I GO TO SEEK A GREAT PERHAPS: THE QUEST TO OBTAIN AN APPROXIMATE UNDERSTANDING OF STEROID HORMONE RECEPTOR SIGNALLING

Prof Ann Louw

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Prof Ann Louw
Department of Biochemistry
Faculty of Science
Stellenbosch University

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ABOUT THE AUTHOR

Ann Louw (née Ramsay) was born on 30 March 1957 in Vereeniging, where she grew up until the age of 13 when her parents relocated to Mexico City. She started her schooling at Drie Riviere Laerskool and Drie Riviere Hoërskool in Vereeniging, continued it at the Greengates School in Mexico City and completed Grade 13 at Forest Hill Collegiate Institute in Toronto, Canada. She returned to South Africa for her tertiary education, completing her BSc with majors in biochemistry and physiology in 1977 and her BSc Honours in 1978 at Stellenbosch University, the alma mater of both her mother, Una Ramsay (BA in 1947), and her grandfather, Eben Dönges (BA in 1918, MA in 1919). She then continued studying towards her MSc in Biochemistry while working for the Research Department of the Western Province Blood Transfusion Service (1979–1985), completing the degree *cum laude* in 1984. She married her husband Albé in 1980. In 1986 her son Niel was born, and she needed some time to acclimatise to parenthood. Thus she accepted part-time employment: first as part-time technical officer in the Department of Medical Biochemistry at the University of Cape Town (1987–1988) and in 1988 as part-time technical officer in the Department of Biochemistry at Stellenbosch University, where she has remained ever since. In 1990, the year her second son, Ramsay, was born, she was appointed as lecturer in the Department of Biochemistry and continued her studies under the supervision of Prof Kirsten van der Merwe and Prof Pieter Swart, receiving her PhD in 1998, the year her daughter Una was born. She was promoted to senior lecturer in 1999, to associate professor in 2009 and to full professor in 2014. Under her supervision and co-supervision, 12 MSc students and seven PhD students obtained their degrees. She currently leads a group consisting of five MSc students, one PhD candidate and one postdoctoral fellow.

Prof Louw's research focuses on signal transduction via steroid hormone receptors, specifically the glucocorticoid receptor (GR) and the oestrogen receptor (ER), which mediate the intracellular actions of the stress hormone cortisol and the sex hormone oestrogen, respectively. Recent work on the phyto-oestrogenic activity of *Cyclopia* or honeybush tea has highlighted ER subtype-specific signalling, indicating a potential use in breast cancer prevention or treatment, while recent work on the GR is focussing on the implications of loss or gain of dimerisation. She has co-authored 26 peer-reviewed articles in international journals (h-index of 12), serves on the editorial board of *Steroids* and has twice received the Rector's Award for General Performance from Stellenbosch University (2011 and 2012).

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INTRODUCTION

Scientific research is a curious endeavour: on the one hand, we yearn for the certainty that we believe science can offer while on the other hand, we acknowledge the uncertainties inherent in "dealing with new phenomena at or beyond the boundaries of current knowledge" (Nature Medicine, 2001).

In thinking about what science and specifically scientific research at an academic institution such as Stellenbosch University means to me personally, I was finding it difficult to distil the essence of my own experience. During a conversation with my daughter Una, who was reading *Looking for Alaska* by John Greene, she introduced me to the phrase, "I go to seek a Great Perhaps".[1] This so perfectly articulated my own experience that I decided to use it as the title for this inaugural lecture.

Scientific research is concerned with questions; it is in essence a quest to understand how things work, a quest to make sense of life,[2] a quest to find 'the truth' within an overwhelming labyrinth of data. It requires curiosity, "a capacity of pleasure in knowing",[3] restless inquiry in the pursuit of a particular knowledge. The image of a quest evoked by the word 'seek' in "I go to seek a Great Perhaps", however, also conjures up images of mystery, the unknown, and troubles and tribulations to be overcome while promising prospects of marvels, wonders and delights to be discovered and a sense of awe to be experienced.

Most scientists are acutely aware of the fact that our knowledge is provisional, precariously awaiting the next discovery to render it obsolete, yet we learn to live with "approximate answers, possible beliefs and different degrees of certainty", as so strikingly expressed by Richard Feynman (Robbins, 1999). We have to embrace doubt, 'the Great Perhaps', yet also endeavour to harness that doubt in seeking out the marvels, wonders and delights within our chosen fields. That promise is perhaps ultimately what motivates and inspires us.

For the last 25 years my quest has involved trying to grasp a little of the mystery of the marvellous structure[4] of the signal transduction system whereby steroid receptors, such as the glucocorticoid receptor (GR) and the oestrogen receptor (ER), provoke a cellular or systemic effect. This quest to understand at a fundamental molecular level how specific compounds or physiological conditions elicit a specific response has been the driving force for my research, that and the promise of 'marvels, wonders and delights'. This article will highlight some of our approximate understandings of the system and describe some of the small instances where 'a perhaps' has guided our endeavours. The 'Great Perhaps' is still awaiting us. First, however, the steroid hormones and their signal transduction system should be briefly introduced.

STEROID HORMONES

Most of us have heard of steroids; anabolic steroid use in sports is frequently reported in the news, and many of us are using steroidal drugs for inflammation or hormone replacement therapy (HRT). The term 'steroid' describes both hormones endogenously produced by the body and artificially produced drugs that duplicate the action of the naturally occurring steroids. Steroid hormones belong to a class of chemical compounds known as steroids,[5] which are lipid-like molecules synthesised and secreted by the adrenal glands, gonads (testes and ovaries) and placenta during pregnancy. These hormones may be divided into five groups: glucocorticoids, mineralocorticoids, androgens, oestrogens and progestogens (Table 1).

1. Although much doubt exists due to lack of documentation, these are generally thought to be the last words of François Rabelais (1483–1553), a major French Renaissance writer.

2. Here I am referring to the property or quality that distinguishes living organisms from dead organisms, manifested in functions such as metabolism, growth, reproduction and response to stimuli or adaptation to the environment originating from within the organism (cf. the Online Merriam-Webster Dictionary (Life, s.a.)).

3. As Ruskin put it. (Davies, 1888).

4. Adapted from a quote attributed to Einstein. The full quote reads as follows: "The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvellous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity" (Miller, 1955).

