposed to be near the sweep volume of an anthracenylmethyl group. A smaller group can still be accommodated by an adjacent hydrophobic pocket of set dimension. However, if the R group is large like the anthracenyl, small distortions in the N1-tether result in a complete loss of PTS selectivity[95].

For the second collection Delcros et al.[95] tested five sets of heterocyclic derivatives of various sizes and complexities coupled by an amidine to determine the influence of the polyamine chain to the quality of cellular transport.

The design of these conjugates includes tetrahydroquino-benzazepine (Figure 33; 4) and tetrahydrobenzo-naphthyridine (5), with their exocyclic nitrogen substituted by alkyl-, hydroxylalkyl- or aminoalkyl-bearing side chains. These conjugates provides significant in vitro cytotoxicity on ovarian carcinoma cells and in vivo anti-tumour activity when given in association with DFMO[107]. The study established that the tetrahydrobenzo-naphthyridine 5b shows a greater overall DNA-binding affinity than its isomeric counterpart 4b.

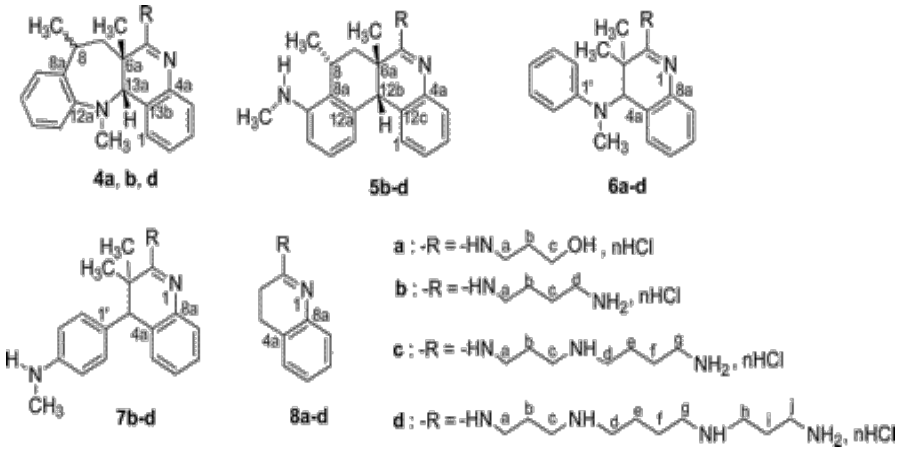


Figure 33: Heterocyclic derivatives (Delcros et al., 2006)[95].

The affinity for DNA varied as follows: spermine (tetraamine) > spermidine (triamine) > putrescine (diamine). Increasing the number of amino groups on the side chains in all series enhanced the affinity for double-stranded DNA. The tetracyclic conjugates 4a, 4b, and 5b bind only weakly to DNA via the minor groove rather than through an intercalative process.

Putrescine and spermidine, conjugated to bulkier substituents (e.g., 4−7b, c) were not taken up by the PTS. Thus, it was confirmed again that large substituent would not fit in the hydrophobic pocket of set dimensions, adjacent to the PTS polyamine binding site[95].

Breitbeil III et al. presented a modelling study of the PTS shape preferences of the motuporamines (Figure 34; 1-4), their mimic (5), and potential new mimics (7). Each of compounds 1-3 consists of a large hydrophobic heterocycle connected to a polyamine motif. Compounds 1-3, 4a, 5, 6a and 7a have 3,3-triamine sequences, while 4b, 6b-d and 7b have 4,4-triamine sequences. 4a was shown to have high cytotoxicity against in vitro breast cancer cells and good anti-invasive properties with rumour cells[108]. 5 was shown to have good anti-invasive properties[109]. It was assumed that an alternative architecture like 7 could elicit the same response as 4a, due to its similarity to carbazole 5, which act akin to 4a. Concerning affinity to PTS, this study showed again that the methylene spacer units (Figure 34; 6:x, y, z), the size of the N1-substituent, and what is new, the degree of N1-substitution all influence PTS mediated delivery[108]. Further, dihydromotuporamine C adducts (4a and 4b) and N1-ethyl, N1-antracenylmethyl-homospermidine 7b do not use the polyamine transporter for cellular entry[110]. PTS selectivity and motuporamine mimicry are related. For example, N1-ethylation of 6b (a highly selective PTS ligand) creates 7b, which is not PTS selective. In addition, N1-

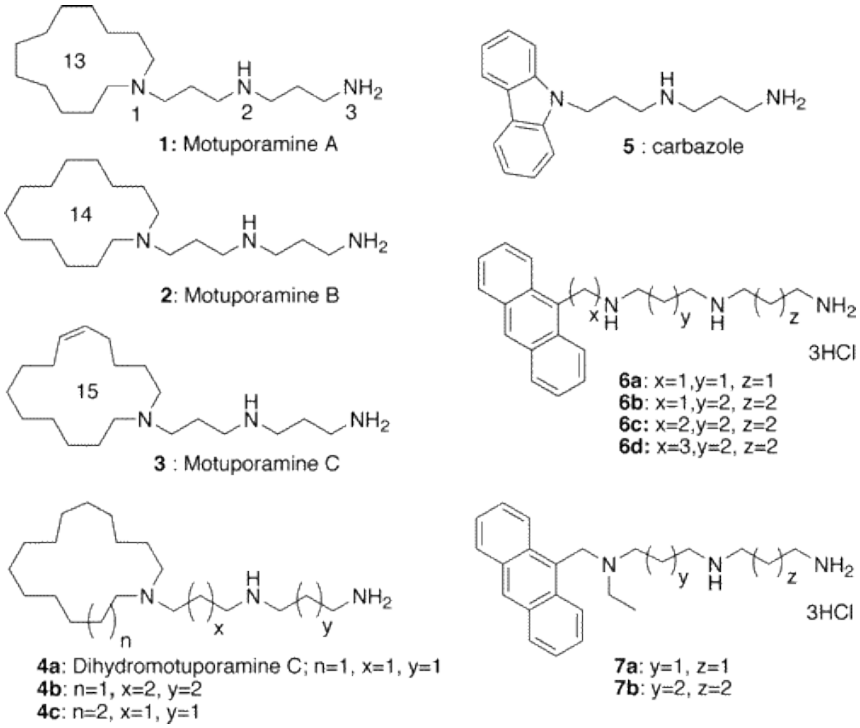


Figure 34: Structures of motuporamines A–C (1−3) and polyamine derivatives 4−7 (Breitbeil III et al., 2006)[108].

ethylation of 6a creates 7a, which has similar shape and initial biological activity as dihydromotuporamine C (4a). Thus, N1-ethylation was shown to be responsible for both loss of PTS selectivity and inducement of 4a mimicry[108].

In another study Phanstiel et al. used a derivative of aminoacridine, amsacrine, which acts by inhibiting topoisomerase II and is utilized in the treatment of acute leukaemia[62].

Thereby lengthening of the polyamine tail (Figure 35; 2) by an additional aminopropyl group directly enhanced the potency of the altered conjugate (16). These results suggest that the monoalkylated tetraamine motif (16) may be the best vector to access the PTS. The three-ringed structure of aminoacridine is believed to be responsible for the ability of amsacrine to inhibit topoisomerase II.

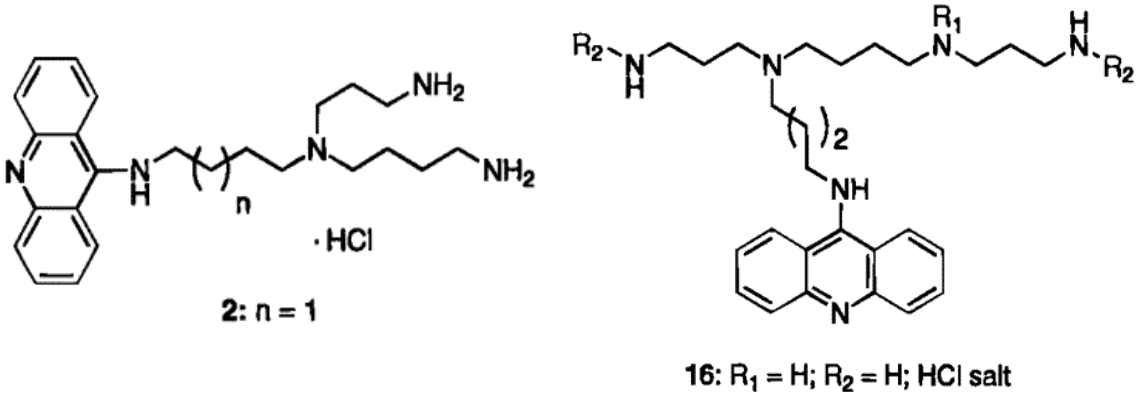


Figure 35: (2) Acridine derivative with polycationic tail (16) Lengthening of the polyamine tail of 2 by an additional aminopropyl group (Phanstiel et al., 2000)[152].

As such, the use of the linear three-ringed aminoacridine and anthracene was choice for the intercalators. Both demonstrated increased DNA-binding ability and inhibition of topoisomerase II when attached to polyamines and both utilize the PTS for uptake[111]. Delcros, however, found that despite the spermine-aminoacridine conjugates having a high affinity for the PTS, uptake was low[112]. Phanstiel also demonstrated that spermidine anthracene conjugates, although being more cytotoxic, had a lower affinity for the PTS than the spermine anthracene conjugates[113]. This can be explained by the fact that whereas spermine conjugates bind tightly to the cell, they not gain

entry, thus explaining their apparent higher affinities. They reasoned that the increased positive charge of the spermine vectors resulted in tighter binding to other components of the cell membrane, resulting in slowing of their transport[106].

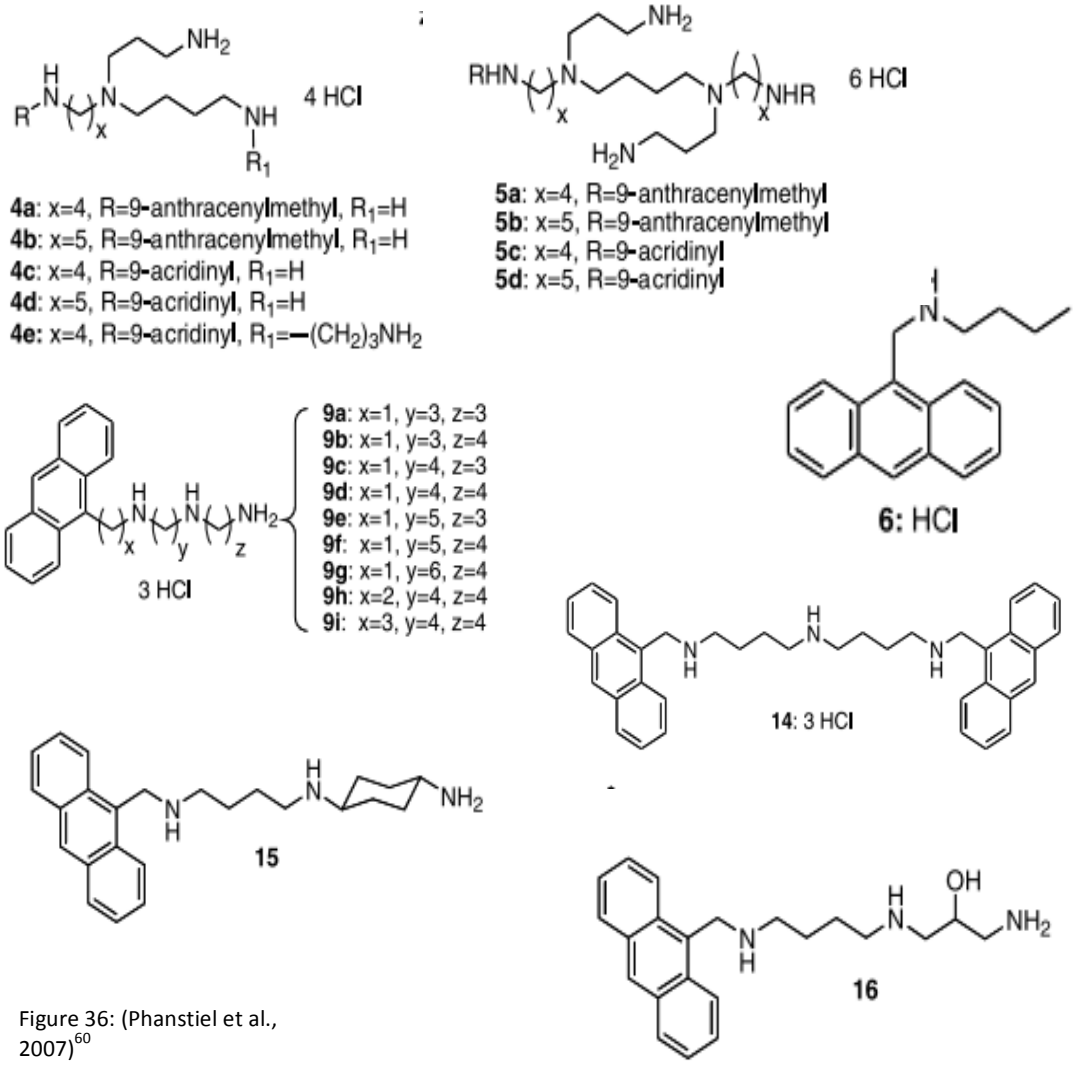


Figure 36: (Phanstiel et al., 2007)[60]

In terms of the regiochemistry of the N-substituent, Phanstiel et al. designed branched polyamines (Figure 36; 4) due to the former observation that N4-benzyl-spermidine was well recognized by the PTS. Acridine and anthracene were chosen as polycyclic aromatic nuclei. Initial evaluations of those branched systems (4a – 4e) demonstrated that the anthracenyl compounds (4a-4b) were more cytotoxic than acridinyl conjugates (4c-4d). However, bis-substituted adducts 5a-d containing large terminal N-alkylaryl groups, did not use PTS for their cellular entry. Thus, monosubstituted poly-

amines were identified as preferred ligands for the PTS. It was also found that neither substitution at both terminal ends of the polyamine (e.g. 14) nor at the N^5-position (e.g., 6) provided PTS selectivity. Terminally monosubstituted, linear polyamine motifs (e.g. 9d) showed dramatic differences in cytotoxicity: suggested that PTS targeting is influenced by both the polyamine sequence and the location of the N-substituent, terminal aminobutylation of 4,4-triamine (9d) to give 4,4,4-teraamine significantly reduced its cytotoxicity. This happens due to the phenomenon, wherein tetraamines indiscriminately bind to cell surface receptors, destabilize the membrane and possibly inhibit their own import. Alteration of the triamine sequence in 9a-91 demonstrated a superior PTS targeting in the (4,4)-, (5,4)-, and (4,3)-triamine compounds (9d, 9f, 9c). Overall homospermidine (4,4-triamine sequence) was optimal.
Subtle changes in the distances surrounding the N1-position also resulted in dramatic changes in cytotoxicity. For example, maintaining the terminal anthracenylmethyl moiety, but altering the central alkylene tether length sequentially from butylene (9d), pentylene (9f) and hexylene (9g) resulted in decreasing PTS-selectivity and less toxicity[60].

Prior work by others demonstrated that conformational-restricted polyamines can significantly alter cytotoxic response. Introduction of cis and trans butane spacers instead of the more flexible, internal butylene chain generated tetraamines, which were less cytotoxic to cells than their aliphatic parent[114]. Thus, Phanstiel designed a 1,2-cyclohexylene adduct (15) which showed to be significantly less PTS selective than the free butylene adduct 9d.
Additionally compound 16 revealed that a hydroxylated spermidine motif results in a dramatic loss of PTS selectivity, compared to its parent 4,3-triamine 9c[60].

A further vector is provided by Tomasi's benzazepine- and azepine-conjugates. The amidin function leads to rapid access of various chain moieties and allows keeping of the positive charge in comparison with amid derivatives. Both families are PTS targeting compounds. The azepine-spermidine derivative 14 (Figure 37) is a very specific substrate of PTS, which accumulates various isotopes for cancer treatment. The nitrobenzazepine-spermine 28 prevents the reversion of the DFMO-induced cytostatic effect by

exogenous polyamines at physiological concentrations. This may serve as an adjuvant in DFMO anti-cancer therapy, which is seriously impaired by the exogenous polyamines, that are imported via PTS[115].

