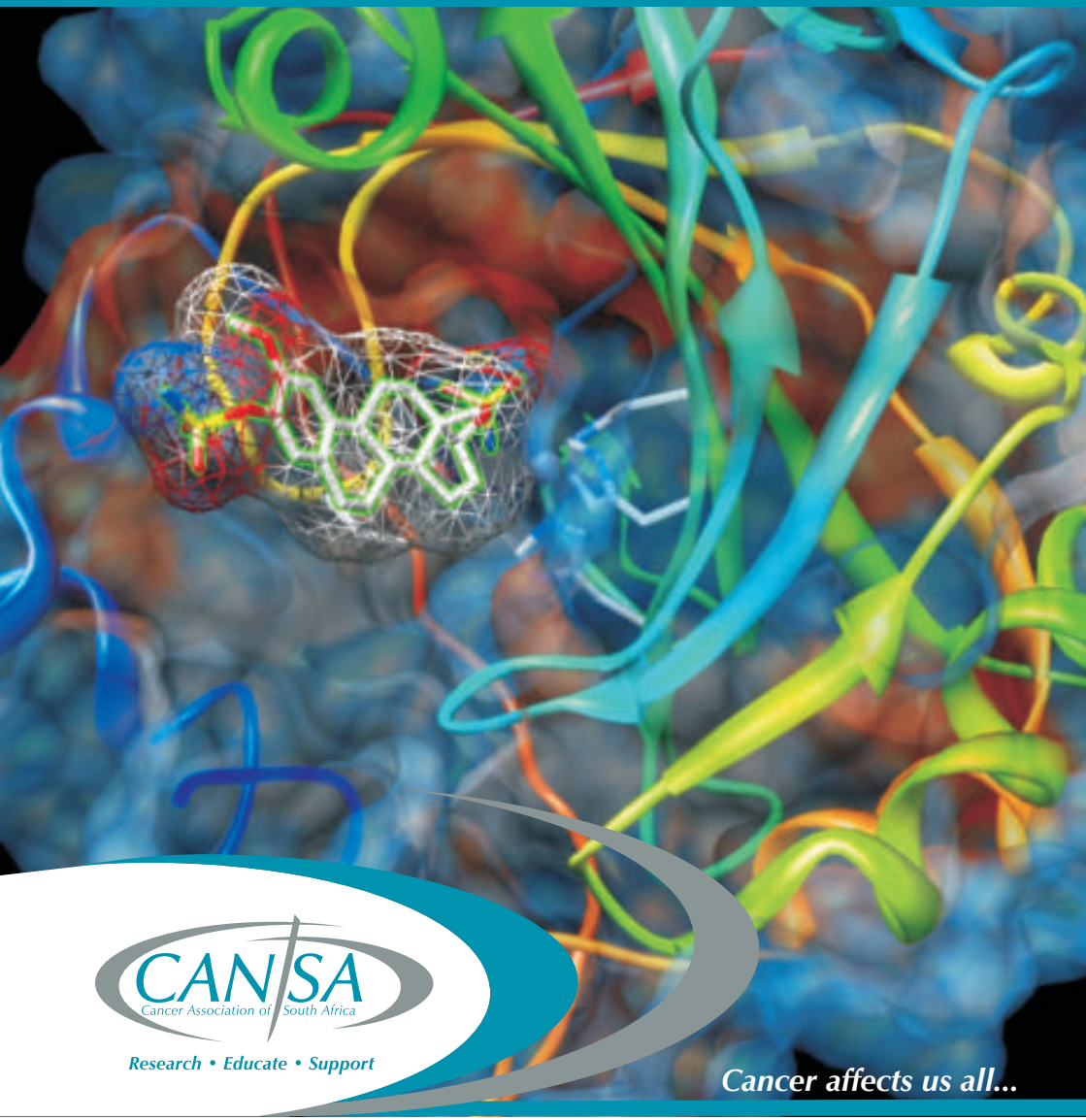
CANSA DETECTIVES

'Designing Weapons Against Cancer' *in Silico* Analysis, Synthesis and *in Vitro* Evaluation of Novel Anticancer Agents



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CANCER DETECTIVES

Booklet 3

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Targeting cancer cells

Cancer cells are abnormally dividing cells without homeostatic control. In the fight against cancer, antimitotic agents are one of the most successful groups of chemotherapeutic compounds currently used for anticancer treatment. The cell's mitotic spindle is composed of long and hollow structures of microtubules that are intimately involved in mitosis and cell division (Figure 1). Since microtubules exert a pivotal role during cell division, this characteristic renders them the most highly validated anticancer targets identified to date.