Table 1: Classes of steroid hormones, endogenous and exogenous examples, and their functions and uses

Steroid hormone class	Endogenous		Exogenous (drugs)	
	Example	Function	Example	Use
Glucocorticoids	Cortisol	Stress response	Cortisone Dexamethasone Prednisolone	Anti-inflammatory Asthma Arthritis
Mineralocorticoids	Aldosterone	Salt and water balance	Fludrocortisone	Addison's disease
Androgens	Testosterone	Male reproductive tissues and secondary sex characteristics	Testosterone Nadrolone	Testosterone replacement therapy Anabolic steroids
Oestrogens	Estradiol	Female secondary sex characteristics	Premarin (CEE[a]) Ethinyl estradiol	HRT and contraception
Progestogens	Progesterone	Maintains pregnancy	MPA[b] and NET[c]	HRT and contraception

[a]Conjugated equine oestrogens; [b]medroxyprogesterone acetate; [c]norethindrone

VARIATIONS ON THE SAME THEME: STEROID HORMONE SIGNAL TRANSDUCTION

Steroid hormones can act as chemical messengers in a wide range of species and target tissues. They are transported in the blood from the tissues in which they are synthesised to target tissues where they will mediate their effects. Because of their lipophilic[6] nature, steroid hormones in blood are bound to carrier proteins such as albumin (the most abundant protein in blood plasma that non-specifically binds lipophilic molecules with low affinity[7]) and the high-affinity carrier proteins, corticosteroid-binding globulin (CBG) and sex hormone-binding globulin (SHBG) (Table 2). These carrier proteins not only act as facilitators of transport but also modulate the bioavailability of the steroid hormones as only the free, unbound fraction of a steroid hormone can transverse the cell membrane.[8] For example, in blood, only 3.5% of cortisol is in the free, unbound form with the majority of the cortisol transported bound to CBG (90%) and a small amount transported bound to albumin (6.5%). Thus the bioavailability of the free, unbound fraction of cortisol may be modulated by the concentration of CBG in blood, and if CBG levels increase, the percentage free cortisol will decrease and *vice versa*.

Table 2: Classes of steroid hormones, their high-affinity carrier proteins and cognate receptors

Steroid hormone class	Carrier proteins	Receptor	
			Abbreviation
Glucocorticoids	CBG	Glucocorticoid receptor	GR
Mineralocorticoids	CBG	Mineralocorticoid receptor	MR
Androgens	SHBG	Androgen receptor	AR
Oestrogens	SHBG	Oestrogen receptor	ER
Progestogens	CBG	Progesterone receptor	PR

5. Steroids comprise a group of cyclical organic compounds whose basis is a characteristic arrangement of carbon atoms in a four-ring structure linked together to form three six-carbon rings followed by a five-carbon ring and an eight-carbon side chain on carbon 17.

6. 'Lipophilic' from the Greek for 'fat loving' refers to the propensity of a compound to dissolve in oils, lipids and nonpolar compounds. The axiom that like dissolves like generally holds true, and thus steroid hormones as lipophilic substances do not dissolve particularly well in plasma (the liquid component of blood), which is hydrophilic (water loving).

7. 'Binding affinity' refers to the tendency of a compound (ligand) to bind to a protein, which may be a carrier protein or a receptor. We can quantify this binding tendency by determining the equilibrium dissociation constant for binding (Kd), which informs us of the concentration of ligand required to half-saturate the protein. The smaller the value of Kd, the higher the binding affinity as less ligand is required to bind to half the binding sites.

Having diffused across the cell membrane (Figure 1A, Step 1), steroid hormones bind specifically and with high affinity to their matching or cognate receptors (Table 2).

These receptors are part of a larger family of receptors, the nuclear receptor (NR) superfamily, that act as ligand-activated transcription factors. This denotes that upon binding of a ligand (the specific steroid hormone), the cognate steroid hormone receptor is empowered to regulate the transcription[9] of genes. This process, from ligand binding to regulation of transcription, is called the signal transduction pathway of steroid hormones. Although variations do occur, a central theme for the signal transduction pathway of steroid hormones is discernible and generally the individual steps involved in steroid signalling are sufficiently similar for a preliminary understanding. Specifically, the individual steps of steroid hormone signal transduction include binding of the steroid hormone to the cognate steroid receptor (Figure 1A, Step 2), which is tethered in the cytoplasm bound to chaperone molecules. Ligand binding results in a conformational change of the receptor, which allows for dissociation from the chaperone molecules, homodimerisation[10] and translocation into the nucleus (Figure 1A, steps 2, 3 and 4). Once in the nucleus, the activated NR homodimer binds to specific regions on DNA called nuclear receptor response elements (NREs), situated in the promoter[11] of steroid hormone-responsive genes (Figure 1A, Step 5), and recruits coactivators and the basal transcription machinery, which initiates transcription of the gene (Figure 1A, Step 6). Variations on the central theme of the signal transduction pathway of steroid hormone receptors include binding of steroid receptor monomers to negative-response elements (Figure 1B), thereby preventing initiation of transcription. In addition, a tethering mechanism whereby a steroid receptor monomer binds to another transcription factor and in so doing either attenuates or potentiates the transcription

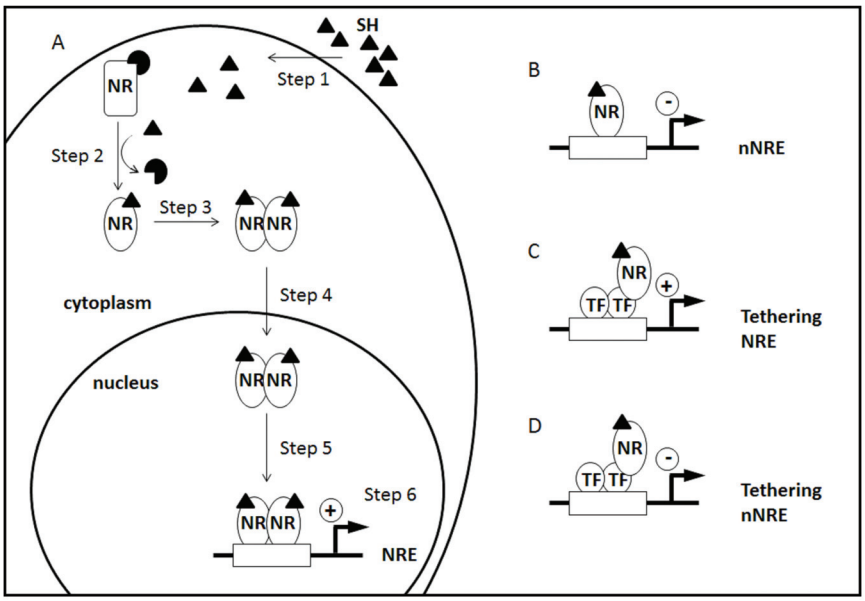

Figure 1: The signal transduction pathway of steroid hormones. (A) The central theme of transactivation through binding of an NR dimer to an NRE, which results in transcription of the gene. Step 1: free, unbound steroid hormone (SH) diffuses across cell membrane; Step 2: binding of SH to NR, conformational change and dissociation of chaperone molecules; Step 3: dimerisation; Step 4: nuclear localisation; Step 5: binding of a ligand-activated, dimerised NR to an NRE in the promoter of a responsive gene; and Step 6: transactivation or initiation of transcription (indicated with a + sign). Variations on the theme include (B) binding of an NR monomer to a negative-response element (nNRE), which prevents transcription (indicated with a - sign), and (C) positive and (D) negative tethering whereby an NR monomer binds to another transcription factor (TF) already bound to the promoter, thereby potentiating or attenuating the response of the originally bound transcription factor.

8. The cell membrane consists of a double layer of lipids, and thus the lipophilic steroid hormones, unbound to carrier proteins, can diffuse across the membrane into the cytoplasm of the cell.

9. Genes are transcribed to messenger RNA, which in turn is translated to form proteins. This process is often called the Central Dogma of Biology.

10. Dimerisation in this context involves a protein complex formed by two non-covalently bound steroid hormone receptors. A homodimer would be formed by the dimerisation of two identical bound steroid hormone receptors.

elicited by the initial transcription factor is a common regulatory variation seen in the signal transduction pathway of steroid hormones (Figure 1C and D).