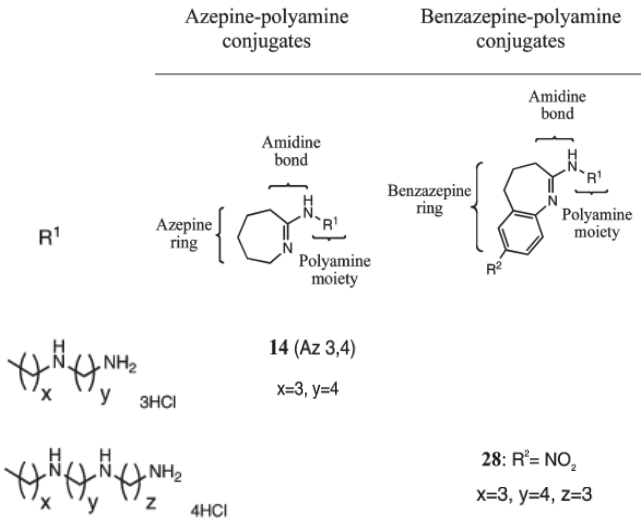


Figure 37: Tomasi et al., 2010[115].

Based on the former work of Phanstiel's putrescine-anthracene conjugates (Ant 4), Palmer et al.[116] demonstrated for the first time an uptake via the PTS in human cell lines. Thereby it was found that these molecules have additional effects outside their original design (delivery) by depleting the polyamine pool. This may occur due to polyamine analogue like actions. Additionally the compounds were found to trigger cell death via the induction of apoptosis, which might be caused by DNA damage and oxidative stress[116]. Recently Phanstiel et al.,[117] linked a homospermidine and homospermine to an anthracene (Ant-4,4 and Ant-4,4,4, respectively) and established that regardless of the cell line used, all responded with Ant-4,4 showing great promise. Small concentration of Ant-4,4 induces significant decreases in cell number of treated cell lines. Indeed, Ant-4,4 effects were seen to be significantly more potent than Ant-4 under the same conditions. On the other hand HL-60 growth inhibition in response to Ant-4,4 treatment was accompanied by a reduced intracellular polyamine content, an effect also seen with Ant-4. The reason why these drugs are interfering with polyamine metabolism remains unclear. One could speculate that feedback mechanisms are being activated by the polyamine backbone to compensate for the uptake. By means of removing Ant-4,4 it was shown for the first time that treated HL-60 cells are able to recover from Ant-4,4 treatment. Drug-induced cytotoxicity was being exerted in the first hours but gradually disappeared over time. Moreover an increase in total cell number as well as protein content, associated with an increase of polyamine concentration, were observed over time up to 96 h. It have been also suggested that Ant-4,4 induces a decrease in G1-S phase transition, i.e., cell cycle perturbation, of the treated cell line, resolved after

drug removal. Anti-tumour effects were also established via activation of apoptotic cell death, the accumulation of ROS and subsequent oxidative stress[117]. Furthermore the Ant-4 study of Palmer et al. agree with this recent findings[116]. For the first time, it was shown that despite being an efficient way of delivering anthracene to cancer cells more effectively, the Ant-4,4 conjugate does not exert irreversible harmful effects[117].

3.2.3 OLIGONUCLEOTIDES

This further class of major groove binders is discussed within chapter 3.4.1

3.2.4 INTERCALATORS

Functional intercalators, those capable of disrupting DNA metabolism, bind to DNA through a non-covalent interaction that requires at least partial planarity, which is facilitated by the formation of at least one hydrogen bond. Considering only these general and minimum requirements, thousands of compounds might be suitable therapeutic agents. Only a few natural intercalating products reach the anti-tumour drug status because of vast side effects associated with less specificity. Apart from that, research has also been concentrated in design of new structures – developed due to improved knowledge about certain DNA topology and the structural requirements for molecules, binding on refined specific DNA targets, respectively.

3.2.4.1 NOVEL ANTHRACYCLINE DERIVATIVES

The activity and toxicity of most commonly used anthracyclines are meant to indicate that a better anthracycline has yet to come. It is therefore not surprising that relatively old drugs like DOX and DNR remain the focus of clinical and preclinical research, aimed at improving our appraisal of their mechanisms of activity or toxicity and at identifying new strategies for better use in cancer patients[20].

3.2.4.1.1 New Anthracyclines with clinical Approval

Only a few more anthracyclines have attained clinical approval; these include pirarubicin and aclarubicin (Figure 38).

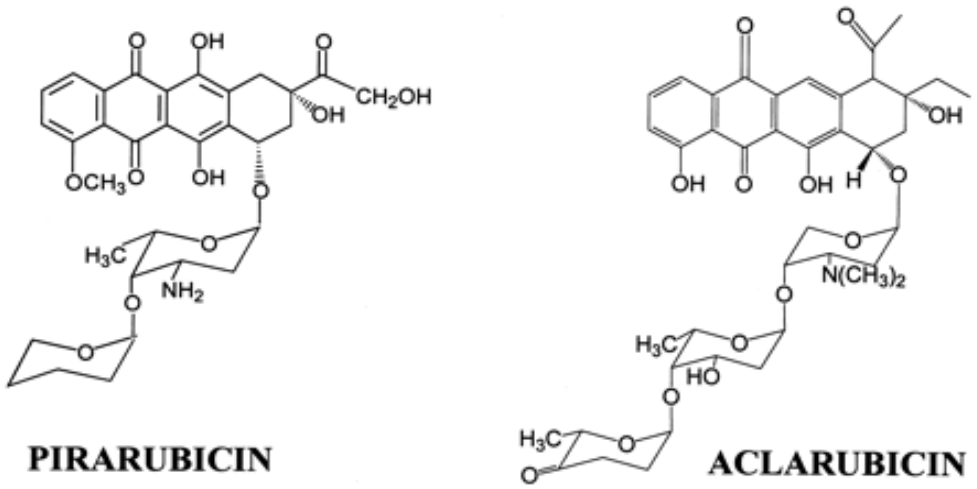


Figure 38: Structures of pirarubicin and aclarubicin (G. Minotti et al., 2004)[20].

Both pirarubicin and aclarubicin demonstrate only modest improvements over DOX and DNR in terms of drug resistance. Pirarubicin, a 4-tetrahydropyranyl doxorubicin, has been reported to induce less cardiotoxicity than DOX in animal models. However, studies in women with metastatic breast cancer have indicated that it may cause significant decrease in LVEF or CHF at cumulative doses of 460 mg/m^2 or >500 mg/m^2, respectively.

Aclarubicin, a trisaccharide anthracycline[20], was found to be topoisomerase II catalytic inhibitor and therefore induces DNA damage during the S phase by preventing relaxation of supercoiled DNA ahead of the replication fork. A comparison of the structure of DOX and aclarubicin shows, the latter contain two additional sugar moieties. These sugars block topo association with the minor groove of DNA[118].

Aclarubicin was shown to be active and cardiotoxicity-tolerable in adult patients with acute myeloblastic leukaemia. However, it induced late cardiac events in a phase II study of adult patients with refractory acute myelogenous or lymphoblastic leukaemia and proved to be inactive in women with metastatic breast tumours[20].

3.2.4.1.2 ANTHRACYCLINE-FORMALDEHYDE CONJUGATES

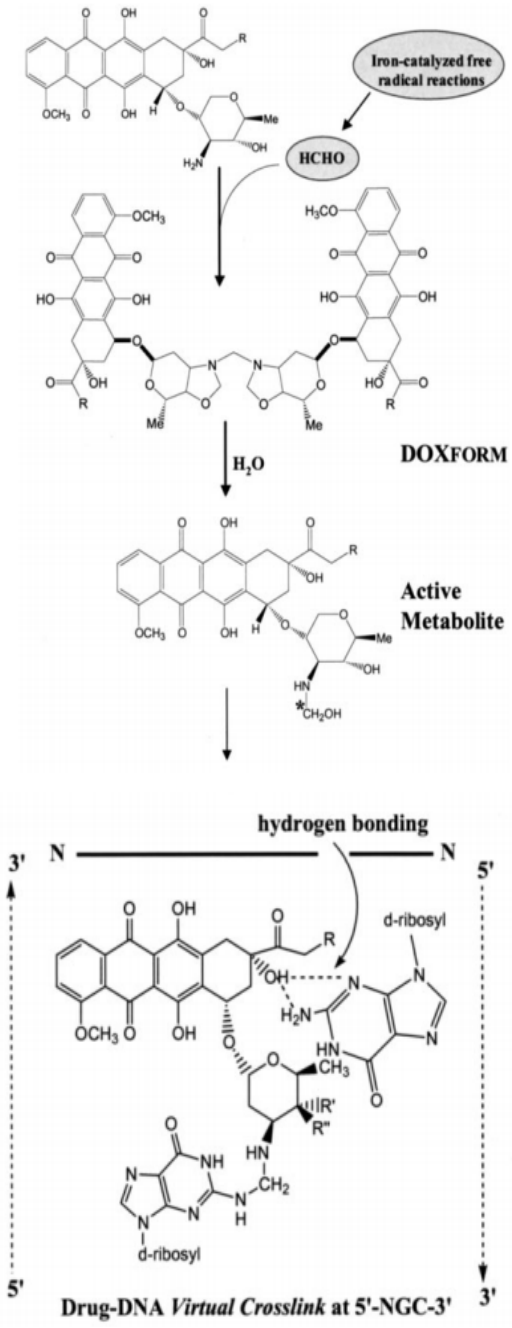


Figure 39: Formation of DOXFORM, its hydrolysis to active metabolite, and mechanisms of drug-DNA virtual cross-link. DOX reacts with HCHO.Two molecules of DOX bind via three methylene groups, two forming oxazolidine rings and one binding the oxazolidines together at their 3'-amino nitrogens. Similar reactions occur with EPI or DNR. DOXFORM then hydrolyzes to give a monomeric metabolite in which the carbon of HCHO (asterisk-marked) is recovered bound to the aminogroup at C-'3 in daunosamine. In this mechanism two DOX are each covalently bonded to a 2-amino group of a G-base on one strand of DNA via a methylene group and hydrogen bonded to a G-base on the opposing strand, virtually cross-linking the DNA. R = -OH (DOX, EPI) or -CH3 (DNR). (Minotti et al., 2004)[20].

Anthracyclines interact with DNA within two ways of covalent bonding: more stable drug-DNA cross-links and less stable drug-DNA adducts[38]. Studies have shown that redox reaction of free radical reactions allow anthracyclines to produce formaldehyde (HCHO) from endogenous carbon cellular sources like spermine and lipids. Anthracyclines, like DOX, and HCHO form a conjugate (DOXFORM) in which two anthracycline molecules bind together with three methylene groups: two forming oxazolidine rings and one binding the oxazolidines together at their 3'-amino nitrogens (Figure 39)[20]. DOXFORM eventually hydrolyses to give an active monomeric metabolite in which the carbon of HCHO is recovered in the form of a Schiff's base at the amino group of daunosamine. Similar reactions take place by EPI and DNR but not with anthracyclines lacking a 3'-amino group.

The interest of such conjugates lies in their unique ability to intercalate into DNA by covalent bonding of the Schiff's base with the 2-amino group of a G-base in the minor groove of DNA. If the interaction with DNA occurs at the trinucleotide 5'-NGC-3', then the drug intercalates between N and G and covalently bonds to the G-base on one

strand using HCHO, and to the G-base on the other strand, using hydrogen bonds. The benefits of such conjugates are huge: improved activity in both anthracycline-sensitive cells and resistant cells (due to overexpression of P glycoprotein or reduced redox mediating enzymes (NADPH)). Advances concerning Pgp-overexpressing tumours were based on two factors: HCHO-dependent reduction of the pK_a of the protonated amino residue of anthracyclines, making this residue unprotonated at physiological pH and thus, decreasing anthracycline affinity for Pgp. Moreover the binding of anthracycline-FORM conjugates to DNA in competition with Pgp happens much faster. In agreement with such expectations of improved activity, both DOXFORM and EPIFORM or DNR-FORM were shown to exhibit enhanced toxicity to anthracycline-sensitive and -resistant tumour cells, in combination with increased DNA targeting of the conjugates, accumulation in DNA, extended cellular retention, and reduced cellular release of anthracyclines.

In a recent program of NCI EPIFORM has shown to be more active than EPI. Of note, EPIFORM significantly exceeded the toxicity of DOX or EPI to the most resistant breast cancer cell line and to the most resistant prostate cancer cell line. EPIFORM also proved to be more active than EPI in efficacy trials, conducted in a mouse mammary carcinoma model[20].

More evidence for the improved activity of anthracycline-FORMs can be investigated in the review of Minotti et al., 2004[20].

3.2.4.1.3 Anthracyclines/Telomerase-Inhibitor Combinations

As already mentioned telomeres are the ends of linear chromosomes of eukaryotic cells and provides protection of chromosomes from degradation and ligation. The length of telomeres is primarily controlled by telomerase, an enzyme composing of a catalytic protein subunit (telomerase reverse transcriptase), a telomerase-associated protein, and a stably associated RNA moiety, which acts as an intrinsic template for the

elongation of telomeres. In contrast to normal cells, telomerase is activated in the majority of cancer-derived cells and was found to be the key to cell immortalization, tumourigenesis or tumour aggressiveness.

Due to the facts that telomeres accumulate single-strand breaks and shorten faster after exposure to agents inducing oxidative stress and DNA damage a combination of telomerase inhibition with anthracycline treatment has been considering a new option for improved cancer treatment. It was expected that combining DOX and telomerase inhibitor(s) resulted in additive or synergic effects in telomerase-positive/anthracycline-sensitive cells. And truly, DOX induced more apoptosis in breast cancer cells in which telomerase had been muted with telomerase inhibitors and formed more DNA double-strand breaks in neoplastic cells derived from telomerase RNA-null mice.

Present research also concerns variation in types of telomerase inhibitors. Preclinical screens promise telomerase inhibiting actions of a range of natural compounds, e.g., telomestatin (a natural product isolated from *Streptomyces annulatus*) or epigallocatechin gallate (a major tea polyphenol).

These results clearly illustrate telomerase inhibition as a novel therapeutic approach in combination with DOX and other anthracyclines[20].

3.2.4.1.4 Anthracyclines in Combination with Antioxidants

Another way to decline cardiotoxicity has been developed by utilizing agents that improved the antioxidant defences of cardiomyocytes against anthracycline-derived ROS. Most frequently used antioxidants includes probucol - a lipid-lowering drug; piperidine nitroxides; lipophilic spin traps; melatonin; vitamins (A, E, and C) and thiol-containing reducing agents like GSH or *N*-acetylcystein[119]. These compounds did not interfere with anthracycline activity in tumour cells, raising hopes for the improvement of the therapeutic index of anthracyclines. Clinical studies, however, failed in documenting a

cardioprotective efficacy of antioxidants like vitamin E or *N*-acetylcysteine. Therefore promising results have been obtained with rutoside-type flavonoids. In some studies, flavonoids were shown to act by exerting direct positive inotropism[120]. It is believed that they act by scavenging free radicals generated by DOX and/or by chelating iron, which was found be able to mediate the redox reactions of ROS and peroxide radicals, which again provide proapoptotic effects. Moreover flavonoids were shown to inhibit carbonyl reductases, which converts DOX to DOXol, a secondary alcohol metabolite, which is even several times more potent concerning cardiotoxicity compared with DOX. Conversely, however, flavonoids were also shown to stimulate DOXol formation in human heart extracts. Thus, the mechanism of action of flavonoids in laboratory animals might therefore be more complex than previously believed[20]. Thus, further investigations are needed to make final statements of a clinical application.

3.2.4.1.5 Iron Chelators

Back in the 1970s, dexrazoxane was found to prevent histologic lesions and contractile dysfunction induced by anthracyclines in laboratory animals or isolated heart models. Dexrazoxane did not interfere with DOX distribution, metabolism, or excretion, nor did it appear to reduce the anti-tumour potency of anthracyclines. It was leading to limited damage on mitotically active tissues (bone marrow, testes, and gastrointestinal epithelia) and reduced both acute and chronic cardiotoxicity in all animal models tested. Dexrazoxane is a bisketopiperazine, which undergoes stepwise hydrolysis of the two piperazine rings to form one-ring-open-intermediates that in turn eventually hydrolyses, resulting in a diacid-diamide (ADR 925). The latter is structurally similar to EDTA and thus, it chelates iron bound to low-molecular-weight cellular ligands or coordinated within 3:1 anthracycline/Fe complexes (Figure 40). This unique pattern of cellular diffusion anticipates rapid formation of ADR 925 inside the heart and plasma[20]. Recent studies, however, show that it would not contribute to protecting the heart because of its limited access to iron pools in critical cellular compartments or impaired cellular uptake caused by complexation with calcium[121].