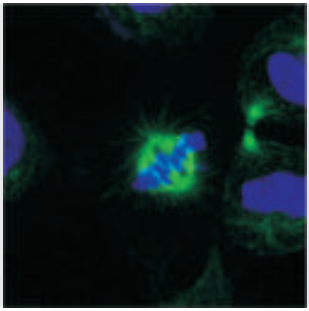


Figure 1: A dividing cancer cell with the spindle indicated in green.

Agents that suppress microtubule dynamics may cause mitotic spindle disruption in dividing cells and may inhibit angiogenesis. Angiogenesis is the formation of new blood vessels which supplies cancer cells with nutrients and oxygen and this enables cancer cells to grow even further. During tumor formation, chemicals are released which promote the formation of these new blood vessels. A clever way to treat cancer is to prevent this formation of new blood vessels that feed the cancer cells. Without nourishment and oxygen, their growth will be limited.

Inhibition of angiogenesis may contribute to excessive reactive oxygen species signaling; thus sensitizing hyperproliferating cells to pro-death signalling. Hyperproliferating cells are fast dividing cancer cells. Pro-death signaling is the switch that initiates cell death. Sensitizing hyperproliferating cells to pro-death signaling means treating these fast dividing cancer cells with specific drugs that will render them more susceptible to undergo cell death.

Another aspect to bear in mind is the importance of finding agents that will target cancer cells, while leaving non-tumorigenic cells unharmed or less affected. Chemotherapeutic and radiotherapeutic treatments of cancer remain works in progress, since; in general, these therapies have undesirable side effects that cause damage to normal cells. Therefore, processes of discovering compounds that are capable of selectively inhibiting the activity of cancer-associated proteins constitute a main component of drug development research. Research is focused on improving treatments to enable the anticancer drug to affect only the cancer cell and at low dosages with less frequent treatment intervals. Thus, our team set out to search for anticancer compounds targeting highly proliferating cells that will impact positively on quality of life - 'Models of Hope: developing molecules for fighting cancer'… The search was on!

In silico-design of novel, potential anticancer agents

In the modern day and age, computers play a vital role in the modeling and analysis of biological data. During 2008, the research group of Prof Annie Joubert at the Department of Physiology (University of Pretoria) collaborated with Prof Fourie Joubert of The Bioinformatics and Computational Biology Unit of the University of Pretoria for the *in silico*-design of novel, potential anticancer compounds. *In silico* design in this sense of the word means development of new anticancer drugs via computer simulation. André Stander, one of Annie's PhD students accomplished this by modeling three-dimensional structures of specific target proteins (tubulin, carbonic anhydrase, kinesin motor proteins) and 2-methoxyestradiol derivatives that bind to them using computer-based molecular docking techniques.

2-Methoxyestradiol analogues

After completing her PhD in Biochemistry in 1998, Annie was appointed as senior technical assistant in the Department of Physiology at the University of Pretoria where she embarked on a journey in cancer research in the laboratory of Prof J Seegers. Prof J Seegers published an article on the effects of 2-methoxyestradiol (2ME) on cell division as early as 1989 (1). They called 2ME the ultimate cytotoxic compound meaning that it caused cancer cells to die, given that they were unable to complete cell division. 2ME is an endogenous metabolite of 17β-estradiol exerting both antiangiogenic effects (inhibition of the formation of blood vessels around cancer cells) and antimitogenic effects (inhibition of cell growth) *in vitro* and *in vivo* (Figure 2). 2ME abrogates microtubule dynamics by binding to the colchicine-binding site of β-tubulin, thereby preventing microtubule assembly. Since then, various articles on 2ME and its effects on different cancer cells have been published (2-10). Currently, ENTREMED Inc is evaluating this drug in phase II clinical trials under the commercial name PANZEM®. Unfortunately, 2ME reveals low oral bioavailability and rapid metabolic degradation.