THE DOUBLE-EDGED SWORD OF STEROID HORMONES: GLUCOCORTICOIDS AND OESTROGENS AS A CASE IN POINT

Steroid hormones are essential for the control of a plethora of regulatory systems in the human body. This is a finely balanced system, and over- or underproduction of these hormones can have serious health consequences, as can the use of exogenous hormones. As my group's focus has mainly been on the signal transduction pathway of glucocorticoids and oestrogens, I will henceforth focus on these two steroid hormones.

Glucocorticoids regulate a broad spectrum of physiologic functions essential for life and play a fundamental role in the maintenance of resting and stress-related homeostasis. Glucocorticoids act on nearly every tissue in the body and play a pivotal role in critical biologic processes, such as growth, reproduction, cognition, behaviour and maintenance of proper cardiovascular tone. In addition, they regulate the intermediary metabolism primarily through catabolic actions in the liver, muscle and adipose tissue, and influence substantially the quantity and quality of the inflammatory and immune responses (Busillo and Cidlowski, 2013). It is this last attribute that has resulted in their commercial exploitation.

Glucocorticoid drugs are widely used and prescribed[12] as immunosuppressant and anti-inflammatory drugs in diseases such as asthma, arthritis and autoimmune diseases. Although amongst the most efficacious drugs to treat inflammation, their efficaciousness is a double-edged sword as prolonged high-dose treatment can result in serious side effects such as glucocorticoid-induced osteoporosis, metabolic syndrome (increased glucose production by the liver leading to hyperglycaemia and increased serum lipids or hyperlipidaemia), muscle wasting (atrophy) and glucocorticoid resistance (when patients no longer respond to therapy).

Oestrogens are the female sex hormones and play an important role in the development of the reproductive tract and secondary sex characteristics and in reproductive behaviour. In addition, oestrogens maintain bone density and skin elasticity and protect against cardiovascular disease. However, because of their stimulating effect on growth and proliferation of certain cells, they are a risk factor for breast and endometrial cancer.[13]

Oestrogen levels in women decline at around 50 years of age and menopause ensues. Menopausal symptoms include hot flashes, night sweats, sleeping problems, vaginal dryness and osteoporosis. HRT has been most effective in controlling menopausal symptoms, yet the double-edged sword of steroid hormones has also had repercussions in that HRT increases the risk for developing breast cancer.[14] Most breast cancers are oestrogen dependent in that they contain the ER and signal transduction via this receptor results in proliferation of cancer cells. Selective oestrogen receptor modulators (SERMs), such as raloxifene and tamoxifen, which counteract the effects of oestrogen in the breast, are used as hormonal therapy in patients with ER-positive breast cancer (Jordan, McDaniel, Agboke and Maximov, 2014).

COMPOUND A: A GROWING TREE OF KNOWLEDGE

The story of Compound A (CpdA) illustrates my quest of 'seeking a Great Perhaps'. It is a quest that started during my PhD research and has continued to date, a quest that has brought unimagined possibilities and glorious prospects. Like a growing tree of knowledge, each new shoot evolved from a tiny bud to a growth of novel possibilities and fresh avenues to explore.

11. A promoter is a section of DNA that generally occurs upstream of a gene. The promoter is involved in regulating the transcription of the gene and is the site where transcription factors bind.

12. Advair Diskus from GlaxoSmithKline, a drug prescribed for the treatment of asthma, chronic bronchitis and chronic obstructive pulmonary disease, contains the synthetic glucocorticoid fluticasone propionate and is amongst the top 10 drugs by total sales and total prescriptions in the United States of America (Top 100 Most Prescribed, Top Selling Drugs. Medscape. May 13, 2014).

The first bud: Cytochrome P450c11

Gannabos (*Salsola tuberculatiformis* Botschantzev) is a Karoo shrub that causes *grootlamsiekte*[15] in sheep and contraception in rats (Figure 2A). When I joined the department in the 1990s, significant progress under the direction of Prof Kirsten van der Merwe had been made in unravelling the mechanism responsible for the actions of *S. tuberculatiformis* and in identifying the type of compound involved. Specifically, Proff Pieter and Amanda Swart had identified inhibition of adrenal cortisol biosynthesis as a potential site (the first *perhaps*) of action,[16] had developed two useful bioassays for evaluation of activity[17] and had produced an active but labile fraction, S2, from the shrub (Swart, Swart, Louw and van der Merwe, 2003). A putative structure of the active compound in the S2 fraction was proposed: a labile hydroxyphenyl aziridine precursor compound that could cyclise to form an aziridine (2-(4-hydroxyphenyl)-1-methyl-aziridiniumchloride). In an attempt to produce a more stable analogue, Dr Schalk de Kock synthesised a whole series of hydroxyphenyl aziridine precursor compounds (compounds A to Z).

I evaluated the series of compounds for activity and established that the first in the series, CpdA (2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride), had biological activity. The next step was to establish whether CpdA (Figure 2B) was a good analogue for the active compound(s) in the shrub *S. tuberculatiformis* and for the labile S2 fraction isolated from the shrub. Vaginal smears of nulliparous female Wistar rats[18] that had been administered either *S. tuberculatiformis* (orally) or CpdA (orally and intraperitoneally) indicated equivalent contraceptive activity characterised by a significant decrease during

A

B

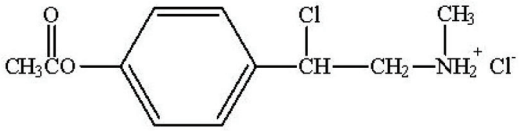

Figure 2: S. tuberculatiformis Botschantzev (A) and CpdA (2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride) (B), an analogue of the active compound in the shrub

13. Increased lifetime oestrogen burden, for example in women with early menarche and late menopause, is one on the strongest factors in risk of breast cancer in women.

14. Recent large randomised clinical trials on HRT, such as the Women's Health Initiative trial in the United States of America, and observational studies, such as the Million Women Study in the United Kingdom, have modified the risk-benefit perception of HRT, specifically regarding an increased risk of breast cancer and cardiovascular disease. The Endocrine Society statement of 2010 now recommends use of HRT with the lowest effective dose and for the shortest duration possible.

15. Grootlamsiekte is an Afrikaans term for a syndrome of prolonged gestation and foetal postmaturity first described amongst Karakul sheep feeding on *S. tuberculatiformis* and literally translates as 'big lamb disease'.

16. Initiation of parturition in sheep had been shown to be dependent on a foetal surge in cortisol, and ablation of this surge would result in prolonged gestation.

17. The first bioassay was based on the spectral and catalytic properties of cytochrome P450c11, the final enzyme involved in cortisol production, while the second bioassay involved a Pap smear (daily inspection of vaginal smears from female rats) to establish contraceptive properties.

administration in the number of oestrus days per 16-day period from 4.00 for control rats to 2.92 and 2.33, respectively (Louw, Swart, de Kock and van der Merwe, 1997; Louw and Swart, 1999). The contraceptive effect was reversible, and on cessation of treatment, the oestrus cycle of the rats returned to that of the control rats. In addition, the P450c11 spectral assay indicated that CpdA inhibited the final step in glucocorticoid synthesis in a manner similar to that of S2. Specifically, CpdA, like S2, elicited a Type II difference spectrum,[19] indicative of the presence of a nitrogen moiety, and inhibited the Type I difference spectrum elicited by deoxycorticosterone, the natural substrate of cytochrome P450c11 (Figure 3) (Louw *et al*., 1997; Louw, Allie, Swart and Swart, 2000; Swart *et al*., 2003). Comparison of CpdA's inhibitory capacity with that of other well-known inhibitors of cytochrome P450c11, however, indicated that CpdA was not a particularly effective inhibitor (Louw, Swart and Allie, 2000).