Originally it was investigated as an anti-cancer agent, and it functions as a catalytic/noncleavable complex-forming inhibitor of topoisomerase II, and thus priming leukemic cells to differentiation or apoptosis – therefor it has been devised for optimizing its use as a single active agent or cardioprotective agent in combination with DOX, which was approved by the FDA[20].

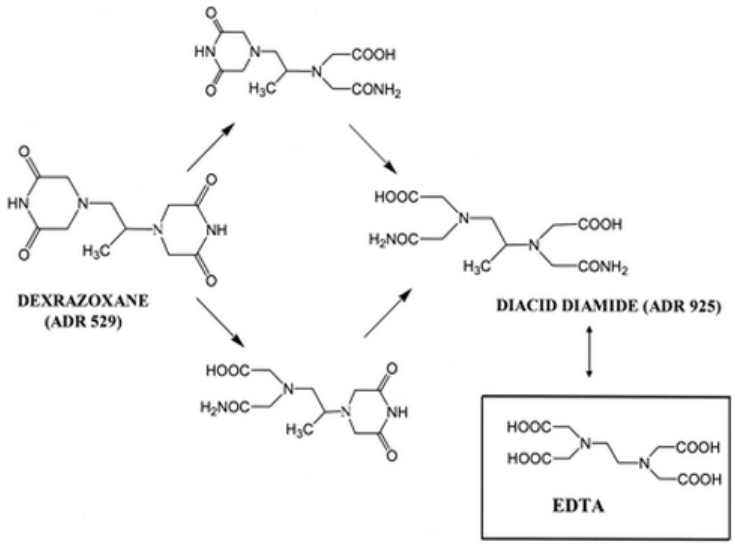


Figure 40: Two-step hydrolysis of dexrazoxane to an EDTA-like diacid diamide. (Minotti et al., 2004)[20].

3.2.4.1.6 Liposomal Formulations as Carriers of Anthracyclines

Compared with micro-spheres or nanoparticles, liposomal incorporation of drugs represents the leading method to passively target anthracyclines to tumours. Liposome-encapsulated anthracyclines are characterized by:

- Reduced V*d* and CL and extended half-life;
- Limited conversion to aglycones or secondary alcohol metabolites
- Preferential accumulation in areas (like tumours) characterized by discontinuous "leaky" microvasculature or in organs containing the macrophages of the reticuloendothelial system (liver and spleen)

- Prolonged release of the drug within the tumour environment
- Partial circumvention of Pgp-mediated tumour resistance, and
- Limited accumulation in healthy tissues (like the heart) with a normal endothelial barrier[122].

Attainment of such numerous goals depends on the interplay between the vesicle size of the liposom, its rate of plasma clearance, and the stability of the liposome-drug association in the bloodstream or in the tumour microenvironment. The tumour microenvironment contributes to destabilizing the lipid carrier through the action of the slightly acidic pH of interstitial fluids, the release of lipases from dying tumour cells, and the release of enzymes and oxidizing agents by tumour-infiltrating inflammatory cells. However, the exact drug releasing mechanisms remains still unclear.

Benefits in the pharmacokinetics of liposomal DOX, resulting in a delivery of greater quantities of the drug to tumour cells and may also affect patients refractory to previous anthracycline treatment. Partial reduction of drug resistance has been observed too when liposomal DOX or DNR were given to cell cultures.
The three main liposome-encapsulated anthracycline formulations that have been assessed in experimental models and clinical settings and found to be superior to free DOX in terms of efficacy and cardiac tolerability are a sterically stabilized polyethyleneglycol-coated formulation containing DOX (Doxil), an uncoated formulation in which citrate is included for increasing DOX encapsulation above the levels predicted by the maintenance of a transmembrane pH gradient (TLC D-99), and liposomal DNR (DaunoXome)[20].

POLYETHYLENGLYCOL-COATED ("PEGYLATED") LIPOSOMAL DOXORUBICIN (PLD)

The liposomal anthracycline causes less nausea, vomiting, alopecia, and stomatitis than conventional DOX. However, a frequent adverse event occurring with PLD is skin toxicity, consisting of a hand and foot syndrome (palmar-plantar erythrodysesthesia syndrome), which depends on the given dose and doses interval.

PLD is active by a fail of first-line treatment of ovarian cancer with platinum and paclitaxel and has shown good activity also in AIDS-related Kaposi's.

A study of the distribution PLD showed a significantly higher accumulation of the drug in brain tumour lesions than in the normal brain, showing that liposomal DOX overcomes the blood-brain barrier in the tumoural areas[20].

PLD leads to less cardiotoxicity compared with conventional DOX[123], releasing very little free DOX in cardiomyocytes. Moreover cardiac safety of high-dose PLD is consistent[20].

UNCOATED CITRATE-CONTAINING LIPOSOMAL DOXORUBICIN

This formulation has pharmacokinetic parameters less pronounced than PLD but still significantly more favourable than those of free DOX. A principal indication of uncoated citrate-containing liposomal DOX is metastatic breast cancer. When used as first-line therapy, this formulation gave the same objective response as conventional DOX but significantly lower cardiotoxicity. Other toxicities of DOX are also reduced by the liposomal formulation, as well as palmar-plantar erythrodysesthesia.

LIPOSOMAL DAUNORUBICIN

The pharmacokinetics of liposomal DNR also more favourable compared with free DNR. DaunoXome has been approved by the FDA as a first-line agent of AIDS-related Kaposi's sarcoma. Moreover it shows activity and tolerability as a single agent or in combination with cytarabin for refractory or relapsed acute myeloblastic leukaemia. In combination with dexamethasone (for multiple myeloma treatment) and cyclophosphamide/vincristine/prednisolone (as salvage therapy in poor-prognosis non-Hodgkin's lymphoma), improved cardiac tolerability and activity in both solid and haematological malignancies have been investigated.

This, raise hopes for broader clinical applications of DaunoXome in the near future.

IMMUNOLIPOSOMES

Within immunoliposomes the specificity of whole monoclonal antibodies or FAB' fragments with the favourable pharmacokinetics and drug delivery of liposomes are com-

Carrier	Conjugate	Investigational or Approved Indications
Liposomes		
Pegylated (Doxil - Caelyx)	–	Ovarian cancer, AIDS-related Kaposi's sarcoma, multiple myeloma, high grade gliomas, glioblastoma or secondary brain tumors, head and neck squamous cell cancer, breast cancer, squamous cell carcinoma of the cervix
Uncoated, citrate-containing [Evacet - Myocet (TLC D-99)]	–	Breast cancer
Uncoated (DaunoXome)	–	AIDS-related Kaposi's sarcoma, acute myeloblastic leukaemia, multiple myeloma, non-Hodgkin's lymphoma, breast cancer
Immunoliposomes		
Pegylated or uncoated	Fab' fragments of trastuzumab	Breast cancer
Pegylated	anti-MUC-1 monoclonal antibody	Breast cancer
Pegylated	anti-CD19 monoclonal antibody	Multiple myeloma
Pegylated	Anti-GD(2) ganglioside whole antibodies	Neuroblastoma
Pegylated	Anti-aminopeptidase-N NGR peptide	Neuroblastoma
Peptides or copolymers		
L-377,202	*N*-glutaryl-[4-hydroxyprolyl]-Ala-Ser-cyclohexaglycyl-Glu-Ser-Leu	Prostate cancer
CPI-0004Na	(*N*-Succinyl-Ala-Ala-Leu)	Breast cancer, prostate cancer
Uncoded	Integrin-targeted RGD-4C peptide + plasmin-targeted (Ala-Phe-Lys)	Fibrosarcoma
PK2	tetrapeptide linked to to galactosamine-containing *N*-(2-hydroxypropyl) methacrylamide copolymers	Primary or metastatic liver cancer

All formulations contain DOX, except DaunoXome, which contains DNR.
MUC-1, mucin-1 antigen.

Table 1 taken from Minotti et al., 2004[20].

bined. The conjugation of uncoated or pegylated liposomes to Fab' fragments of trastuzumab is used to create immunoliposomes targeting HER2/neu-overexpressing cancers. Anti-HER2/neu liposomal DOX selectively binds to HER2/neu-positive cells, benefiting internalization of DOX in the target cells, together with less accumulation of liposome-encapsulated DOX in the interstitium. In animals xenografted with human breast cancer cells, anti-HER2/neu PLD showed higher anti-tumour efficacy than DOX, reduced systemic toxicity, and the same extended circulation of nontargeted PLD without any apparent drug leakage or monoclonal antibody dissociation[20].

More such conjugations and a short summary of the given examples and its indications are listed in the following table.

All such findings clearly call for further refinements in investigational settings, especially with reference to an improved cardiac safety of antiHER2/neu PLD versus conventional DOX/trastuzumab regimens, but their development in clinical studies is highly attractive[20].

3.2.4.1.7 EXTRACELLULAR TUMOUR-ACTIVATED PRODRUGS (ETAP)

Additional liposomal preparations involve the synthesis of prodrugs that are unable to enter normal cells but are proteolytically activated by peptidases secreted of cancer cells. An example is L-377,202, a prodrug obtained by covalently linking DOX to *N*-glutaryl-[4-hydroxyprolyl]-Ala-Ser-cyclohexaglycyl-Glu-Ser-Leu (Figure 41). In the presence of prostate cancer cells secreting the serine protease prostate-specific antigen (PSA), the peptide moiety of L-377,202 is hydrolysed to release DOX or Leu-DOX, the latter being freely diffusible and activated to DOX inside the target cells. Thereby the new agent is several times more active than conventional DOX in human prostate cancer xenografts while also inducing less systemic or cardiac-specific toxicity[20].

Another ETAP with well prospects of clinical development is CPI-0004Na (*N*-succinyl-β-Ala-L-Leu-L-Ala-L-Leu-DOX), which does not undergo hydrolysis in blood or does it enter the cells. Activation occurs through tumour peptidases to give *N*-(L-Leu-DOX), which eventually diffuses inside the cells as described for PSA-hydrolysed L-377,202. In mice it was found to be less toxic than DOX due to its lower accumulation in heart and other normal tissues[124]:

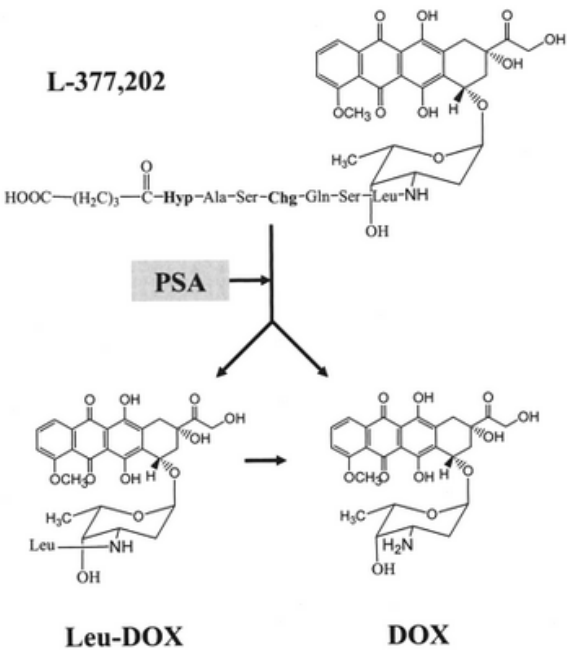


Figure 41: PSA-dependent hydrolysis of L-377,202 (Minotti et al., 2004)[20].

The newest member of the ETAP family is a DOX prodrug that incorporates both a tumour-specific recognition site and a tumour-selective enzymatic activation sequence. The first tumour-specific sequence is the bicyclic CDCRGDCFC peptide that selectively binds to αvβ3 and αvβ5 integrins highly overexpressed in cancer endothelial cells. The second tumour-specific sequence is a D-Ala-Phe-Lys tripeptide recognized by the tumour-associated protease plasmin, an important factor of tumour invasion and metastasis. An aminocaproyl residue was incorporated as a

spacer between the two peptide sequences, whereas a self-eliminating 4-aminobenzyl alcohol spacer was inserted between the plasmin substrate and DOX. In vitro, this prodrug shows plasmin-dependent cytotoxicity for endothelial cells and HT1080 fibrosarcoma cells, but improvements in water solubility and bioavailability are needed for prospective clinical applications[125].

3.2.4.1.8 POLYMER-BOUND DOXORUBICIN

An additional improvement of targeted DOX-delivery provides the linkage of the anthracycline to polymers recognized by receptors expressed in on the tumour cell. A good example here is PK2, a structure in which DOX is linked via a lysosomally degradable tetrapeptide sequence to *N*-(2-hydroxypropyl)methacrylamide copolymers bearing a galactosamine residue that is recognized by the hepatic asialoglycoprotein receptor (Figure 42).

A study of body distribution of the radiolabeled polymer showed that PK2 selectively accumulated in the liver, allowing for the attainment of higher concentrations of DO, also in primary hepatocarcinomas. In rats PK2 induced no or considerably less severe cardiotoxicity than equivalent doses of free DOX, suggesting that selective tumour targeting and improved cardiac safety might be observed in future clinical studies[20].

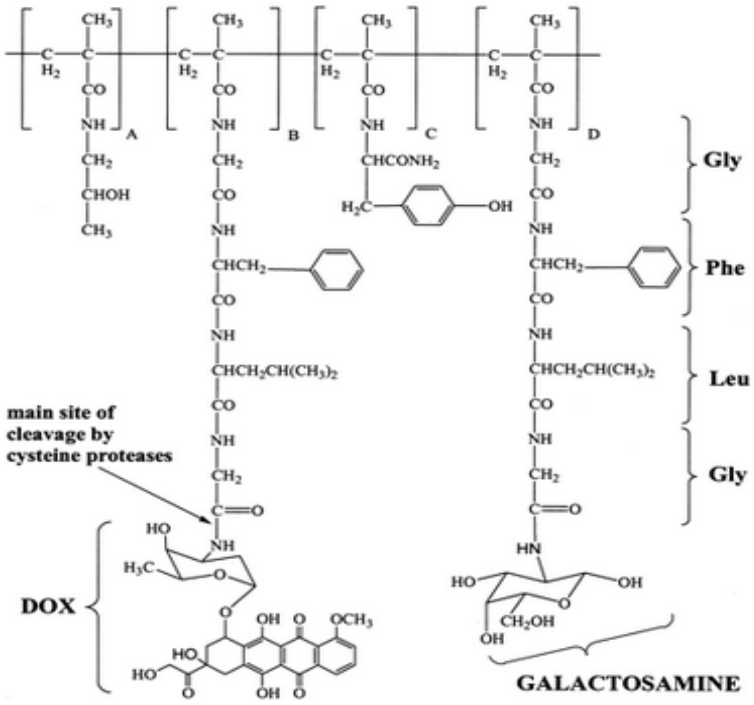


Figure 42: Structure of PK2. PK2 is a copolymer of *N*-(2-hydroxypropyl)methacrylamide bearing DOX and galactosamine, the latter being recognized by the hepatic asialoglycoprotein receptor. DOX is released after cleavage of the bond between Gly and the aminogroup at C-'3 in daunosamine, mediated by lysosomal cathepsin-type cysteine proteases (Minotti et al., 2004)[20].

3.2.4.1.9 ANALOGUES OF ANTHRACYCLINES

The role of the aminosugar in stabilizing drug intercalation into DNA has been taken great influence in the new generation of analogues. Promising agents include anthracyclines with morpholinyl or alkyl substituents at the amino group at C-3', and disaccharide anthracyclines where the amino group has switched to the second sugar. Another class of anthracyclines are designed by combining modifications at C-14 with modifications of the aminosugar. This latter strategy has produced congeners that no longer target DNA but potentiate apoptotic events modulated by protein kinase C (PKC).

For those anthracyclines targeted at DNA, preserving the quinone-hydroquinone chromophore will remain a structural requirement. For those anthracyclines targeted

at PKC or other extranuclear sites, preserving a canonical chromophore might not represent an absolute requirement[20].

NUCLEAR-TARGETED ANTHRACYCLINES

MORPHOLINYL ANTHRACYCLINES

Morpholinyl derivatives of DNR (MX 2 or KRN 8602), morpholinyl, methoxymorpholinyl (MMRA), or cyanomorpholinyl derivatives of DOX (Figure 43) bind to DNA through a mechanism comprising rearrangement/opening of the morpholino ring which subsequent binds to DNA with a structure reminiscent of *N*-(2-hydroxyethyl)DOX. In the case of cyanomorpholinyl anthracyclines, the cyano group is released.