However, sulfamates, including sulfamoylated derivatives of 2ME have increased bioavailability. These molecules are able to overcome the biotransformation encountered by liver metabolism. This is attributed to the ability of sulfamoylated derivatives to reversibly bind to carbonic anhydrase II (CAII) in red blood cells after which they are then slowly released into the blood circulation system. Carbonic anhydrases are zinc enzymes that catalyze the conversion of carbon dioxide and water to carbonic acid. Tumor cells have a lower extracellular pH than normal cells. The acidotic environment promotes the action of growth factors and proteases involved in tumor progression. Carbonic anhydrase IX (CAIX) is overexpressed in a variety of tumors. CAIX overexpression in cancer cells leads to changes in nutrient transport, disruption of cell contact and the exclusion of some chemotherapeutic drugs. Selective inhibition of CAIX therefore provides a valuable strategy for curtailing the development of metastatic processes associated with acidotic microenvironmental conditions in tumors and renders this a valuable anticancer therapy model to explore.

Promising sulphamoylated analogues of 2ME were designed in Annie's laboratory (Figures 3-6). *In silico* docking revealed that the 2-ethyl derivatives displayed increased binding affinity for tubulin binding, thus interfering with microtubule dynamics when compared to other C2-modified analogues. Since these are novel compounds and since they are not commercially available, synthesis was conducted by Ithemba Pharmaceuticals (PTY) Ltd (Modderfontein, Gauteng).

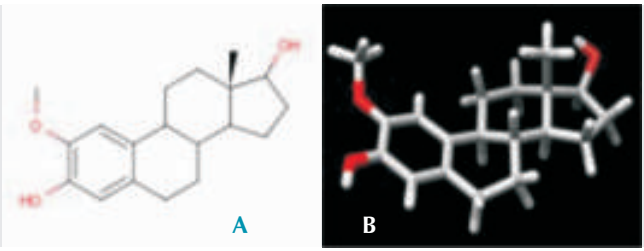


Figure 2: Chemical structure (A) and three-dimensional structure (B) of 2-methoxyestradiol.

(Courtesy: BA Stander, PhD student).

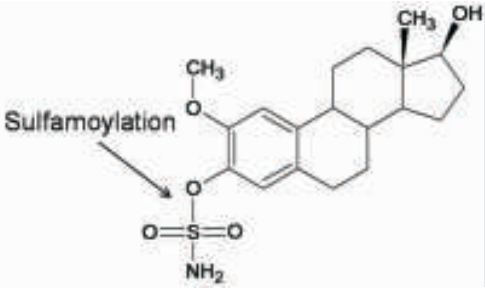


Figure 3: Stick model of a typical sulfamoylated version of 2ME with an arrow indicating where the sulfamoylation has occurred.

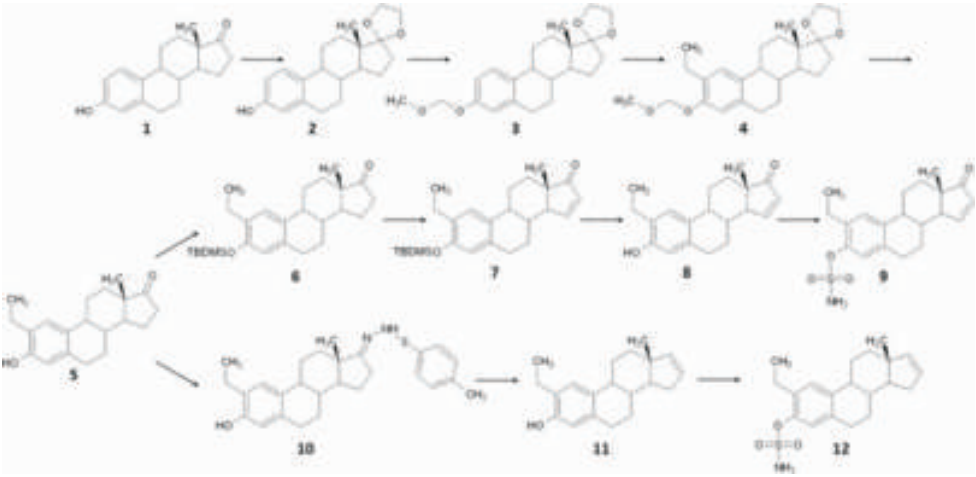


Figure 4: Synthesis scheme of 2-ethyl estrone derivatives.

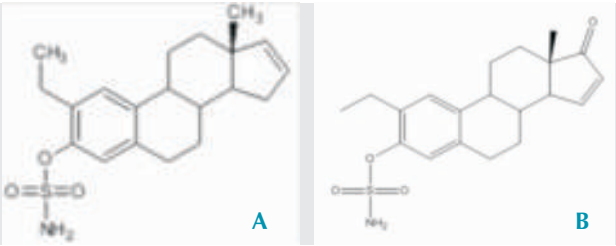


Figure 5: Chemical structure of compound 12 (C12) (A) and compound 9 (C9) (B).