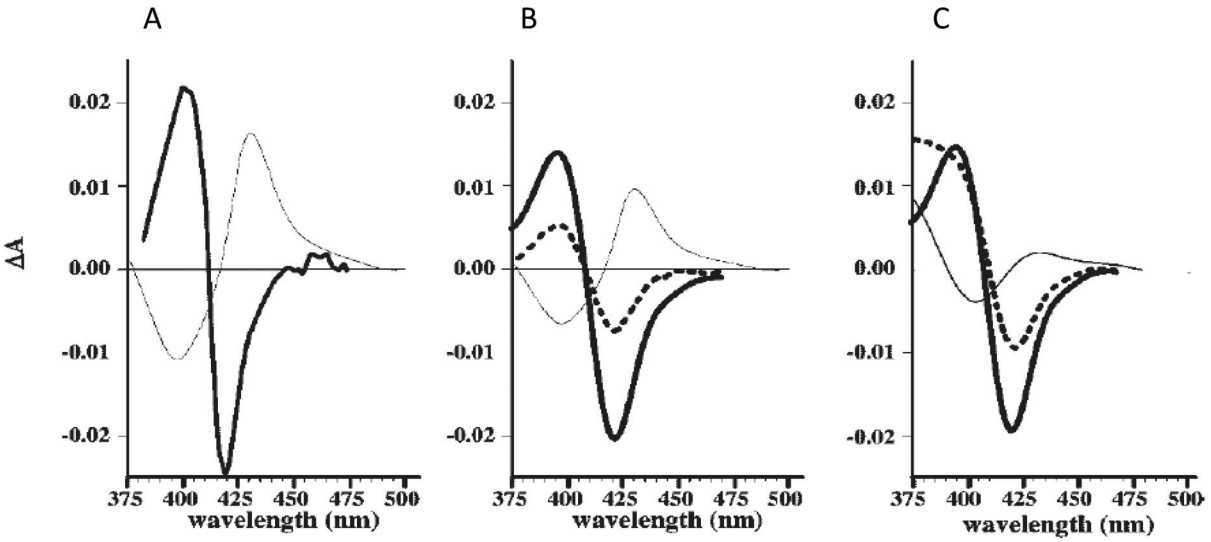

Figure 3: The P450c11 bioassay. (A) Examples of cytochrome P450 Type I (bold solid line) and Type II (solid line) difference spectra. (B) Type I difference spectrum (bold solid line) of a sheep adrenal cytochrome P450 preparation induced by deoxycorticol (DOC) and Type II difference spectrum (solid line) induced by either S2 (B) or CpdA (C). Inhibition of DOC-induced Type I difference spectrum (bold dotted line) by either S2 (B) or CpdA (C) (Swart et al., 2003).

The second bud: Corticosteroid-binding globulin

The spectral studies of the interaction of CpdA with cytochrome P450c11 were most challenging as the effects of CpdA on both the Type II and Type I spectral properties attenuated over time. The question that arose at this stage was, If CpdA was such a labile compound, how could it possibly mediate its effects physiologically? Prof Pieter Swart, my PhD promoter, suggested that *perhaps* a component in blood plasma stabilised CpdA.

I investigated this possibility and indeed found that although CpdA's inhibitory capacity was attenuated in buffer by 30 minutes, addition of rat or sheep plasma could stabilise the inhibitory activity of CpdA (Louw *et al*., 1997). Electrospray mass spectrometry confirmed our results by indicating that in buffer, the cyclisation of CpdA to its corresponding aziridine followed a similar time course to that of the attenuation of cytochrome

18. Wistar albino rats, developed at the Wistar Institute in 1906 for use in basic biological and medical research, are notably the first rat strain developed to serve as a model organism. The oestrous cycle of the rat lasts four days on average and comprises distinct phases characterised by different cell types in vaginal smears (proestrus, oestrus, metestrus and diestrus). The classical Papanicolaou technique, developed by George Papanikolaou, is used to stain the vaginal smears and identify the cells. This is the same procedure used for cervical cancer screening in gynaecology (known as the Pap smear).

19. All cytochromes P450 have a heme iron in their active sites. Interaction of certain inhibitors or substrates with the heme iron in the active site gives rise to unique spectral properties. A Type I difference spectrum (absorbance maximum at 385–395 nm and absorbance minimum at 420 nm) is elicited by the natural substrate for the enzyme (e.g. deoxycorticosterone for P450c11) whereas a Type II difference spectrum (absorbance maximum at 430 nm and absorbance minimum at 390 nm) is often observed with ligands that contain a nitrogen moiety.

P450c11 inhibition and that addition of sheep plasma could retard this cyclisation. Furthermore, the electrospray mass spectrometry results established that CpdA and not its corresponding aziridine was the active agent inhibiting cytochrome P450c11 activity.

In serum or plasma, steroid hormones are either free in solution or bound to albumin, CBG and SHBG (Table 2). Thus we thought that *perhaps* these proteins could be involved in the stabilisation of CpdA's activity in plasma. To test this hypothesis, we heated sheep plasma, which denatures CBG and SHBG but not albumin, and established that heated plasma abrogated the stabilising effect observed in native plasma (Louw *et al.*, 1997). As rat plasma does not contain SHBG, CBG was selected for further investigation. Studies of rat and sheep plasma indicated that CpdA could displace endogenously bound glucocorticoids (cortisol in sheep or corticosterone in rats) but not progesterone from CBG, and concomitantly, a significant increase in free glucocorticoids but not progesterone was measured (Louw, Swart and Allie, 2000). In addition, we established that competitive inhibition of glucocorticoid binding occurred, which would suggest direct competition with the endogenous ligands, and that CpdA probably bound at the same ligand binding site on CBG (Louw, Swart and Allie, 2000).

Although the studies establishing that CpdA binds to and is stabilised by CBG in plasma answered the troublesome question of how such a labile compound could have a physiological effect, the results also raised the possibility that *perhaps* the contraceptive effects of CpdA were not mediated exclusively through inhibition of glucocorticoid synthesis (cytochrome P450c11 inhibition), as previously thought. We were thus faced with two contrasting scenarios (Figure 4), both of which had been shown to elicit a contraceptive effect in rats:

1. The first scenario mimics adrenalectomy (removal of adrenal glands) in that glucocorticoid biosynthesis in the adrenal is inhibited by CpdA.
2. The second scenario mimics a state of chronic stress with increased free glucocorticoids in that CpdA displaces glucocorticoids from CBG with a concomitant rise in free glucocorticoid levels.