Cytotoxicity induced through this DNA binding and alkylation does not associate with topoisomerase II-mediated DNA cleavage but involves enhanced topoisomerase I-controlled DNA cleavage and DNA cross-linking (~100-fold higher potency then DOX). Morpholino-derivatives of DOX or DNR share properties like circumvention of multidrug resistance- gain of activity after metabolism by CYP3A4, and reduced cardiotoxicity in animal models.

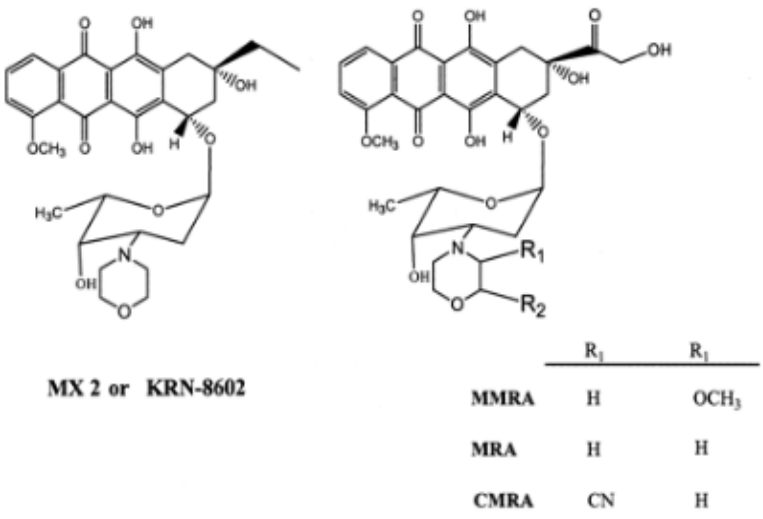


Figure 43: Structures of morpholinyl, methoxymorpholinyl, or cyanomorpholinylderivatives of DOX or DNR (Minotti et al., 2004)[20].

Studies of different chemical modifications and the ability to overcome resistance showed that an insertion of morpholinyl or methoxymorpholinyl groups at position 3' of the sugar moiety confers the ability to overcome resistance, in vitro and in vivo, while dislocation of the morpholinyl residue to C'4 was accompanied by loss of activity in vivo. These observations formed the basis to say that position 3' in the sugar moiety plays a crucial role in the ability to overcome resistance[20].

Only MX 2 (KRN 8602) and MMRA (PNU-152243, also referred to as nemorubicin) have been evaluated in clinical trials. In adult patients with currently diagnosed acute myelogenous leukaemia, MX 2/cytarabin has been as effective as DNR/cytarabin but induced more severe gastrointestinal and central nervous system toxicity, likely, due to the ability of MX 2 to cross the blood-brain barrier. Moreover there was no cardiotoxicity in MX 2/cytarabin patients, opposed to a dose-dependent incidence of arrhythmias, decrease of LVEF, and overt cardiac failure induced by DNR in combination with cytarabin[126].

MMRA has been evaluated in a phase II and pharmacokinetic study of patients with chemotherapy resistant solid tumours whereas an i. v. bolus exhibited extensive clearance as well as rapid and extensive distribution into tissues. The response rate was low, but hematologic or hepatic toxicities were frequent[20]. Further evaluation in this field is needed.

Alkyl Anthracyclines

In alkyl anthracyclines an alkylating substituent was placed at position C-3' of the aminosugar of IDA. PNU-159548 is characterized by an aziridinyl moiety at C-3' and esterification of -OH at C-4' with a methylsulfonic group (Figure 44). Alkyl anthracyclines do not interact with topoisomerase II but do cause DNA intercalation via the anthracycline backbone and bind covalently to guanines at the N7 position, and adenines at the N3 position via the reactive alkylating group in the sugar. PNU-159548 shows great anti-tumour efficacy *in vivo* after both i.v. and p.o. application against rapidly proliferating murine leukaemia or several human tumour xenografts. PNU-159548 is also able to cross the blood-brain barrier and to delay the growth of intracranial implanted tumours. As expected, PNU-159548 is effective in tumours bearing

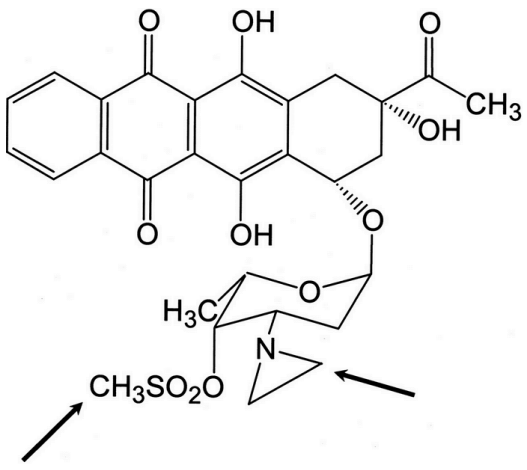


Figure 44: Structure of PNU-159548. The arrows indicate esterification of -OH at C-4' with methylsulfonate and substitution of an aziridinyl moiety for the amino-group at C-3'(Minotii et al., 2004)[20].

alterations of topoisomerase II levels and activity. It also affects cells overexpressing the MDR-1 gene and tumours, resistant to classical alkylating agents.

Due to its promising profile of activity and cardiac safety, PNU-159548 has already entered clinical evaluation[20].

DISACCHARIDE ANTHRACYCLINES

Animati et al.[127] designed 4-methoxy or desmethoxy analogues of DNR in which the sugar attached to the aglycone is in lack of the aminogroup, and daunosamine is dislocated to become the second sugar via an α(1-4) linkage. Replacing daunosamine with a disaccharide moiety dramatically reduced the cytotoxic potency of the drugs in the 4-methoxy series. Conversely, in the 4-demethoxy series, the C-4 axial configuration (but not the equatorial configuration) conferred a cytotoxic potency and anti-tumour activity, several fold higher than that of DNR or IDA and virtually comparable with DOX. The configuration as well influenced the ability of disaccharide anthracyclines to cause topoisomerase IIα-mediated DNA cleavage. Only glycosides with the axial orientation proved to be active, whereas methoxy-substituted analogues were significantly less effective than desmethoxy analogues.

Thus, MEN 10755 as a 4-demethoxydoxorubicin was designed characterized by insertion of 2,6-dideoxy-L-fucose between the aglycone and daunosamine, with axial configuration of the disaccharide moiety (Figure 45). Unfortunately, MEN 10755 did not overcome the MDR phenotype. However, it was remarkably active in xenografts of breast carcinoma cells, which show modest or delayed apoptotic responses to DOX[20].

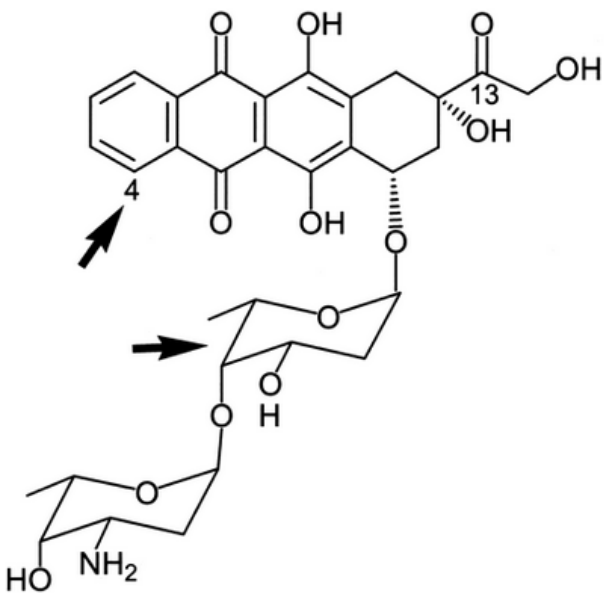


Figure 45: Structure of MEN 10755. The arrows indicate the lack of the methoxy group at C-4 in the aglycone and the presence of 2,6-dideoxy-L-fucose betweenthe aglycone and daunosamine (Minotti et al., 2004)[20].

Despite its reduced cell penetration and lower nuclear concentration, MEN 10755 has been as potent as DOX in eliciting DNA single- and double-strand breaks, G_2/M cell arrest, and apopto-

sis. Moreover, morphologic and functional abnormalities induced by DOX worsened with time, while the effects of MEN 10755 were not progressive. Studies in nude mice xenografted with human tumours showed that MEN 10755 had limited distribution not only in tumour tissue, but also, more remarkably, in heart and other normal tissues. So it seems that a weak drug-uptake into cells does not preclude MEN 10755 from eliciting anti-tumour activity, but it strongly diminished its cardiotoxicity.

The cardiotoxicity of MEN 10755 is also limited by its hindered conversion to the secondary alcohol metabolite MEN 10755ol. Furthermore a reduced formation of MEN 10755ol might contribute as well to optimize the anti-tumour activity of MEN 10755 compared with DOX[20]. In fact, studies of cancer cells overexpressing carbonyl reductases prove this principle by establishing that secondary alcohol metabolites diminish sensitivity to anthracyclines[128].

NON-NUCLEAR-TARGETED ANTHRACYCLINES

14-O-ACYL-ANTHRACYCLINES

14-O-acylanthracyclines are obtained by combining modulation in the aminogroup at C-3' with addition of alkylesters, preferably 5-carbon moieties, at C-14. As shown in figure 46 there are two leading compounds: N-benzyldoxorubicin-14-valerate (AD 198) and N-trifluoroacetyldoxorubicin-14-valerate (AD 32, valrubicin). The valerate moiety results in an in-

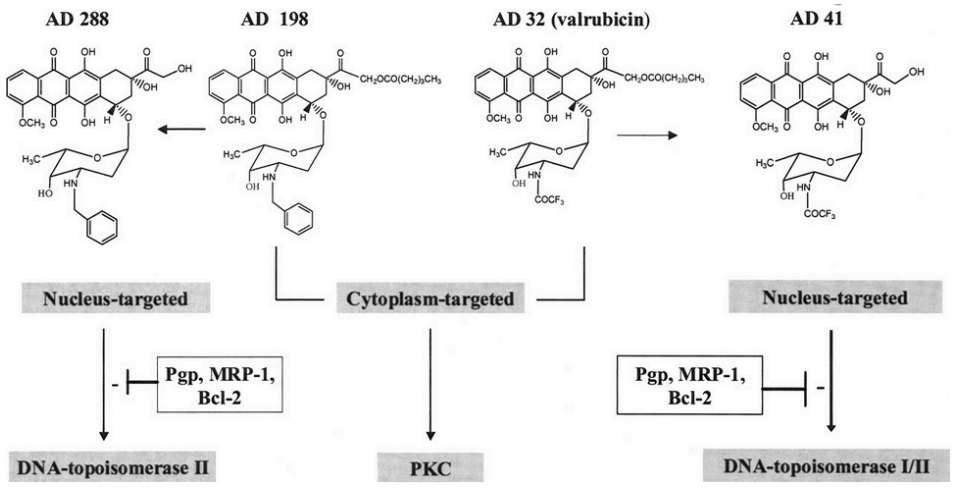


Figure 46: Nucleus-versus cytoplasm-targeted 14-O-acylanthracyclines. AD 198 and AD 32 (valrubicin) localize to perinuclear cytoplasm, induce apoptosis through PKC modulation, and escape counter effects mediated by Pgp, MRP-1, and Bcl-2. Their valerate-free metabolites (AD 288 and AD 41) localize to nucleus, act through DNA-intercalation and topoisomerase II inhibition, and are counteracted by Pgp, MRP-1, and Bcl-2 (Minotti et al., 2004)[20].

crease in lipophilicity and rapid cellular penetration, an effect contributed to also by derivatisation of the charged aminogroup with benzyl or trifluoroacetyl moieties. Moreover, valerate substitution at C-14 modifies the pattern of subcellular localization of the anthracyclines, favouring localization of AD 198 and AD 32 in the perinuclear cytoplasmic region rather than in the nucleus. Despite their poor nucleus penetration, both AD 198 and AD 32 induce cytotoxicity which was at least equal to, if not greater than DOX. AD 198 induced tumour cell apoptosis by mechanisms distinct from those of its valerate-free metabolite N-benzyldoxorubicin (AD 288), a DNA-intercalating agent and topoisomerase II inhibitor. AD 198 has little topoisomerase II inhibitory activity and did not effectively intercalate into DNA. It acted via targeting cell constituents located in the cytoplasm. One such has been identified in the regulatory domain of PKC isozymes. Due to structural homologies with PKC activators AD 198 is able to induce rapid translocation of proapoptotic proteins, like PKC-δ intact cells. Thus, rapid translocation and activation of PKC-δ in mitochondria causes apoptosis.

Moreover AD 198 is lipophilic enough to escape extracellular transport by Pgp or MRP-1. This occurs only if both N-benzyl and 14-O-valerate are attached to the anthracycline.

AD 32 (valrubicin) is similar to AD 198 concerning preferred localization in the cytosol and lack of topoisomerase inhibitory activity. Valrubicin may be a catalytic inhibitor of PKC. However, the valerate-free metabolite of AD 32 [N-trifluoroacetyldoxorubicin (AD 41)] is a nuclear-targeted topoisomerase I/II poison whose activity may be hampered by resistance mechanisms typical of conventional anthracyclines. Thus, AD 32 and AD 41 act by distinct mechanisms that resemble the different modes of action of AD 198 and AD 288, respectively.

After initial studies assessing the safety and efficacy of its intraperitoneal administration, valrubicin has been approved in the United States for the topical treatment of bladder cancer in patients who fail standard therapy whereas first-line therapy is also considered[20].

3.2.4.2 Novel Acridine and Acridone Derivatives

Recent understanding of the mode of action of acridines leads to continuous and exciting research in this heterocyclic family. Indeed, biological targets such as topoisomerases I and II, telomerase/telomere and protein kinases emerge and allow the design of novel acridine-based patterns[41].

3.2.4.2.1 Quinacrine

Quinacrine (IUPAC name 4-N-(6-chloro-2-methoxyacridin-9-yl)-1-N,1-N-diethylpentane-1,4-diamine) is a heterocyclic three-ring compound - a derivative of 9-aminoacridine. Recently it was observed that the anti-cancer mechanism of quinacrine is complex, with many potential cellular targets. The following sections describe the different anti-cancer mechanisms elicited and signaling pathways modulated by quinacrine:

- Quinacrine intercalates into DNA: Due to its diaminobutyl side chain, its not only able to stack between base pairs via planar acridine "backbone", but also to interact with the minor groove of the DNA and to be involved in the stabilization of the double helix against thermal strand separation.
- Telomerase inhibition: The exact mechanism remains unclear
- Topoisomerase inhibition: Although no sure evidence for this fact exists. A number of recent studies indeed support the role of other nuclear enzymes in the anti-tumour effect observed by quinacrine, for instance UV endonucleases, or DNA helicases, but not topoisomerases.
- Sensitizing cells to killing via x-rays and prevention of repair of single strand breaks
- Inhibition of DNA/RNA polymerase: DNA/RNA and the synthesis of other proteins are inhibited. However, studies found that the cytotoxicity and anti-tumour effects of quinacrine achieved at lower dose well below those needed to inhibit polymerase activity must be attributed to other cellular mechanisms.

- Interaction with and inhibition of proteins involved in multidrug resistance: Quinacrine was found to enhance the activity of paclitaxel in hormone-refractory prostate cancer cells both in vitro and in vivo. Thus, a combination therapy of quinacrine and paclitaxel would be suggested.
- Disruption of the arachidonic acid pathway: Agents, which inhibit this pathway, have been demonstrated to hold promise in the chemoprevention of prostate, gastrointestinal, lung as well as oesophageal cancer. One such putative target is PLA_2 (Phospholipase A_2), which is inhibited by quinacrine via intercalation into the membrane. This results in inhibition of leukotriene and COX activity, as well as eicosanoid activity. Recent reports implicate an enhanced activity of arachidonic acid pathway proteins in the prevention of apoptosis and promotion of tumour progression in head and neck cancer. Quinacrine also inhibits PGE_2 (Prostaglandin E_2), which is associated with angiogenesis, tumour growth and invasion, apoptosis resistance and suppression of anti-tumour immunity via suppression of T- and natural killer cells. An upregulation and pro-oncogenic actions of PGE_2 have been observed in head and neck cancer as well.
- Induction of p53 and inhibition of NF-kB and AKT pathway

According to this wide range of targets, quinacrine is an attractive drug in the use of different cancer types. In addition, as inflammation is now being considered as a further hallmark of cancer, quinacrine's anti-inflammatory effects seem to increase its potential. Also its effects on multiple key signaling pathways make quinacrine an exciting candidate as a chemotherapeutic agent in new types of combination treatments. Continued research into the mechanisms of this drug is clearly warranted[129].