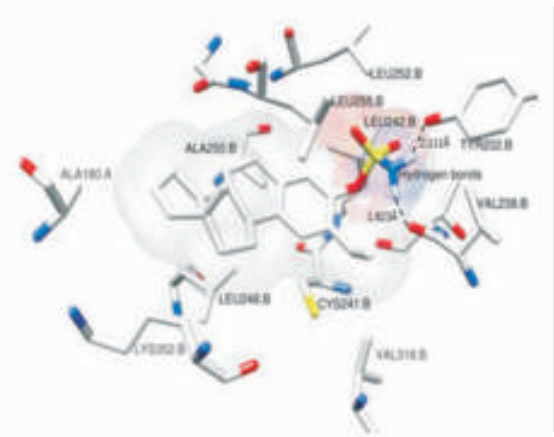


Figure 6: Molecular docking of compound 12 in the colchicine-binding site of tubulin.

In vitro analysis of 2ME analogues

In vitro cellular and molecular studies are currently being conducted to elucidate each compound's signal transduction mechanism and to confirm their potential antitumor activity *in vitro*, having already been tested *in silico* (11-16). Signal transduction refers to the cell's response when a drug is being administered. The potential anticancer efficacy of these analogues are being investigated on human breast adenocarcinoma (MCF-7) cells (Figure 7) that are derived from a pleural effusion of human breast adenocarcinoma, the MCF-12A cell line that is a non-tumorigenic epithelial cell line produced by long-term culture of normal mammary tissue and MDA-MB-231 that is described as an estrogen receptor negative metastatic breast cancer cell line. SNO cells are squamous oesophageal carcinoma cells and the HeLa cell line is a human cervical carcinoma line.

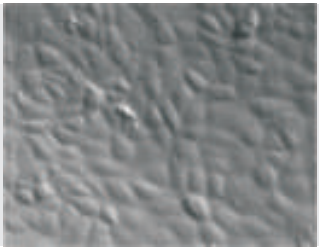


Figure 7: Optical transmitted light differential interference contrast (PLASDIC) image of MCF-7 cells.

Two of the newly synthesized and novel compounds, C9 and C12, are five to eight times more potent *in vitro* on various cell lines when compared to 2-methoxyestradiol. C9 is also being tested in conjunction with a glycolysis inhibitor, dichloroacetate. Preliminary results are promising and demonstrate a synergistic effect on cancer cells. This strategy thus also targets the deregulated metabolism associated with cancers.

Computational methods for discovering targets and identifying lead compounds against these targets are playing an increasingly important role in discovery and development of new pharmaceutically relevant drugs. The use of *in silico*-virtual screening (VS) methods can in principle identify lead compounds that are likely to succeed in further downstream assays and screens. Making use of *in silico*-VS methods such as virtual docking, assists scientists in identifying novel compounds and can significantly lower the cost of drug development by negating the need to synthesize unnecessary compounds that could not be removed prior to virtual screening.

The *in silico*-designed drugs were provided in powder form. To enable testing of the drug it is necessary to first dissolve the powder in a liquid that will solubilize the drug. This liquid is named a 'vehicle'. To ensure that the drug is the cause of cell death rather than the solubilization liquid, we have to treat the cells with the liquid without the drug in a separate sample as well. Hitherto, as already mentioned, some of these compounds were proven to be five to eight times more potent *in vitro* when compared to 2ME. These demonstrate extremely low concentrations for a drug to have anticancer activity when compared to conventional current treatments.

A few examples of our findings are presented below. Figures 8-10 demonstrate the induction of cell death via apoptosis, abnormal mitotic spindle formation, as well as tubulin disruption after treatment with C12.

Figure 8: Vehicle-treated MDA-MB-231 control stained cells with DAPI and Alexa-488 anti-tubulin. Cells in interphase, metaphase and normal mitotic spindle formation are observed.