We postulated that Scenario 1 should result in a decrease in total and thus also free glucocorticoid levels and that this decrease in free glucocorticoid levels would attenuate the negative feedback of glucocorticoids on the synthesis of several other proteins, most notably adrenocorticotropic hormone (ACTH)[20] and CBG,[21] resulting in their increased production. In contrast, Scenario 2 would result in an increase in free glucocorticoids with a concomitant decrease in ACTH and CBG due to increased negative feedback. Administration of *S. tuberculatiformis* and CpdA at a contraceptive dose resulted in a significant increase in free glucocorticoids and a decrease in ACTH and CBG levels (Louw and Swart, 1999). These results then favour the second scenario, which mimics stress and entails binding of *S. tuberculatiformis* and CpdA to CBG, thereby displacing endogenous glucocorticoids and resulting in an increase in free glucocorticoids that feedback negatively on, amongst others, the pituitary (site of ACTH synthesis) and liver (site of CBG synthesis).

20. ACTH is a hormone produced by the anterior pituitary and is part of the hypothalamic-pituitary-adrenal axis (HPA-axis). The HPA-axis controls the secretion of glucocorticoids: stress activates the hypothalamus in the brain to secrete corticotropin-releasing factor (CRF), which activates the pituitary (a small gland near the base of the brain) to secrete ACTH that in turn activates the adrenal glands (situated above the kidneys) to secrete glucocorticoids. Glucocorticoids have a negative feedback effect on the hypothalamus and pituitary, decreasing the secretion of CRH and ACTH, respectively. This negative feedback system is a common motive in biology and is designed to limit the secretion of hormones.

21. CBG, synthesised in the liver, is regulated by glucocorticoids. CBG biosynthesis is stimulated during adrenalectomy and inhibited during exogenous glucocorticoid treatment.

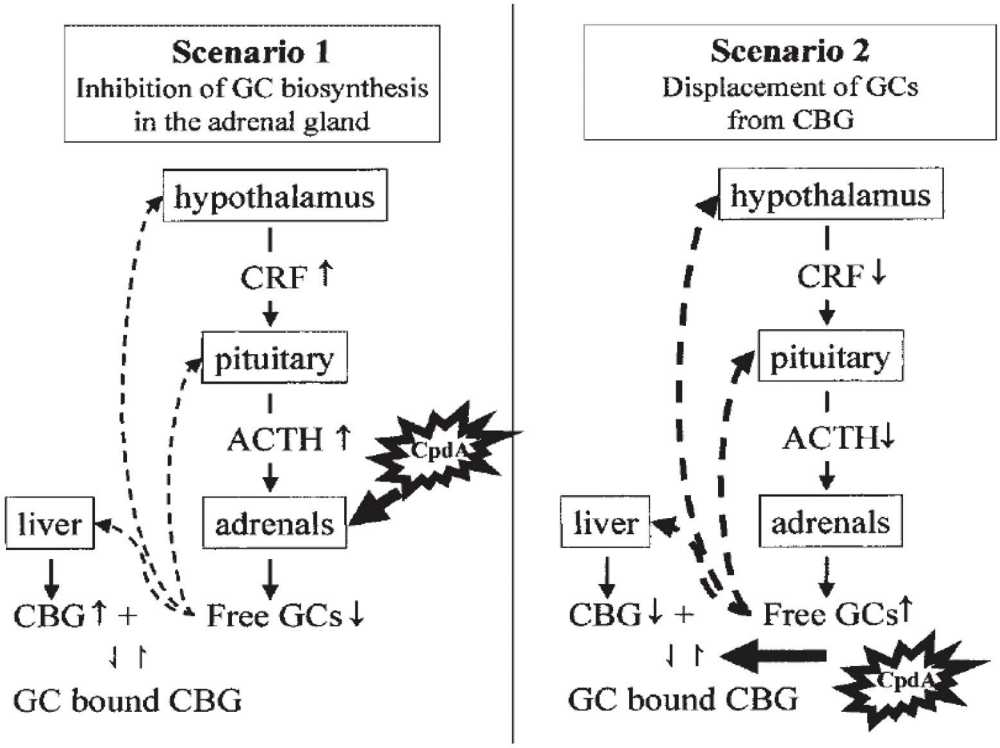

Figure 4: Schematic of the two possible scenarios that could explain the contraceptive effect of S. tuberculatiformis Botsch. or CpdA in rats. Diagram depicts the HPA-axis, which shows negative feedback (dashed arrows) by free glucocorticoids (GCs) and the liver, which synthesises CBG and is also under negative feedback from free GCs. The intensity of the dashed arrows reflects the strength of the negative feedback. In addition, for both scenarios, the effect on hormones (CRF, ACTH and GCs) and binding proteins (CBG) is indicated by ↓ if the levels decreased and by ↑ if the levels increased (Swart et al., 2003).

Stress, as exemplified by increased levels of free glucocorticoids, has been shown to profoundly suppress the reproductive system at multiple levels, including the hypothalamus, pituitary and gonads, and thus our results suggest that this scenario could account for the contraceptive mechanism of action of *S. tuberculatiformis* and CpdA. In addition, our results could also explain the syndrome of prolonged gestation in sheep. Parturition is sheep is accompanied by activation of the foetal HPA-axis (suggested to be due to the stress of foetal hypoglycaemia resulting from placental insufficiency at this late stage of gestation), resulting in a progressive prepartum surge in glucocorticoids and ACTH. Despite this, free glucocorticoid levels do not increase as the foetal liver increases production of CBG.[22] In addition, local CBG production in the foetal pituitary attenuates the negative feedback on ACTH production. Taken together, these observations suggest that foetal CBG buffers the prepartum increase in glucocorticoids, thus contributing to continual HPA-axis signalling and ACTH production. Just before parturition, the free glucocorticoid levels rise significantly, presumably due to saturation of CBG by the continued surge in glucocorticoid production. These high levels of free glucocorticoids are required to activate an enzyme that reduces maternal progesterone to concentrations at which uterine activity is increased and labour ensues. Ingestion of *S. tuberculatiformis* during the last 50 days of gestation results in prolonged gestation, and we could *perhaps* suggest that displacement of glucocorticoids from CBG with a concomitant increase in free glucocorticoid levels could increase systemic CBG levels,[23] thereby

22. The sheep foetal liver responds differently from the adult liver in that glucocorticoids stimulate rather than inhibit CBG production.

23. Interestingly, the dose of glucocorticoids required to increase foetal CBG production is lower that the dose required to initiate parturition.

delaying the prepartum surge in free glucocorticoids required for parturition. Eventually, however, increased trophic drive from the hypothalamus will overcome these factors and parturition, albeit delayed, will occur.[24] The above discussion is speculative at present and would require further research to establish a definitive mechanism.

The third bud: The glucocorticoid receptor

Having shown that CpdA can bind to two glucocorticoid-binding proteins, the cytochrome P450c11 enzyme involved in glucocorticoid biosynthesis and CBG, the high-affinity glucocorticoid transport protein in plasma, we wondered whether *perhaps* CpdA could also interact with the protein involved in transducing the glucocorticoid signal, the GR.

Serendipitously, at this stage Prof Janet Hapgood, a researcher with extensive signal transduction expertise, joined the department and we embarked on a study to investigate CpdA's ability to interact and signal via the GR. Not long after, Guy Haegeman[25] from Gent University in Belgium contacted us and we established a very fruitful collaboration on CpdA.