3.2.4.2.2 Acridines as Novel Telomerase-Inhibitors

Novel approach in telomerase inhibition involves the stabilization of higher-order quadruplex structures (explained within Chapter 3.3.3) formed by the guanine-rich telomeric DNA primer, which is based on the requirement to maintain single-strandedness when the terminal bases are recognized by the complementary se-

quence of the RNA template of telomerase, the essential first step prior to the synthesis of further telomeric DNA repeats[130]. For this DNA folding small molecules can be used to bind and stabilise the quadruplex DNA, which inhibits telomerase. A vary of such compounds have now been described[131], whereas the more potent in vitro quadruplex-binding ligands produce telomere shortening and senescence in cancer cells. It was found that trisubstituted acridine compounds have potent and selective activity against telomerase, yet with up to 20-fold less short-term cytotoxicity.

Compounds based on planar tricyclic chromophore frameworks consisting of anthraquinones, fluorenones, or acridines (central planar pharmacophore) disubstituted with appropriate aminoalkylamido groups, can both stabilize G-quadruplex structures and inhibit telomerase activity[130]. The size of the amine substituents is also important, with bulky amines disrupting quadruplex binding and reducing potency of enzyme inhibition. Based on this facts a trisubstituted series of acridine derivatives have been designed, showing high potency against telomerase[131].
A member of this family is BRACO-19 (Figure 47), which shows *in vivo* activity against a human tumour xenograft. Thereby the cytotoxic agent taxol was used to produce tumour regression and BRACO-19, given after debulking, was able to suppress tumour regrowth[132].

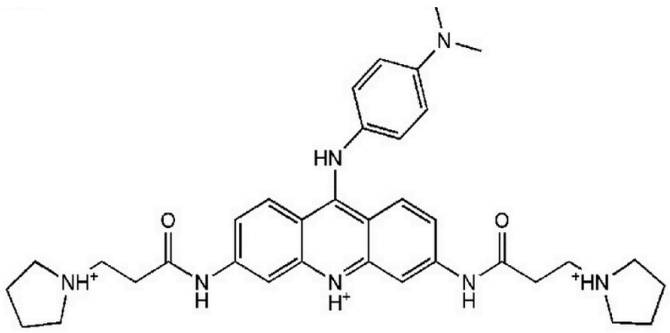


Figure 47: BRACO-19 (Burger et al., 2005)[132].

Harrison et al. investigated further derivatisation at the 9-position of the 3,6-disubstituted acridine ring system. The studies agreed with Burger et al., by showing

that 3,6,9-trisubstituted compounds are the most potent of the three regioisomeric series (2,6,9 and 2,7,9 acridines, respectively) examined[130]. Basing on this advances the synthesis and evaluation of a group of 2,6-, 2,7- and 3,6-bis-aminoalkylamido acridones are reported, which show a similar level of activity against telomerase in vitro compared to their acridine counterparts (Figure 48)

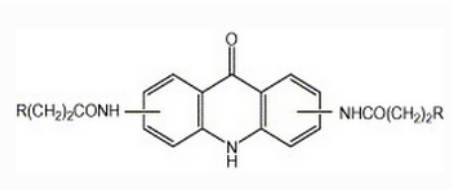


Figure 48: Chemical formula and numbering scheme of the disubstituted acridones (Harrison et al., 2004)[131].

The compounds were supposed to bind to the quadruplex DNA via stacking onto and between G-quartet bases, with side chains residing in opposite quadruplex grooves. Almost all investigated acridone isomers show significant in vitro telomerase inhibition. Thereby all of compounds possessing strongly cationic terminal substituents on the side chains, like piperidine, piperazine or dimethylamin, show significant *in vitro* telomerase inhibition. The 2,6 derivatives have been the most potent acridones amongst the tested series[131].

A summary of several more distinct developments can be found in the citing list of references 9 and 10 in Harrison et al.[131].

3.2.4.2.3 Acridines as Novel Topoisomerase I Inhibitors

Camptothecin derivatives are the only FDA-approved topo I targeted anti-cancer drugs. However, two families of non-camptothecin topo I inhibitors (indenoisoquinoline and dibenzonaphthyridinone derivatives) are in clinical development. Two derivatives of indenoisoquinolines are currently in clinical trials, indotecan (LMP400) and indimitecan (LMP776). In addition to their chemical stability, they offer several advantages over the camptothecins: they target additional genomic sites, their cleavage complexes are markedly more persistent than for camptothecins, they overcome mul-

tidrug resistance drug efflux pumps, and they produce less bone marrow suppression at equal anti-tumour activity.

The dibenzonaphthyridinone derivative Genz-644282 has equivalent or superior activity in xenograft models compared with standard drugs and has a favourable cytotoxic profile in vitro in bone marrow and tumour cell colony forming assays. Genz-644282 has comparable topoisomerase I targeting activity as the indenoisoquinolines. Both the indenoisoquinolines (LMP776 and LMP400, indimitecan and indotecan) and Genz-644282 appear active and relatively well tolerated in phase I clinical trials[133].

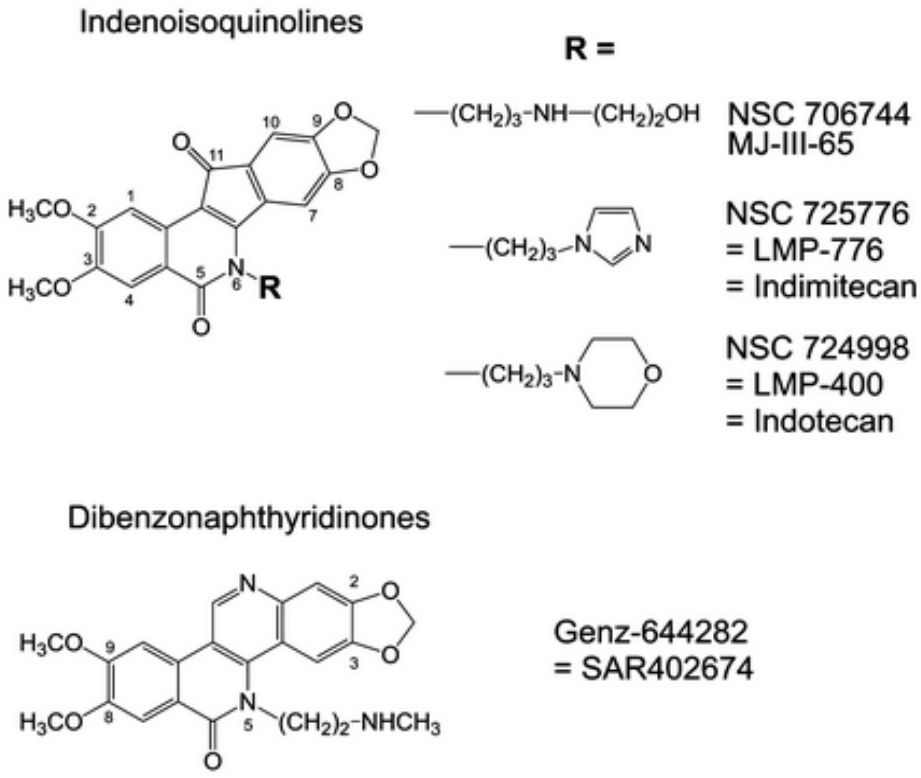


Figure 49: New non-camptothecin topoisomerase I inhibitors (Pommier et al., 2013)[133].

A new class of compounds has been synthesized by Lafayette et al. by coupling acridine and a thiazolidine or imidazolidine nucleus to obtain thiazacridine and imidazacridine derivatives, respectively. Topoisomerase I inhibition activity of several derivatives has been described. Experimental results indicate that the derivatives can bind to DNA both by intercalation of external binding. This intercalation power is suggested to be

crucial for topo I inhibition of these compounds. The study indicates that coupling of acridine with another nucleus can produce topo I inhibitory agents[134].

Ghosh et al.[135] tested the biological activity of 9-phenylacridine (ACPH) and revealed that ACPH binds in the minor groove of DNA and bends it. Moreover it was proved to bind and inhibit topoisomerase I. Therefore, ACPH may be proposed as a potential lead compound for development of new anti-tumour drugs.

3.2.4.2.4 NOVEL TOPOISOMERASE II INHIBITORS

Current topo II inhibitors are classified as topo II catalytic inhibitors or topo II poisons, with most of the current inhibitors being poisons. Topo II poisons have been well studied and act by stabilizing the "cleavable complex" resulting in the covalent linkage between the enzyme and DNA, ultimately leading to irreversible double strand breaks. Catalytic inhibitors have received far less attention. Previous studies indicate that topo II poisons primarily cause irreversible damage during the S phase (when topo II levels are high), ultimately leading to an accumulation of cells in the G_2 phase. Catalytic inhibitors result in cellular arrest at the G_2 phase by stopping the cell cycle at the catenation checkpoint from G_1 to S.

Goodell et al. demonstrated that several substituted 9-aminoacridine derivatives appeared to have novel topo II catalytic inhibitory activity. On the basis of their cytotoxic potency and their effectiveness in inhibiting human topo II catalysed relaxation reactions investigated compounds were found to not only inhibit pancreatic cancer cell proliferation but also induce apoptosis, which is a further characteristic of catalytic topo II inhibitors[118].

3.2.4.2.5 ACRIDONE DERIVATIVES AGAINST MULTIDRUG RESISTANCE

A series of acridone derivatives has been found to have inhibiting properties to the recently discovered p-glycoprotein transporter ABCG2, present in many types of breast cancer cells leading to multidrug-resistance. The spectrum of ABCG2-effluxed anti-

cancer drugs includes mitoxanthrone, camptothecin derivatives, methotrexate and the intercalating anthracyclines[136]. Elacridar (GF 120918) as an acridonecarboxamid is a potent inhibitor of the breast cancer resistance protein (bcrp/ABCG2)[137], which acts as a multidrug resistance efflux transporter, evidenced in combination with doxorubicine[138]. Almost the same properties provide tectochrysin, and the hydrophobic flavone 6-prenylchrysin. Both 6-prenychrysin and tectochrysin were able to sensitize ABCG2-transfected cells to mitoxanthrone, with low intrinsic cytotoxicity. In contrast to GF120918 which strongly inhibits the ABCG2, both of them bind on different sites of ABCG2. Thus, a combination of these different classes might therefore lead to synergistic effects[136].

3.2.4.3 4-AMINOQUINOLINE DERIVATIVES

3.2.4.3.1 4-AMINOQUINOLINE BASED HYBRIDS

To fight an array of diseases and counter drug resistance problems hybrid molecules has been synthesized by covalently connecting two (or more) pharmacophores to generate a new 'dual-drug' agent. These dual-drugs inhibit two biological targets at the same time. Chloroquin (CQ, Figure 50) and other 4-aminoquinolines have antimalarial activity. 4-Aminoquinoline based compounds are also known for their antimicrobial, anti-prion, anti-trypanosomal and anti-cancer activities. Thereby the anti tumour mechanisms of both agents is not exactly known. Similarly, several other 4-aminoquinoline based derivatives were also reported for their

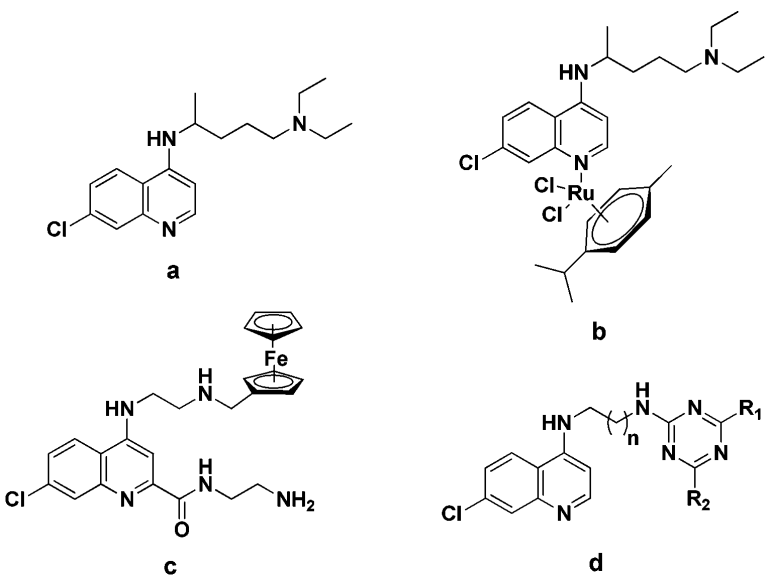


Figure 50: (a) Chloroquine (CQ); (b) ruthenium(II) complex of CQ; (c) ferrocenyl-aminoquinoline-carboxamide based bio-organometallics; (d) general structure of 4-aminoquinoline-triazine based hybrids (Manohar et al., 2013)[139].

activity against different cancer cell lines. Earlier studies have shown ruthenium (II) complexes of CQ (Figure 50; b) to inhibit the growth of colon cancer cells[139], and ferrocenyl-aminoquinoline-carboxamide based hybrid bioorganometallics (Fig. 50, c), which displayed inhibitory activity against colon and breast cancer cells[140].

Previous reports showed that hybrids of 4-aminoquinoline and triazine moieties when covalently connected via different alkyl chain linker (Figure 50; d) showed improved antimalarial activity and are 3–4 times more potent than the reference molecule CQ. Encouraged by these results great interest has been layed into exploration of eventual anti-cancer activity of these hybrids. Therefore, a series of 4-aminoquinoline-triazine based hybrids were initially screened and the two representative compounds SM334 (Figure 51; 11) and SM332 (Figure 51; 14) showed significant inhibition against the growth of in vitro cell lines. It was found that an intrinsic apoptotic pathway may be the mode of action for this coumpouns[139].

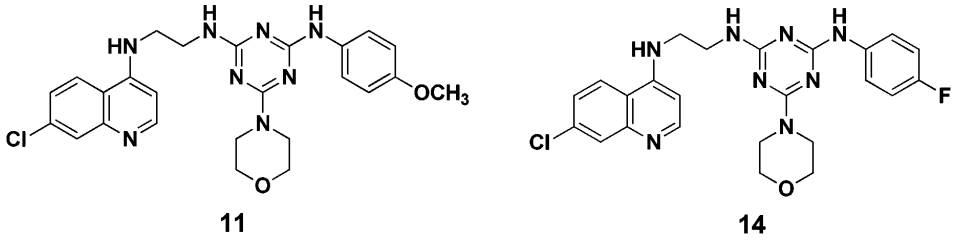


Figure 51: 4-Aminoquinoline-triazine based hybrids (Manohar et al., 2013)[139].

More advances of hybrid molecules examined during the past few years can be obtained from a collection of citations listed within reference 3 of Manohar et al[139].

3.2.4.3.2 CRYPTOLEPINE

Occasionally researchers come across new molecules with unexpected sequence preferences that can be exploited to design new compounds, provided that an illustrative structure is available. This is the case of cryptolepine.

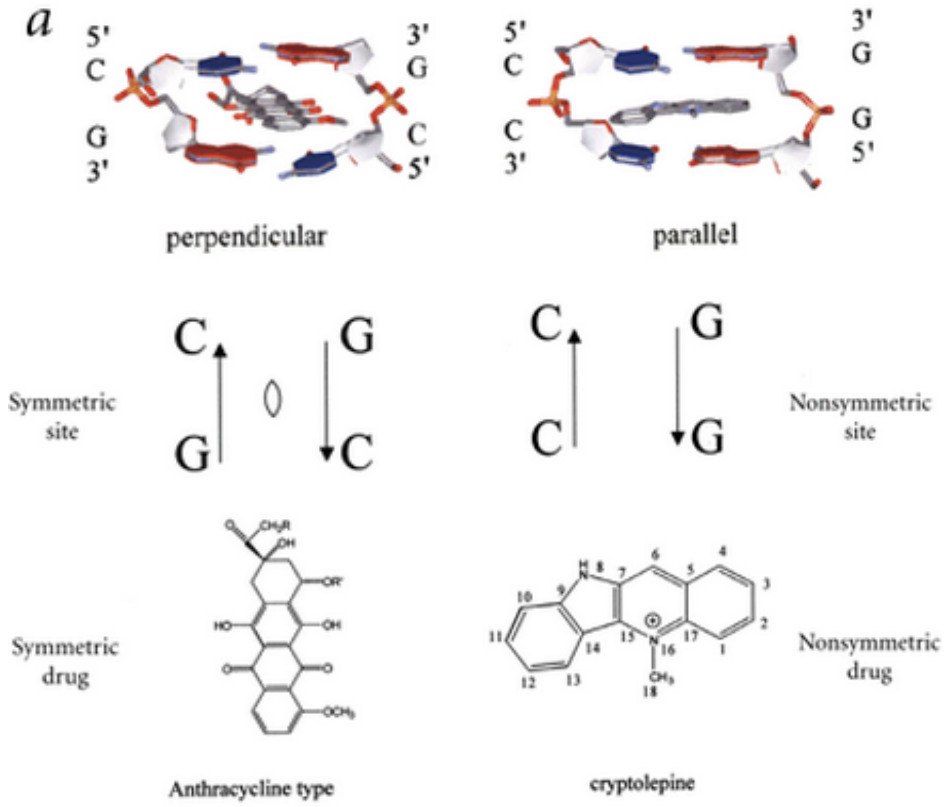


Figure 52: Cryptolepine compared with the anthracycline type: The cryptolepine site does not have two-fold symmetry (Lisgarten et al., 2001)[141].