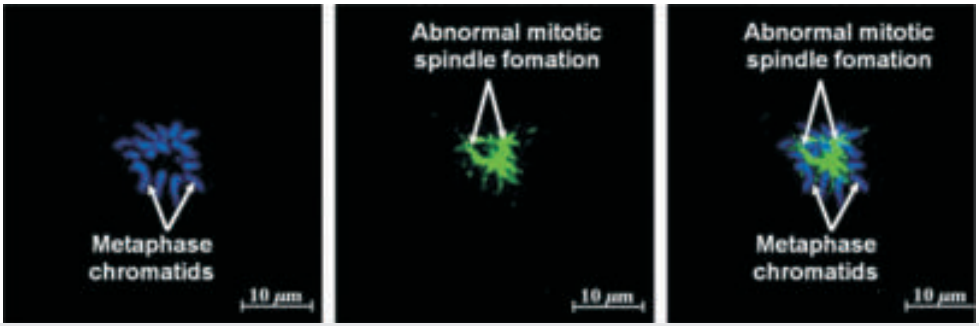
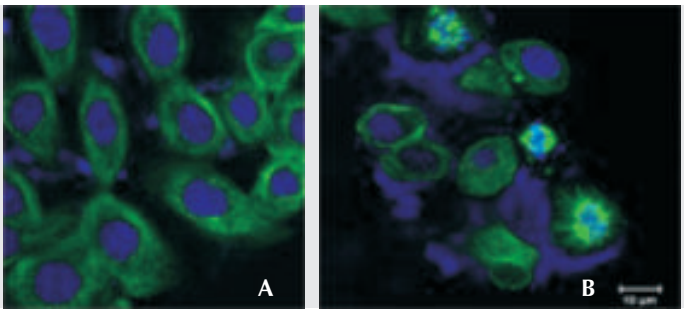


Figure 9: C12-treated (200nM) MDA-MB-231 cells stained with DAPI and Alexa-488 anti-tubulin. Abnormal mitotic spindle formation is clearly visible.

Figure 10: Confocal microscopy of MCF-7 cells stained for tubulin (A, vehicle-treated control, B; C9 treated; 130 nM) after 24h of exposure. Tubulin disruption is distinctly noticeable in B.



Antimitotic characteristics were also confirmed by means of X-ray crystallography in collaboration with the group of Robert McKenna and Sippel from the Department of Biochemistry and Molecular Biology, McKnight Brain Institute (University of Florida, Miami, USA). X-ray crystallography is a method used to determine how molecules bind to specific proteins. Compounds can be designed to bind to the colchicine-binding site in and X-ray crystallography can be used to confirm binding and thus to demonstrate interference with tubulin dynamics.

The road ahead

The newly designed compounds are the first antimitotic compounds that are capable of selectively inhibiting carbonic anhydrase IX, a cancer-associated protein, and in doing so, is capable of inhibiting cell proliferation. So far and noteworthy, is the fact that these compounds exert differential effects on specific cancer cell types when comparing their influence on normal cells and, as already mentioned they show increased anticancer potential *in vitro* when compared to their parent molecule, 2ME. Their signal transduction mechanism and the cause of differential cell susceptibility are now being addressed in our laboratory via several high throughput analyses, including gene microarray and protein array technology. The DNA of each cell stores information that also control cells growth. Gene expression microarrays allow researchers to monitor the levels of genes being affected. Data from gene microarray studies assist researchers to understand the response of cell on a specific drug. Proteins arrays also contribute to the latter by measuring the intracellular content of various proteins. Cell signaling events associated with these compounds will enable researchers to focus on affected cellular mechanisms, and the identification of possible new targets for therapeutic intervention. We therefore preferred to publish rather than patent our results, as much of the data we are generating may also be valuable to groups working on other cancers and the aspects thereof. We feel that the rapid public distribution of research results is to the benefit of the whole cancer research community. In summary, the initial results from *in vitro* studies suggest that the compounds have potential anticancer activity and that they are more selective towards tumorigenic and metastatic cancer cells when compared to non-tumorigenic cells. The facilities to conduct basic medical research on the mechanism of action, as well as animal studies are available in South Africa. However, clinical trials are expensive and input form pharmaceutical companies are of importance to further drug development into the clinical phase.

Acknowledgements

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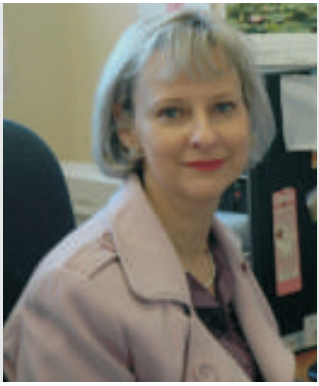

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Prof Annie Joubert and some of her postgraduate students

Allies in the fight against cancer

Prof Annie Joubert and some of her postgraduate students

André Stander (PhD student)

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