We established that CpdA bound reversibly to the GR, albeit at an affinity lower than that of dexamethasone, a well-known GR agonist[26] (De Bosscher, Vanden Berghe, Beck, Van Molle, Hennuyer, Hapgood, Libert, Staels, Louw and Haegeman, 2005; Robertson, Allie-Reid, Vanden Berghe, Visser, Binder, Africander, Vismer, De Bosscher, Hapgood, Haegeman and Louw, 2010). In addition, CpdA was found to slow the rate of binding of radioactively labelled dexamethasone to the GR but to have no effect on the dissociation rate of dexamethasone from the GR (Robertson *et al.*, 2010). Partial protease digestion, a relatively crude technique to compare the tertiary[27] structure of proteins, indicated that CpdA resulted in a conformational change similar to that of dexamethasone, which corroborated the binding data by indicating that CpdA did indeed interact with the GR (De Bosscher *et al.*, 2005).

Binding of a ligand to a receptor does, however, not give any indication of the ability of the ligand to signal through the receptor, nor does it provide information regarding the type of signalling that occurs, in other words, whether the ligand is an agonist or an antagonist. Having shown that CpdA bound to the GR, we were thus curious to know whether it signalled through the receptor and investigated this using promoter reporter assays[28] for transactivation and transrepression (refer back to Figure 1). Interestingly, CpdA could not transactivate via a glucocorticoid response element (GRE, similar to the NRE of Figure 1); however, transrepression via a tethering nGRE (similar to the tethering nNRE in Figure 1) did occur and was as effective as that of the agonist dexamethasone (De Bosscher *et al.*, 2005). This was very exciting news as at that stage, the focus of research for new anti-inflammatory drugs that were as effective as glucocorticoids but with a reduced side effect profile (remember the double-edged sword of glucocorticoids) had shifted to a search for drugs that could dissociate the transactivation capacity of the GR from its transrepression capability. The reigning paradigm[29] at that stage consisted of the idea that transrepression mediated the positive anti-inflammatory action of glucocorticoids while transactivation mediated the negative side effect profile of these drugs. Several so-called dissociated glucocorticoids were being described at around this time, many of them with only a slight preference for transrepression over transactivation while others behaved well *in vitro* yet failed to maintain their dissociated profile *in vivo*. What made CpdA so remarkable was that it displayed complete dissociation of transrepression from transactivation. In addition, follow-up studies of endogenous genes and their protein products indicated that CpdA could inhibit the transcription of proinflammatory genes and

24. It is interesting to note that an increased maternal ratio of cortisol to CBG has been identified as a marker for preterm labour in humans.

25. Guy actually saw a poster on CpdA's interaction with GR presented by a student at a South African Society of Biochemistry and Molecular Biology conference at which neither Janet nor I was present as we were both heavily pregnant, Janet with Sammy and I with Una. Our collaboration was formalised in two bilateral agreements between South Africa and Belgium and also included researchers from the Catholic University of Leuven.

26. In steroid receptor signalling, an agonist will mimic the action of the endogenous hormone (i.e. cortisol for human GR) while an antagonist will antagonise the effect of the agonist. Thus an antagonist does not display activity unless in the presence of an agonist.

27. The primary structure of a protein consists of the sequence of amino acids contained in the protein, the secondary structure refers to the general three-dimensional form of local segments of proteins while a protein's tertiary structure refers to its global geometric shape.

28. Promoter reporter assays are commonly used in signal transduction research as they are simple, quick and specific. They are artificial in that the signalling of an artificial construct that contains only the promoter element of interest (i.e. an NRE) coupled to the gene for an easily assayed reporter protein (i.e. luciferase) is measured. This is in contrast to an endogenous promoter that may contain several promoter signalling elements besides the one being investigated.

decrease the levels of proinflammatory proteins (i.e. interleukin 6) without transactivating genes involved in the metabolic side effects of glucocorticoids (i.e. tyrosine amino transferase). Furthermore, in a mouse model of inflammation, CpdA preformed as well as dexamethasone in preventing inflammation without giving rise to increased blood glucose levels such as seen with dexamethasone (De Bosscher *et al*., 2005).

These exciting attributes of CpdA opened up glorious prospects for us as researchers, and we set out to systematically investigate the signal transduction pathway of the GR as regulated by CpdA and to compare this with regulation by dexamethasone. We have found several similarities and tantalising differences between the signal transduction elicited by CpdA and that elicited by dexamethasone (De Bosscher *et al*., 2005; Robertson *et al*., 2010; Tanner, Louw, Rombauts, Hapgood and Claessens, 2003; Visser, Smith and Louw, 2010). However, I would like to focus on the attribute of CpdA that we now consider to be fundamental to its dissociated activity, which is also the attribute that has opened up new vistas for us to explore. This attribute involves the third step in Figure 1A: GR dimerisation. The established model, as illustrated in Figure 1A, was that agonists such as dexamethasone resulted in dimerisation of the GR, yet we found that CpdA not only did not result in dimerisation, it effectively abrogated the basal levels of GR dimerisation observed in the absence of ligand (Robertson *et al*., 2010). We were so astounded by this finding that we used three separate techniques to confirm that indeed CpdA did not result in GR dimerisation (Figure 5). We also followed this up by comparing the activity of CpdA via the wild-type GR (wtGR) with the behaviour of dexamethasone via a mutant GR, GR^{dim}, which is unable to dimerise.[30] We argued that if loss of GR dimerisation was mechanistically the essential difference between GR signalling via CpdA and signalling via dexamethasone, we should be able to recapitulate the critical features of CpdA signalling by using dexamethasone and GR^{dim}. We could indeed mimic the dissociated behaviour of CpdA via wtGR in transrepression and transactivation assays by using dexamethasone and GR^{dim} (Robertson *et al*., 2010).

CpdA has now emerged not only as a possible lead compound for future therapeutic applications but also as a powerful analytical tool to investigate the implications of GR dimerisation in the signal transduction pathway of glucocorticoids.

29. This paradigm has since been found to be an oversimplification as induction of several important anti-inflammatory signals has since been shown to be mediated via direct binding of GR to DNA. In science, such modifications of reigning paradigms occur when additional information that does not fit the original paradigm becomes available and thus necessitates modification of a paradigm or adoption of an entirely new paradigm.

30. Holger Reichardt, currently at the University of Göttingen in Germany, produced this mutant and has also established a transgenic mouse model with GRdim. We plan to collaborate in an investigation of the relevance of GR dimerisation.