Cryptolepine is an antimalarial and cytotoxic drug that intercalates into DNA at cytosine-cytosine sites. This binding to non-alternating bases is exceptional among intercalators, most of which have a CG intercalation site, a few intercalating GC sites and some TG sites[25]. Two main kinds of DNA-drug intercalation are observed: the first is perpendicular intercalation (Figure 52; anthracycline type), in which long fused-ring molecules penetrate perpendicularly to the base pair hydrogen bonds. Parallel base-stacking intercalators, such as actinomycin and acridine-type drugs, intercalate parallel to the base pair hydrogen bonds and stack their aromatic rings into the DNA-bases[141]. The key for cryptolepine's non-alternating site preference is its intercalation parallel to base pairs combined with the asymmetry of the drug molecule, which enhances its fitting in non-alternating sites CC-GG by allowing a distinct stacking interaction with either CC or GG[25].

3.2.5 Covalent Ligands

Interstrand DNA alkylation is the predominate mode of action of chemotherapeutic agents. Recently, a synthetic interstrand cross-linked DNA was devised and structurally characterized, which reveals little variation compared to other known structures. This topic is only noted to complete the subject of DNA binding modes and is not further discussed in this thesis.

More details shown by D. M. Noll et al.[142], as well as by M. C. Swenson et al[143].

3.2.6 Combinations of Binding Modes

It is to be expected that combinations of distinct binding modes will improve the stability of recognition and enhance target specificity with respect to both DNA structure as

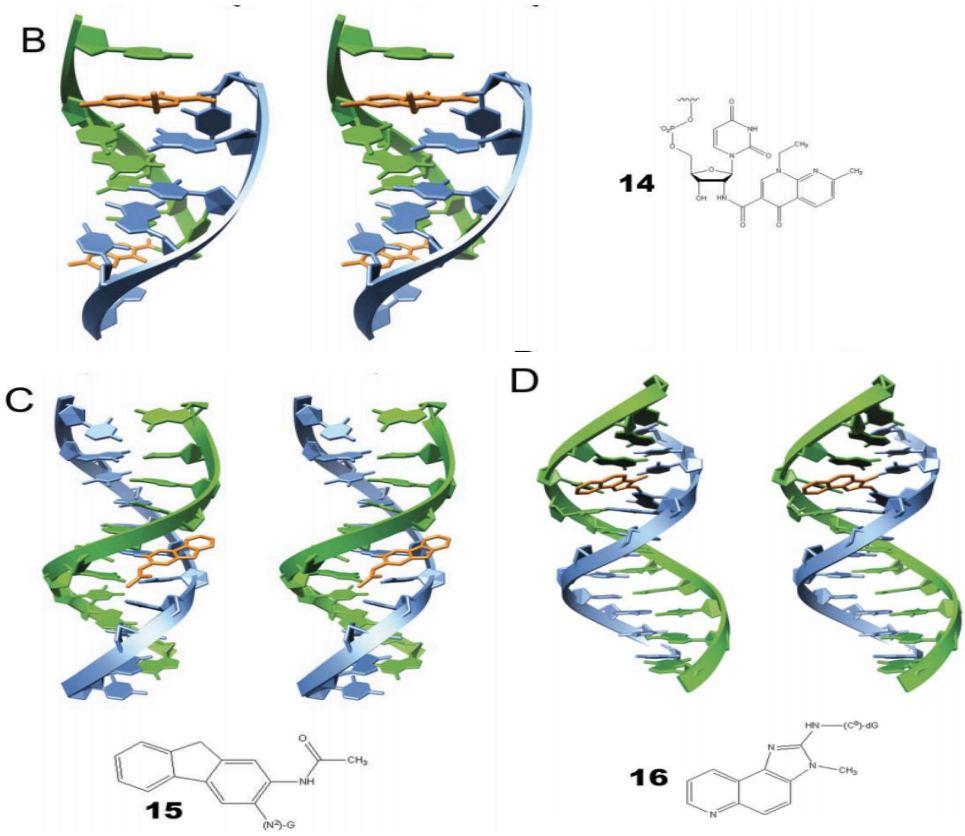


Figure 53: (B) Covalent addition of the quinolone derivative nalidixic acid 14 in the major groove (C) Covalent addition of AAF 15 to the minor groove. (D) Covalent addition of IQ 16 in the minor groove causing subsequent displacement of the Watson–Crick paired bases (D.R. Boer et al., 2008)[25].

well as sequence.

For example, a structure of an intercalating quinolone derivative (Figure 53; 14) covalently linked to the 5-phosphate of one of the strands of a B-DNA duplex has been resolved. The covalent bond helps to stabilize the intercalation, which would not have been stable without the covalent anchor to an uracil base that was incorporated in the DNA sequence.

A further example is the structure of a covalent complex between an aminofluorene (AAF) derivative (Figure 53; 15) and DNA, which reveals a minor groove binding mode. The structure shows that the adduct binds deep into the minor groove cleft, which narrows as a result of its presence. The long axis of the covalently attached AAF aligns with the minor groove and its binding is reminiscent of the minor groove binders distamycin and netropsin. The dG(N^2)-AAF adduct stabilizes the DNA analogous to the quinolone-DNA complex described above.

Other structures representing covalent binding of intercalating molecules include those of 2-amino-3-methylimidazo[4,5f]quinolone (IQ) shown also figure 53; 16. The dG(C^8)-IQ adduct results in the displacement of two bases of a base pair and insertion of the IQ Moiety into the resulting space, where it interacts with the opposing DNA base.

The combination of covalent binding with non-covalent recognition represents an entropic advantage over molecules that do not localize covalently to the DNA. Furthermore, most molecules only have weak preference for a certain sequence, and binding to several alternative sites often occurs. Combinations of sequence preferences from various

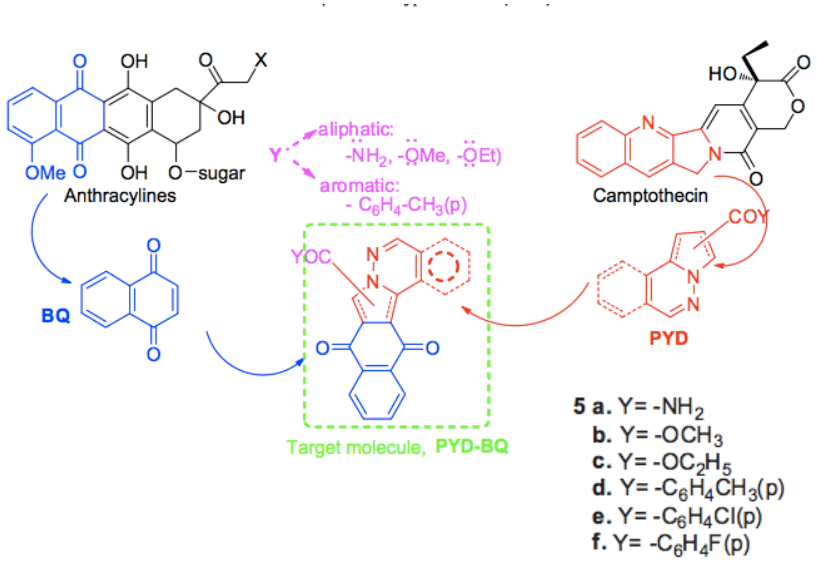


Figure 54: Antoci et al., 2014[144].

party of a single molecule could, however, provide a convenient means of site-specific DNA-targeting[25].

Based on similar hypotheses Antoci et al. very recently developed hybrid anti-cancer derivatives with multiple mechanism of action. The new molecules contain a pyrrolodiazine (PYD) moiety and a benzo-quinone (BQ) one. The Y-substituent (Figure 54) onto the PYD graph is an electron reach group, aliphatic or aromatic. The BQ moiety is fused with the pyrrole ring from PYD, in order to make the molecule as flat as possible. It was hypothesized that PYD will act as a topo I inhibitor (similar to camptothecin) and as DNA Intercalator (via the nitrogen atoms from nitrogen heterocycles and Y-electron reach groups, which will be able to interact via noncovalent bonding with the electron deficient active sites from DNA). The BQ graph will act as a topo II inhibitor (similar to the anthracyclines) and DNA strand breakers via electron transfer mechanism (quinone/semiquinone couple). Overall, the hybrid should act as an anti cancer agent with multiple mechanisms: intercalating the DNA, inhibiting topo I and II and destroying the DNA strand via electron transfer mechanism. Disappointingly results shows that only PYD-BQ 5 (Figure 54) but therefore has a significant anti-cancer activity, which makes the conclusion that the intercalation with the DNA seems to prevail in competition with the other mechanisms[144].

This examples shows that such novel fusions may lead to completely new directions to go, concerning prospective anti-cancer drug design.

3.3 Recognition of unique DNA Conformations

Based on the recognition of higher order forms of DNA, specificity for certain parts of DNA may constitute a new way of designing pharmaceutical agents that act on specific cellular processes and stages and so side effects might are decreased.

3.3.1 DNA THREE-WAY JUNCTION BINDING

Boer et al. described the recognition of a DNA three-way junction (3WJ) by a small molecular helical compound. The interaction mode of this compound involves a highly stable complex between the DNA junction and the "helicate", i.e., a compound that consists of three organic stranded ligands wrapped around two Fe^{2+} atoms, as shown in Figure 55.

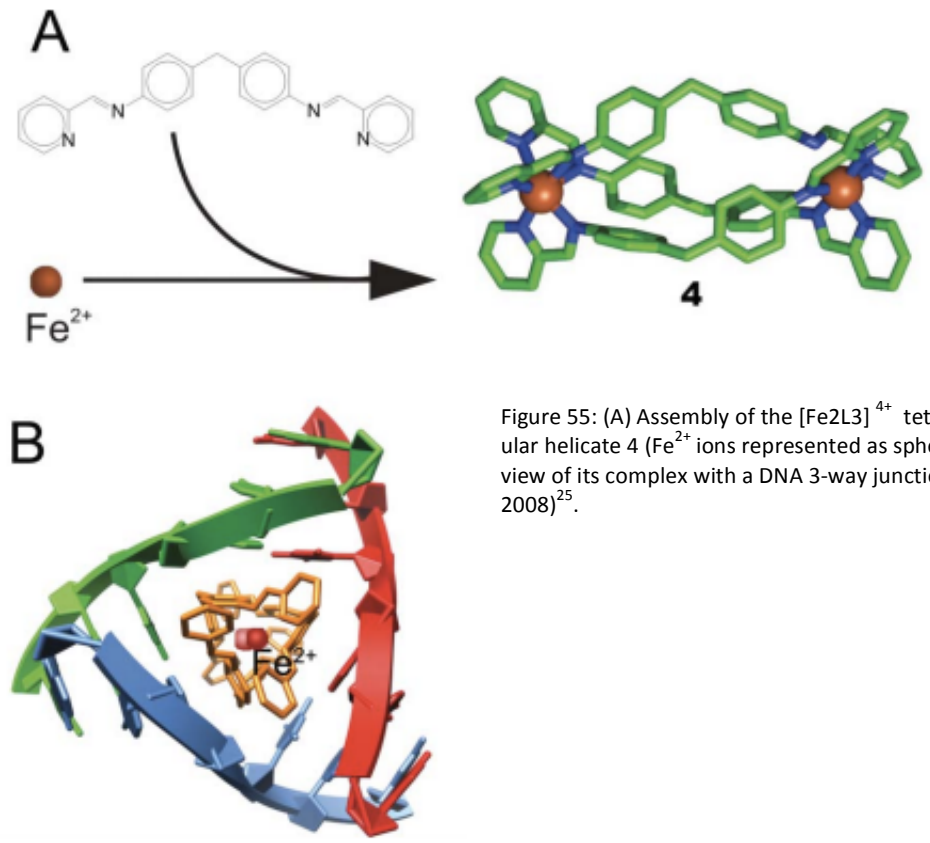


Figure 55: (A) Assembly of the $[Fe2L3]^{4+}$ tetracationic supramolecular helicate 4 (Fe^{2+} ions represented as spheres) and (B) a stereo view of its complex with a DNA 3-way junction (D.R. Boer et al., 2008)[25].

The helicate combines the two positively charged centres with an extensive hydrophobic surface formed by four aromatic rings of each respective ligand. The binding mode consists of electrostatic interactions between the iron ions and the phosphate backbone of the DNA and of pi stacking between the central DNA bases and the two phenyl rings in the center of the ligand strands. In addition, the shape complementarity between the central cavity of the 3WJ and the helicate is extraordinary. The DNA in the 3WJ adopts an open, Y-shaped conformation in which the three B-DNA arms extend

away from the central helicate-filled cavity. The DNA interacts predominantly with one half of the helicate cylinder and its arms curve slightly to the centrals axis, toward the free half. The major grooves of the DNA are facing towards the free half, whereas the minor grooves interact with the pyrimidine rings of the contacting half of the ligand strands through non-conventional hydrogen bonds and staking interactions. It is suspected that the topology of the 3WJ will allow for the adaptation of the helicate to specific sequences through adequate substitutions of the central ligand strands.

3.3.2 DNA HOLLIDAY JUNCTION BINDING

Recognition of a Holliday junction binder has been achieved with the acridine derivative shown in Figure 56 below.

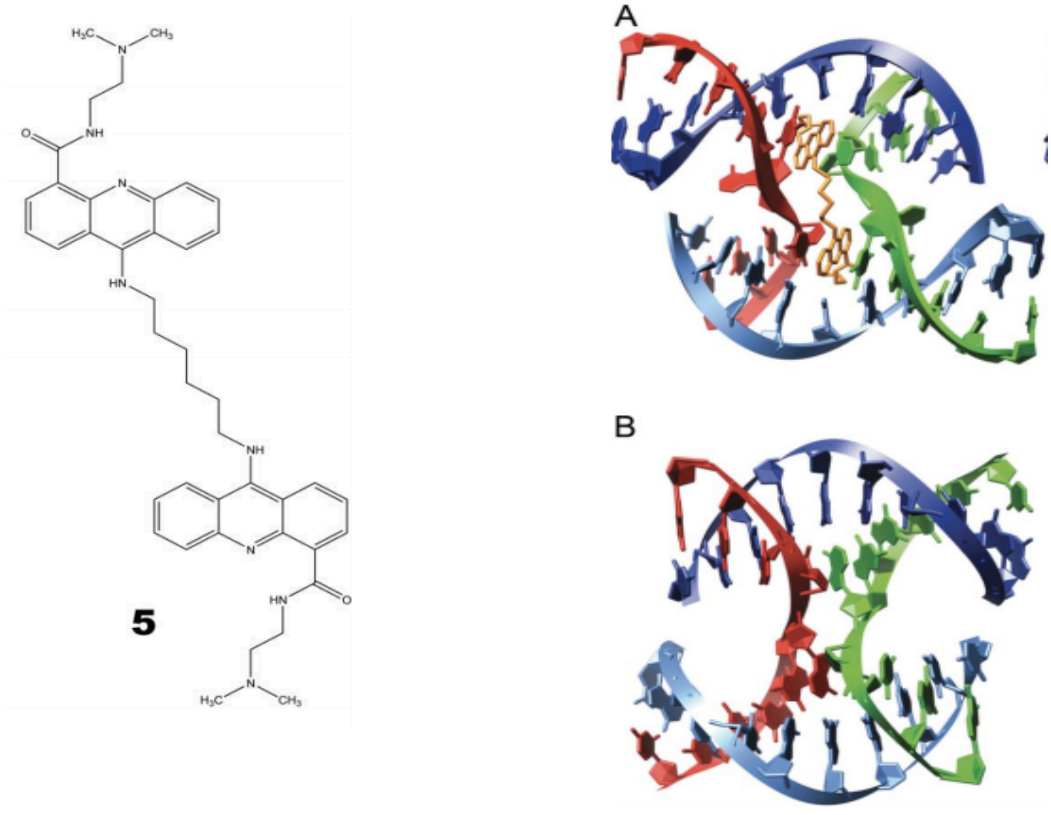


Figure 56: (A) Stereo view of a Holliday junction, complexed to the acridine derivative 5 shown alongside. The first reported, unliganded Holliday junction is shown in panel (B) (D. R. Boer et al., 2008)[25].