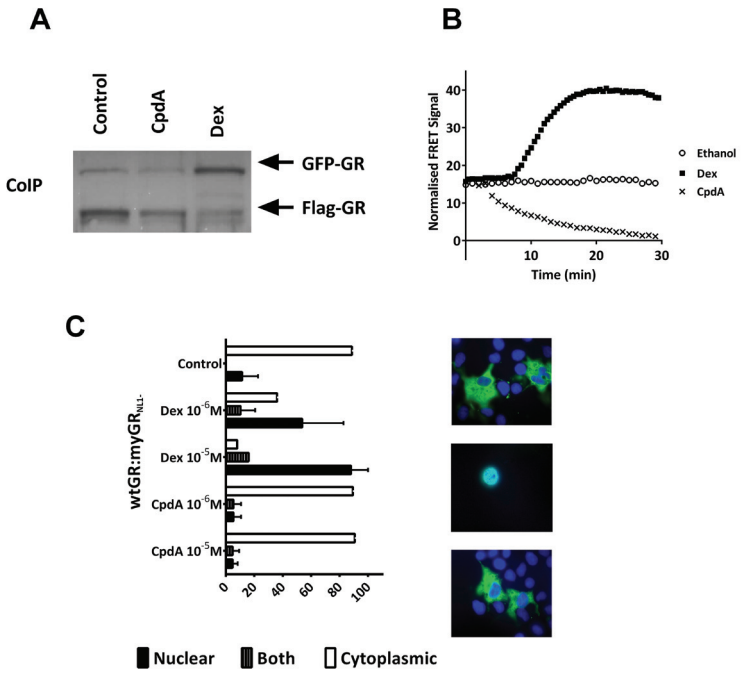

Figure 5: CpdA abrogates dimerisation of the GR. Three experimental techniques were used to confirm that CpdA abolished GR dimerisation.[31] (A) Co-immunoprecipitation of differentially tagged GR: FLAG-tagged GR (mass 96 kDa) and green fluorescent protein (GFP)-tagged GR (mass 128.5 kDa) were transfected into cells and treated with either ethanol (control), dexamethasone (DEX) or CpdA. Cellular extracts were immunoprecipitated with anti-FLAG antibody that precipitates proteins containing a FLAG-tag (such as the FLAG-tagged GR). Any proteins that have associated with the FLAG-tagged GR will be co-immunoprecipitated and may be observed using a second antibody using Western blotting.[32] In this case, the second antibody used was against the GR. The difference in molecular weight between the FLAG- and GFP-tagged GR allows for identification of the two species of GR. The darker band at the GFP-GR position observed with dexamethasone indicates that more GFP-tagged GR was immunoprecipitated and therefore greater dimerisation occurred when dexamethasone was added. CpdA results in a lighter band indicative of less dimerisation. (B) FRET analysis of differentially tagged GR: cyan fluorescent protein (CFP)-tagged GR and yellow fluorescent protein (YFP)-tagged GR were transfected into cells and treated with ethanol (control), dexamethasone or CpdA while FRET[33] intensity was monitored using a fluorescence microscope. An increase in FRET, as observed with dexamethasone, indicates increased dimerisation while the decrease in FRET, as observed with CpdA, indicates abrogation of dimerisation. (C) Nuclear translocation of a GR nuclear translocation mutant: wtGR was cotransfected with a c-myc-tagged nuclear translocation mutant GR (myGRNL1), which is unable to translocate to the nucleus even in the presence of an agonist ligand. However, if myGRNL1 dimerises with wtGR, the nuclear localisation signal present in the wtGR is sufficient for nuclear localisation of the wtGR:myGRNL1 dimer. Localisation of myGRNL1 was visualised by indirect immunofluorescence (an anti-c-myc antibody recognises myGRNL1 while a second antibody, which contains a florescent tag [green], is directed against the first antibody). Nuclei were visualised with a blue stain. From the micrographs (top: control; middle: DEX; bottom: CpdA) and quantification graphs, it is clear that dexamethasone induces nuclear localisation of myGRNL1, which implies dimerisation, while CpdA is unable to induce nuclear localisation of myGRNL1 and thus is also unable to induce dimerisation (adapted from Robertson et al., 2010).

31. Because GR dimerisation is essentially a homodimerisation, which involves two identical molecules, we tag (small peptide sequences genetically grafted onto a recombinant protein) the GR proteins with small molecules that contain attributes that would allow us to track and distinguish one GR from another.

32. Western blotting is a widely used analytical technique to identify a specific protein in a mixture. It relies on separation of proteins according to their molecular weight and interaction with an antibody against the protein of interest. The correlation of expected molecular weight with the presence of a specific antibody-protein interaction confirms the identity of the protein.

33. FRET, an acronym for 'fluorescence resonance energy transfer', is a technique that permits investigation of intramolecular interactions such as GR dimerisation. If two fluorophore-tagged molecules come into close contact, excitation of the donor fluorophore (e.g. CFP-tagged GR) results in an energy transfer from the donor fluorophore to the acceptor fluorophore (e.g. YFP-tagged GR), which may be measured as a specific emission of fluorescence.

One such investigation, emanating from our laboratory in collaboration with Profs Kathy Myburgh and Carine Smit,[34] focussing on the interplay of the inflammatory and stress systems in the liver has yielded some very interesting central paradigms regarding the different outcomes of CpdA and dexamethasone treatment. The liver plays a critical integrative role in inflammation and stress by producing the acute-phase proteins (APPs) required for swift resolution of inflammation as well as by delivering systemic glucose, through gluconeogenesis, required to fuel the stress response (often called the metabolic response to glucocorticoids). Although the repression of inflammatory cytokines by glucocorticoids is well studied as being the basis of the anti-inflammatory action of glucocorticoids, the effect of inflammatory cytokines on glucocorticoid action, especially in the periphery, is less well studied. Having shown *in vivo* that the pro-inflammatory cytokine interleukin 6 (IL-6) antagonises the effect of glucocorticoids in the liver, specifically by limiting autologous down-regulation of GR levels[35] and by preventing full-blown up-regulation of a glucocorticoid-inducible metabolic enzyme in the liver (Smith, Wilson, Louw and Myburgh, 2007), we suggested that *perhaps* the brake on glucocorticoid action provided by IL-6 in the periphery might limit an overshoot of the deleterious endocrine responses of tissues to stress. This could act as a second feedback loop complementing the well-known feedback loop whereby glucocorticoids limit the effects of inflammatory cytokines and in so doing exert a finer control over homeostasis than would be present without this dual system of reciprocal control (Smith *et al*., 2007). Expansion of the study in tissue culture confirmed that glucocorticoids and pro-inflammatory cytokines generally have divergent effects on the GR levels and metabolic enzymes (glucocorticoids upregulate while IL-6 down-regulates the GR and metabolic enzymes) while their functions are convergent on the APPs (both glucocorticoids and IL-6 upregulate positive APPs and down-regulate negative APPs) (Visser, Smith and Louw, 2010). In contrast, CpdA had effects convergent to those of IL-6 on the GR, metabolic enzymes and APPs, thus confirming that CpdA would result in effective control of inflammation while being less likely to lead to glucocorticoid-induced side effects. One of the APPs investigated in the above study was CBG, which is a negative APP down-regulated during the acute-phase response while also being responsible for the transport of glucocorticoids in plasma and for regulating their bioavailability to tissues (Table 2). The molecular signal transduction mechanism whereby glucocorticoids down-regulate CBG has now also been elucidated by our group and involves tethering of the GR to the transcription factor C/EBPβ, a mechanism analogous to that in Figure 1C (Verhoog, Allie-Reid, Vanden Berghe, Smith, Haegeman, Hapgood and Louw, 2014).

CpdA is currently available commercially,[36] and many researchers have used CpdA to investigate aspects of steroid receptor action, from prostate cancer to osteoporosis (as of March 2015 our publication that first discussed the dissociated activity of CpdA [De Bosscher *et al*., 2005] has garnered 124 citations).