Like the 3WJ it is another example of recognition of higher order B-DNA structures. The two acridine moieties function as intercalators, separated by a spacer that places them at the appropriate distance for insertion at two distant sites[25].

3.3.3 QUADRUPLEX-INTERACTIVE DRUGS

Since 1962, special sequences in DNA that can form secondary DNA structures – such as four-stranded structures in which consecutive runs of guanines forms the quadruplex – have been observed. Proteins were discovered, either binding to the preformed secondary structures or are involved in the formation of the same. Their possible role in regulating the activity of telomerase through formation of G-quadruplex structures led to first effort to target these unusual DNA structures[47] due to the behaviour of these regions differs in somatic and cancer cells. The function of telomeres are already mention in the first chapter of this thesis. The extremes of telomeres contain repeated poly G sequences that rearrange into quadruplex structures, i.e. parallel bundles consisting of four DNA strands[25]. This type of DNA form occurs additionally in the promotor regions of the MYC, MYB, FOS and Abl oncogene[47]. These oncogenes have se-

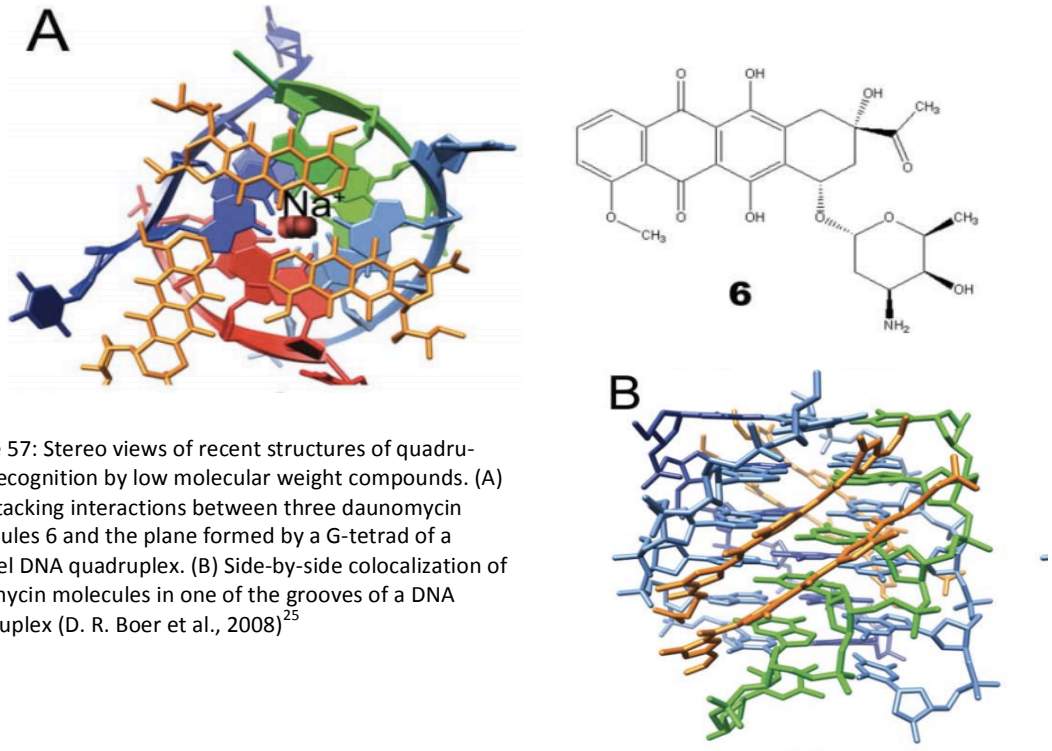


Figure 57: Stereo views of recent structures of quadruplex recognition by low molecular weight compounds. (A) End-stacking interactions between three daunomycin molecules 6 and the plane formed by a G-tetrad of a parallel DNA quadruplex. (B) Side-by-side colocalization of distamycin molecules in one of the grooves of a DNA quadruplex (D. R. Boer et al., 2008)[25]

quence-specific DNA-binding activity and a constitutive expression of them leads to cellular transformations *in vitro* and are often associated with mutations in human tumours that activate this genes[145]. The search of suitable agents had led to several candidates. It was also shown that molecules like daunomycin and distamycin, which are known to bind duplex DNA, are also capable of binding to telomere sequences (Figure 57).

Three daunomycin molecules stack on top of the plane formed by a G-tetrad at the end of a parallel intermolecular DNA quadruplex. This exemplary description provides an explanation as to how the molecule may interfere with telomere modification and maintenance by associated proteins. According to distamycin two molecules bind into one groove on two opposite sites of the quadruplex structure, which corresponds to 4 equivalents of distamycin for each DNA quadruplex. (Figure 57B). Due to the fact that the minor groove environment of quadruplexes differs to those of duplexes, it was suggested that distamycin provides a starting structure for the design of quadruplex groove recognition.

Another compound for specific quadruplex DNA have been found and characterized is the porphyrin TMPyP4 (Figure 58; 7) and an acridine derivative (Figure 59; 8). These structures represent various binding modes corresponding to end-stacking and side-way interactions. TMPyP4 was shown to stack on top of a G tetrad of a parallel DNA quadruplex mimicking the c-MYC oncogene. Here, however, a distinct type of interac-

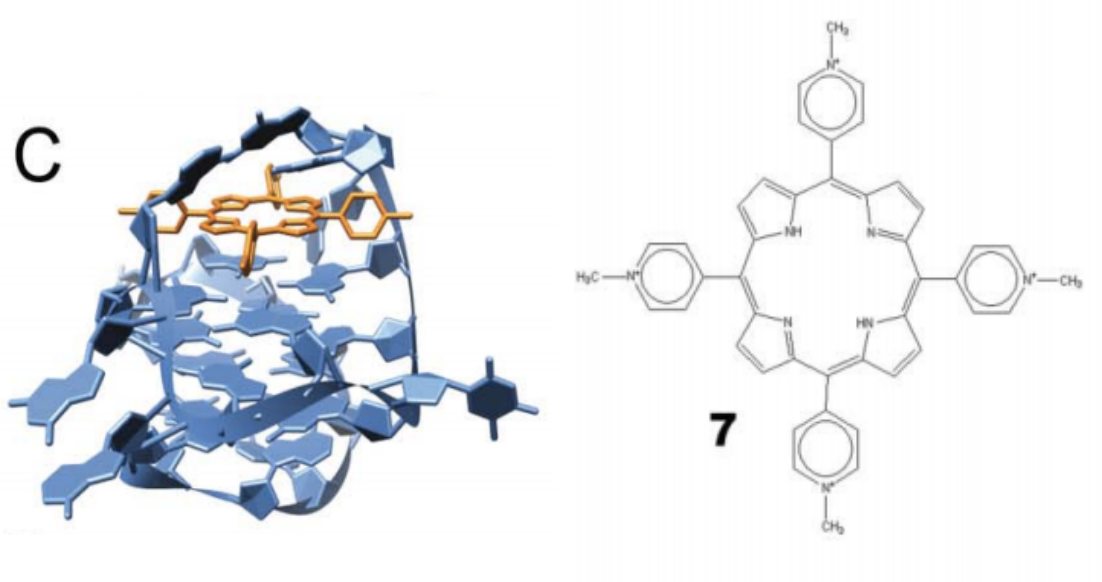


Figure 58: The complex of a porphyrin molecule 7 and a parallel quadruplex representing the c-MYC oncogene DNA (D. R. Boer et al., 2008)[25]

tion was found. Inherent steric hindrance in the porphyrin frame impedes direct interaction with the G-tetrad. As a result, each porphyrin molecule is involved in intermolecular stacking interactions with either non-paired bases or with the Watson-Crick base pairs of various DNA strands. The porphyrin stacking found in the structure of the c-MYC parallel DNA quadruplex, described above, seems to confirm the suggestion that tetrad interactions are more feasible for parallel quadruplexes. A recent structure of a complex of acridine derivative with an antiparallel quadruplex does not contradict this notion: the structure reveals intercalation of the agent between a G-tetrad, covering three of the four bases, capped by a loop that connects two antiparallel DNA strands of the quadruplex (Figure 59; D)[25]. Other acridine compounds, binding quadruplex DNA sequences are shown in chapter 3.2.3.2.2.

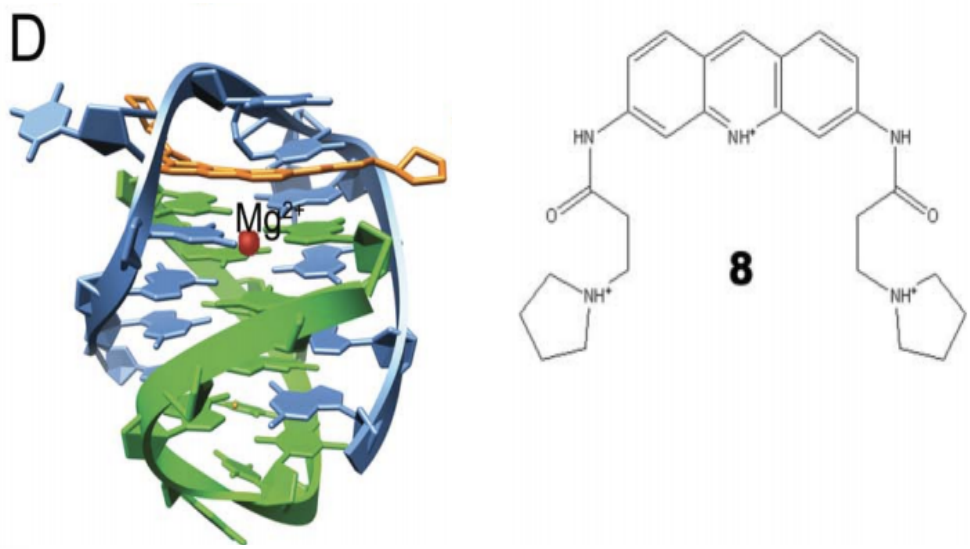


Figure 59: (D) Intercalation-like binding of the acridine derivative 8 into an antiparallel quadruplex (D. R. Boer et al., 2008)[25].

A hudge number of other G-quadruplex-binding ligands have been reported, notably based on various substituted polycyclic compounds[146], triazine derivatives[147],and quinolines[148]. Furthermore, the natural product telomestatin, which comprises one thiazoline and seven oxazole rings in a cyclic arrangement, has been reported to be a potent nanomolar inhibitor of telomerase. Thereby its mechanism of action also involves the formation of a G-quadruplex complex[149].

3.3.4 INTERCALATORS FOR MISMATCHED, BULGED DNA

Mismatched and bulged DNAs are phenomena that have been linked to disease. Mismatches may be caused by mutation of the bases of a Watson-Crick pair. Bulges occur

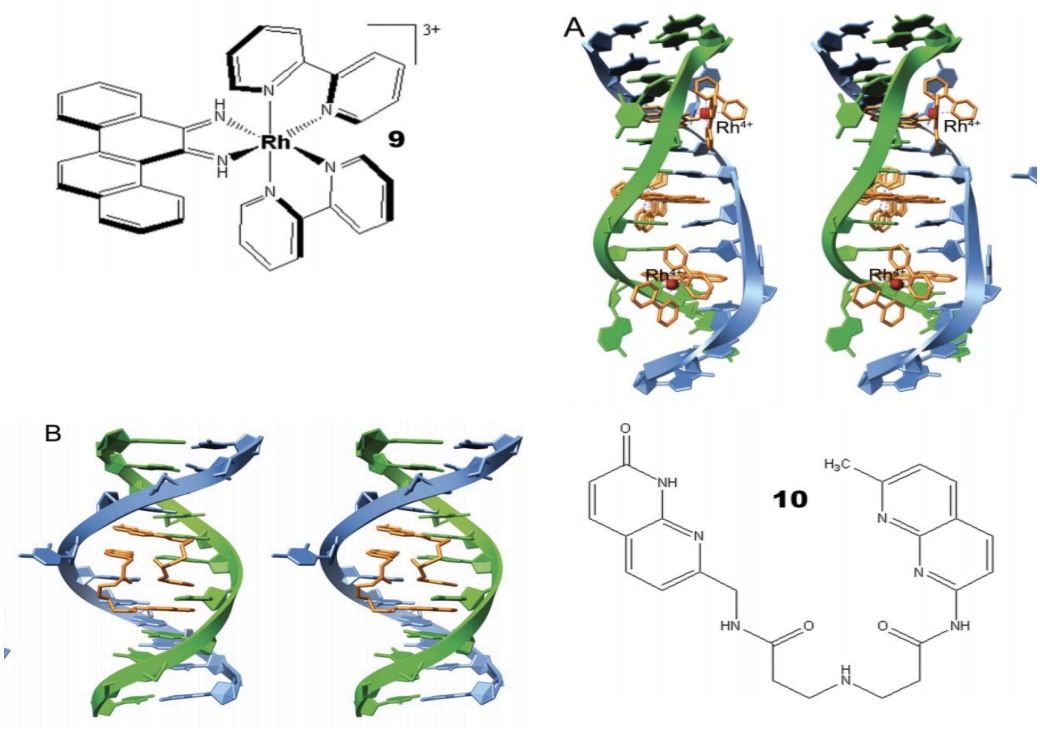


Figure 60: Stereo views of (A) a simultaneous intercalation and intercalation/displacement interactions of a rhodium supramolecular compound 9 (B) the intercalation interactions of two identical intercalators 10, each with chemically distinct intercalating moieties, at a single interaction site (D. R. Boer et al., 2008)[25].

when unmatched bases on one of the strands are present in an otherwise complementary duplex DNA. It was shown that these types of local topology of nucleic acids can indeed be recognized by small molecular weight compounds. Thereby the most straightforward strategy for recognition of a single mismatched base pair would intuitively be through base extrusion and subsequent intercalation. For such single mismatch, an intercalator would function as a replacement of the non-Watson-Crick bases, thus obviating any adjustment of the phosphate backbone to compensate for the

base extrusion from the DNA duplex. A rhodium compound that consists of several intercalating moieties is an example for such a type of interaction (Figure 60; 9). The

structural characterization of this compound in complex with a 12 base pair mismatch duplex DNA shows that, indeed, the compound intercalates at the mismatch site, ejecting the mismatched bases and replacing this pair with the aromatics four-ring system of one of its ligands. The remaining part of the ligand resides in the minor groove. Interestingly, a second molecule intercalates in a classical manner at the major groove, accommodated by a rise in the surrounding base pairs. The extrusion and stabilization of the resulting DNA structure by intercalation can be expected to change the way proteins interact with the DNA and to lead to inactivation of the damaged DNA.

Bulges that are caused by mismatches are thought to occur at extended triple repeat sequences that are present throughout the genome. The sequence repeats allow for DNA-slippage, which causes mismatches if the sequence repeats are not perfectly complementary. A naphtyridine-azaquinolone (NA) derivative (Figure 60; 10) was found to bind to this type of DNA topology. Each NA ligand consists of two intercalating aromatic ring systems connected via spacer. Each of the two chemical distinct ring systems of a single NA molecule, pairs with bases A and G of two distinct DNA strands. The other NA molecule forms a similar interaction with the remaining A and G bases. Thereby the spacers of the NA molecules run parallel to each other through the major groove. Using this structure as a starting point, one could design compounds that form Watson-Crick mimicking base pairs with any of the nucleic acids.

3.3.5 PHOSPHATE BACKBONE BINDING

A lot of research has been done concerning the search of strict sequence specificity, which requires direct interactions with the purine and pyrimidine rings of the bases. However, it may be advantageous to target other parts of the DNA structure in order

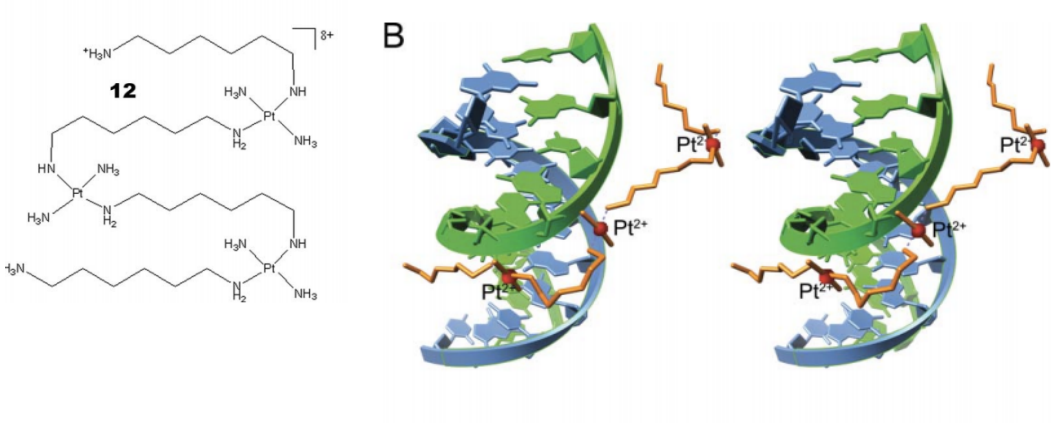


Figure 61: (B) the DNA backbone phosphate tracking polycyclic aromatic hydrocarbons (PAHs) by phosphate clamp 12 (D. R. Boer et al., 2008)[25].

to achieve improved affinity. A recent study describes the targeting of the phosphate backbone by a novel type of compound using a binding mode termed “phosphate clamps”(Figure 61). The DNA is recognized by a spacer-determined adjustment of the distance between three consecutive phosphate binding Pt(II) groups to the distance between the DNA backbone phosphonic acids. The phosphate clamp binding mode is achieved by sequential interactions of these square-planar tetra-amine Pt(II) moieties, separated by the eight-carbon spacer, with the phosphate oxygen atoms that point outwards from the DNA duplex. The ligand spans parts of the minor groove and interacts with several DNA molecules in the distal grid, which serve to stabilize the association of the molecules[25].

3.3.6 Z-DNA Binding

The recognition of DNA by polyamines and its properties concerning anti-cancer affects had already been described in Chapter 3.2.2

3.4 The Use of nucleic Acid Sequences

3.4.1 Triplex-forming Oligonucleotides

Nucleic acids themselves can also be considered sequence-specific DNA-binding drugs that could be classified as major groove binders. In contrast to minor groove binders, which open the major groove thereby granting access to other molecules such as proteins, these so-called Triplex-Forming Oligonucleotides (TFOs) bind to the major groove and block any further access by other molecules. TFOs are single-stranded oligonucleotides that bind to polypurine-polypyrimidine DNA duplexes through some local, sequence-specific and stable hydrogen bonds with bases on the purine strand (Figure 62).

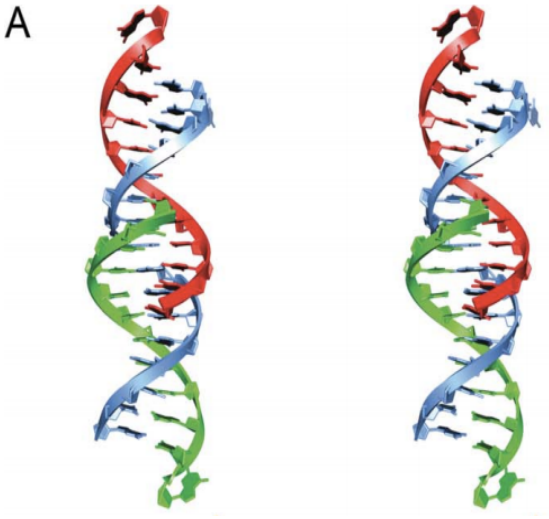


Figure 62: Nucleic acid modifications with potential application in therapy. (A) General stereo view of a DNA triplex X-ray structure (D. R. Boer et al., 2008)[25]

The bases of the third (oligonucleotide) strand connect with the Watson Crick duplex via Hoogsteen-type interactions. The overall conformation of the triplex resembles that of the B-form, but differs from both A- and B-forms. The main restriction of this

binding mode is the requirement for purine-rich tracts in the target duplex, as only this guarantees both sequence specificity and high binding affinity.

In theory, TFOs are versatile tools which, on one hand, can inhibit transcription (by competing with transcription factors, or by blocking RNA-polymerase II), and on the other hand activate it (through the creation, upon binding, of new transcription start sites, or in conjunction with covalently-bound activation domains)[25].

As an example for utilization of TFOs a 14-bp DNA oligonucleotide was conjugated to a Hoechst analogue (see chapter 3.1.1.3). Thereby the TFO moiety was linked via a free –SH group with the Hoechst moiety, which interacts with DNA by its disubstituted phenyl ring and a free primary amino group in the side chain. The intention has been to stabilize DNA triplex helix formation, benefited likely due to the major and minor groove contacts contributed by the TFO and the Hoechst moiety, respectively[46].

However, TFOs have several practical drawbacks that prevent them from being used for medical applications; Nuclease sensitivity, tendency to form self-associating structures, poor discrimination of DNA from RNA, triplex instability and a difficult cellular uptake – these are all relevant hindrances. However, research is in progress to overcome those problems by introducing modifications to the "natural" form of DNA[25].

3.4.1.1 TRICYCLO-DNA

The conformation of the DNA analogue tricycle DNA (tc-DNA) is restricted through the introduction of an ethylene bridge fused to a cyclopropane ring between the C3 and C5 atoms. Explanation for the increased RNA affinity and the superior nuclease resistance relative to DNA is a difference concerning the sugar pucker, together with A-conformation and backbone torsion angles and the distortion introduced by the cyclopropane ring on the tc-DNA backbone, respectively. The latter introduces unpaired steric interactions at the active site of both endo- and exo-nucleases.

3.4.1.2 (L)-A-THREOFURANOSYL-(3-2)-OLIGONUCLEOTIDES

TNAs are other DNA analogues with known structures that could be used in the design of TFOs. TNA is derived from a sugar containing only four carbon atoms and, hence, it is one of the simplest natural nucleic acid derivatives. Despite this, and as a result of the different topology and torsion angles at the TNA backbone, the analogue is still able to maintain the B-DNA conformation that would be expected for a DNA with the same sequence.

3.4.1.3 PEPTIDE NUCLEIC ACIDS

PNAs are among the most widely studied TFO-compatible DNA analogues. They are synthetic and achiral DNA-mimicking compounds[25], binding the major groove[47]. The sugar-phosphate backbone of DNA has been substituted by a pseudopeptide skeleton consisting of N-(2-aminoethyl)-glycine units with a methylene carbonyl linker connecting to the nucleobase (Figure 64). This huge deviation from the natural phosphoribose backbone is both nuclease-resistant and uncharged. The configuration is described as a so-called P-from for PNA. The hydrogen bonds between the DNA backbone and the PNA backbone explained why polypyrimidine PNA sequences form highly stable TNA-DNA complexes[25].

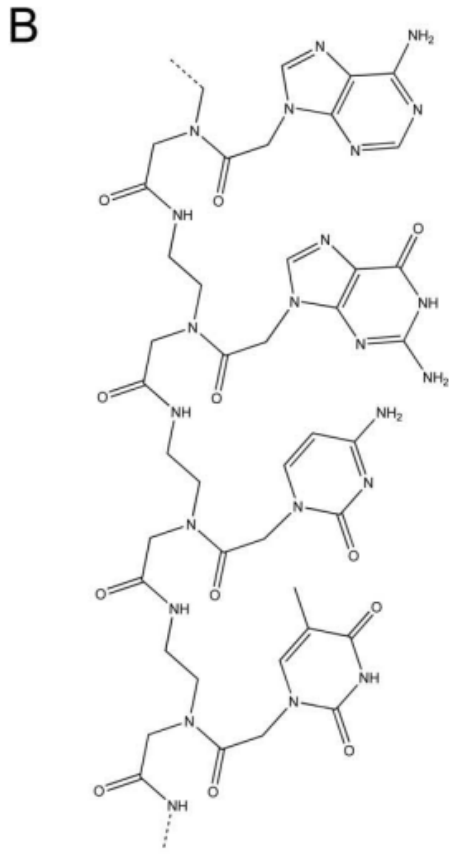


Figure 63: (B) Example of a PNA chain structure with the four canonical DNA bases (from top to bottom: Adenine, Guanine, Cytosine and Thymine) (D. R. Boer et al., 2008)[25]

More recently, a single structure of a partly self-complementary PNA oligomer showed several kinds of interactions, comprising both right- and left-handed Watson-Crick duplexes and a PNA-PNA-PNA triplex. Such diversity demonstrates that the PNA backbone is highly adaptable and that even with limited sequence complementarity these analogues are bale to from complex hydrogen-bonding networks. Moreover, as they are uncharged molecules, PNA binding to DNA and RNA targets is stronger than that of DNA/DNA or RNA/RNA bindings because electrostatic repulsion is lost. Furthermore, as the backbone of these analogues cannot be degraded by nucleases (and is also very resistant to proteases), they are very stable inside the cell. Unfortunately, their delivery into the cell has limited their applications as the uncharged nature of PNA prevents them from reaching their target. This feature could be improved by adding positive charges to the PNA, thereby enhancing the attraction of molecules to the cell membrane.

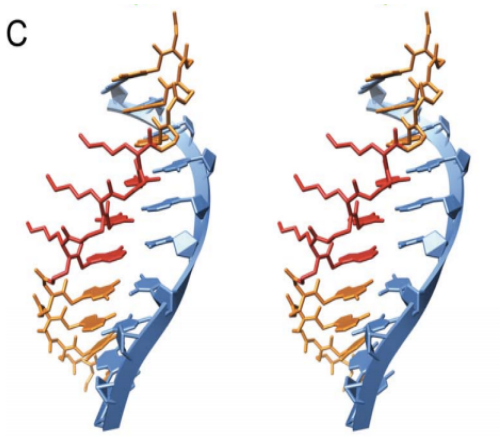


Figure 64: (C) Stereo view of the D-lysine-based PNA (orange) in complex with a complementary DNA (blue) modified D-lysine residues are highlighted in red. D. R. Boer et al., 2008)[25]

Compounds like the D-lysine-based chiral PNA, which forms a duplex with a DNA strand (Figure 63), may indicate one direction to follow. Moreover, this structure highlights the C5-substituted PNAs as powerful candidates to insert functional groups to be further conjugated with suitable compounds and to achieve PNA derivatives endowed with enhanced properties as antigen and antisense therapeutic agents[25].

Such triplex-forming molecules (TFMs) can be conjugated to a drug, such as 10-carboxycamtothecin, that will recruit an enzyme (Topo I) with a DNA-cleaving chemistry. In this manner, the toxic effects of individual drugs might be minimized by markedly increasing their sequence selectivity[47].

3.4.2 APTAMERS

As discussed above, using DNA as a DNA-binding agent is the obvious choice when a reliable code-reading approach is to be followed. Aptamers, however, are a fascinating group of new putative therapeutic agents that take advantage of the capacity of short DNA (or RNA) molecules to fold and bind to specific targets by recognition of their three-dimensional structure, with high affinities and specificities comparable to those of monoclonal antibodies. Aptamers can be selected to bind to unique structures of either DNA or RNA rather than to specific sequences and offer again, the possibility to use the altered-structure recognition strategy to target key events in the nucleic acid metabolism of the cell. This has been base of the development of the DNA and RNA aptamers against the HIV.1 trans-activation-responsive element (TAR), a 59-nucleotide-long stem loop RNA structure that allows a high yield synthesis of the full-length retroviral genome upon binding to the viral protein Tat. A preliminary NMR structure of a motif derived from an anti-TAR DNA aptamer complexed with its target demonstrated the formation of a kissing complex and provided insight into the global fold.

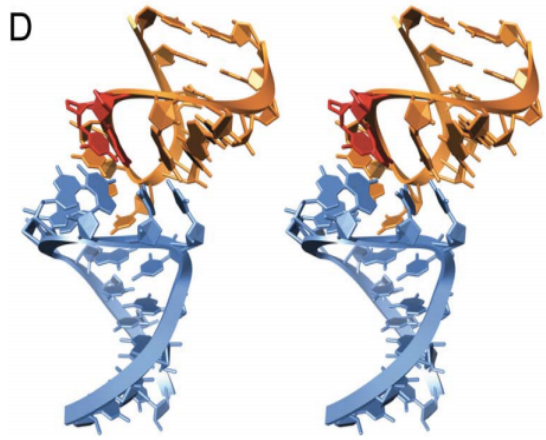


Figure 65: (D) Stereo view of the best representative conformer in an NMR ensemble of the HIV-1 TAR RNA hairpin (blue) in complex with an RNA aptamer (orange) with two locked residues (red) D. R. Boer et al., 2008)[25]

To improve nuclease resistance of the aptamer, RNA derivatives with locked nucleic acid (LNA) residues, containing a methylene linkage between the 2-O and 4-C of the ribose ring, were tested. LNA derivatives of the parent aptamer were much more stable and the NMR structure of the kissing complex eventually described the subtle structural changes responsible for the improvement (Figure 65). This is just one example of how RNA or DNA structures constitute valid targets for designing potential therapeutic agents of interest[25].

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SUPPLEMENTS

ZUSAMMENFASSUNG

Krebs gilt als die häufigste Todesursache in Industrieländern und zweithäufigste in Entwicklungsländern. Allein im Jahr 2012 wurden weltweit 14,1 Millionen Neuerkrankungen gezählt. Die Behandlungsmöglichkeiten von Tumoren beinhalten chirurgische Eingriffe, Bestrahlung und Chemotherapie. Ein sehr hoher Prozentsatz der heutzutage verwendeten Chemotherapeutika entspricht der Kategorie DNA-interagierende-Arzneistoffe. Diese Substanzen stehen mit der DNA mittels drei Bindungsmethoden in Wechselwirkung: interkalieren, groove binding und kovalentes crosslinking. Unser Wissen über die genauen Beziehungen zwischen DNA-Bindung, chemischen Strukturen und biologischer Aktivität, darunter auch Genotoxizität, verbleibt jedoch unvollständig. Enorme Nebenwirkungen stammen von nicht bzw. wenig vorhandener Spezifität der eingesetzten Medikamente. Seitdem jedoch detailliertere Informationen über die DNA-Struktur vorhanden sind, gibt es Hoffnung auf eine Erhöhung dieser Spezifität, um grobe Nebenwirkungen, vor allem an gesunden Zellen, zu vermeiden. In der vorliegenden Diplomarbeit werden neue Ansätze über ein optimiertes Wirkstoffdesign diskutiert. Das Hauptaugenmerk beruht auf dem Versuch heutiger Krebsforschung, mittels verschiedenster Methoden DNA-zielgerichteten Substanzen eine höhere Spezifität dafür zu verschaffen.

Die Entwicklung neuer und verbesserter DNA-bindender Arzneistoffe kommt nicht selten von leichten Variationen bereits bekannter und vielfach verwendeter Therapeutika. Einige Beispiele hierfür sind unter anderem Brostallicin und Lexitropsine, die auf Strukturenbasis von Nepropsin und Distamycin aufgebaut sind. Weitere Beispiele sind Aclarubicin und Pirarubicin als neue Anthrazykline. Multi-anti-Krebs-Funktionen wurden erreicht, indem man Arzneistoffe mit verschiedensten Wirkungsmechanismen miteinander kombinierte, wie zum Beispiel Anthrazyklin-Konjugate mit Formaldehyd bzw. Telomerase-Inhibitoren. Außerdem wurden neue Bindungsmethoden und abgeänderte DNA-Konformationen für ein alternatives Design verwendet: Man entwickelte Hairpin-Polyamid-Chlorambucil-Verbindungen oder liposomale Formulierungen als Träger für Anthrazykline. Weiters wurden Arzneistoffe mit Polyaminketten versehen, um ihre Spezifität für Krebszellen zu erhöhen. Polyamine sind hochpotente Liganden des Polyaminetransportsystems, welches insbesondere auf Krebszellen überexprimiert

vorkommt. Speziell letzteres verspricht Großes in Hinblick auf eine neue Target-optimierte Wirkstoffentwicklung.

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