NEW GROWTH: THE ROLE OF GLUCOCORTICOID RECEPTOR DIMERISATION IN GLUCOCORTICOID SIGNAL TRANSDUCTION

During the time that we were thinking about the wider implications of CpdA's ability to abrogate GR dimerisation and thus by implication the relevance of GR dimerisation in glucocorticoid signalling, we were also attempting to reconcile some puzzling binding results with CpdA. We found that CpdA's ability to bind to the GR appeared to be influenced by the concentration of the GR present while the affinity[37] of dexamethasone (always included as a control) for the GR also appeared to be modulated by GR concentration. This prompted us to wonder whether *perhaps* GR concentration had a greater effect on GR signalling than had previously been thought.[38] A seminal paper presented at our weekly laboratory journal club (Cho, Kagan, Blackford, Szapary and Simons, 2005) added another clue: this work from

34. Both are from the Department of Physiological Sciences at Stellenbosch University.

35. Autologous down-regulation of GR by glucocorticoids is thought to be a mechanism whereby glucocorticoids down-regulate the concentration of their cognate receptor, thus limiting signalling and preventing overshoot of glucocorticoid action, which would threaten homeostasis.

36. Available from, amongst others, ENZO Life Sciences, product number ALX-550-516, Santa Cruz Biotechnology, product number sc-221677, and Calbiochem, product number 346110.

37. The affinity of a ligand for a receptor had always been thought to be an immutable constant and certainly not one that should be affected by receptor concentration.

38. At this point, the assumption in steroid receptor signalling was that more receptors would simply result in a greater efficacy in response to ligand; in other words, only the amplitude of the response would be affected.

the laboratory of Stoney Simons Jr., a well-respected researcher investigating the factors that modulate the potency and efficacy of the dose-response curve[39] of transactivation via the GR, suggested that cooperative ligand binding to the GR might occur at high but not at low receptor concentrations. Interestingly, the researchers had not pursued the matter nor had they considered that cooperative ligand binding presupposed the presence of at least two binding sites.[40] We then hypothesised that wtGR would display cooperative ligand binding at high concentrations and that *perhaps* this was due to ligand-independent dimerisation. We were thus also very interested in the implications of a loss or gain of dimerisation potential and thus employed a continuum of GR species (Table 3).

Table 3: The continuum of GR species potentially available to evaluate loss or gain of dimerisation

GR species	GR monomers unable to dimerise	GR monomers that dimerise in the presence of agonists	GR dimers that elicit cooperative agonist binding
Genetically generated	Mammalian cell culture cells transfected with GR^{dim}	Mammalian cell culture cells transfected with low wtGR concentrations	Mammalian cell culture cells transfected with high wtGR concentrations
Pharmacologically generated	CpdA	Agonists at low levels of wtGR	Agonists at high wtGR concentrations

GR levels vary among tissues and individuals with, for instance, skin cells having 15 times as many GR molecules as lung cells. In addition, GR levels are altered by physiological and pharmacological effectors such as cancer and AIDS that may upregulate GR levels or stress and use of glucocorticoid drugs that may decrease GR levels. We were thus concerned that the levels of GR used in our study should not be supra-physiological and used three physiologically relevant yet statistically different GR concentrations, designated low, medium and high. Luckily the physiological range of GR concentrations is quite wide (4–900 fmol GR/mg protein in healthy individuals and rising as high as 2 777 fmol GR/mg protein in the skin of AIDS patients), so we had sufficient scope for differentiation.

In our first experiment, we assessed the effect of GR concentration on transcription using a promoter reporter assay and found that increasing GR concentration resulted in a much greater increase in efficacy and potency[39] of the GR response than we had expected (Table 4). Increasing wtGR levels, two-fold from low to medium levels and four-fold from low to high levels, resulted in an increase in efficacy (Bmax) of four- and 12-fold, respectively, and furthermore, a massive 650- and 2 600-fold increase, respectively, in potency (EC_{50}) was observed (Robertson, Rohwer, Hapgood and Louw, 2013). In addition, we were intrigued by an observation of an increase in basal induction, which we attributed to ligand-independent transactivation, arguing that *perhaps* at higher concentrations the GR could dimerise and elicit a transcriptional response even in the absence of ligand. We established that at medium and high GR concentrations, the GR indeed dimerised in the absence of ligand, shifting the equilibrium between GR monomers and dimers in favour of more dimers (from 38% dimers to over 60% dimers at higher GR concentrations) (Robertson *et al*., 2013). Furthermore,

39. A dose-response curve (whereby the magnitude of the response, for example induction of a gene or expression of a protein, to different concentrations of ligand is plotted) may be characterised by two parameters: efficacy (Bmax: maximal response achieved) and potency (EC_{50}: the concentration of ligand that elicits a half-maximal response). The Bmax and EC_{50} are useful parameters to compare the signalling between different compounds, such as dexamethasone and CpdA.

40. Cooperative ligand binding is observed in systems that have multiple binding sites. If the affinity of the binding sites for a ligand apparently increases, it is called positive cooperativity while if the affinity decreases, it is called negative cooperativity. In cooperative ligand binding, the binding of the first ligand to the receptor changes the affinity of the second ligand binding to the second binding site.

at high and medium GR concentrations, ligand binding to the GR was shown to be cooperative (Hill slope > 1)[41] and to display a three-fold increased affinity (K_d) for the ligand. We could recapitulate the findings on an endogenous glucocorticoid-responsive gene by also showing the greater-than-expected increase in efficacy and potency, albeit not to the same degree as for the artificial system used in promoter reporter studies. Intriguingly, we could also show that at medium and high GR concentrations, the GR was already present on the promoter of the gene (i.e. loaded onto the DNA), poised to initiate transcription upon arrival of the ligand. To confirm the importance of dimerisation for the observed behaviour of the GR at higher concentrations, we utilised GR^{dim} constructs and established that GR^{dim}, unable to dimerise to the same extent as wtGR, could not elicit cooperative ligand binding or ligand-independent DNA loading, nor could it elicit the same massive shift in transactivation potency as seen with wtGR. We followed up this work by investigating the implications of higher GR concentrations and the ability to dimerise on the nuclear import and export rate of the GR and concluded that the ability of the GR to dimerise resulted in an increased nuclear import rate and a decreased export rate due to an increase in glucocorticoid-dependent GR nuclear foci and that the shorter residence time in the nucleus of the GR^{dim} might be a contributing factor to explain the lowered potency of its glucocorticoid response (Robertson, Hapgood and Louw, 2013).

Table 4: Summary of dose-response parameters measured at low, medium and high GR concentrations

Fold increase in:	**Low GR concentration**	**Medium GR concentration**	**High GR concentration**
GR concentration	1	2	4
Maximal efficacy (Bmax)	1	4	12
Potency (EC_{50})	1	**650**	**2 600**
Basal induction	1	4.5	10

Table adapted from Robertson et al., 2013.

To place our results in terms of what we believe, the physiological implications may be as follows: In tissues with high GR concentrations, such as in skin or during certain diseased states, significant formation of preformed ligand-independent dimers will be evident, displaying positive cooperative ligand binding and ligand-independent priming of DNA, poised for the arrival of ligand. The tissues would therefore be hypersensitive

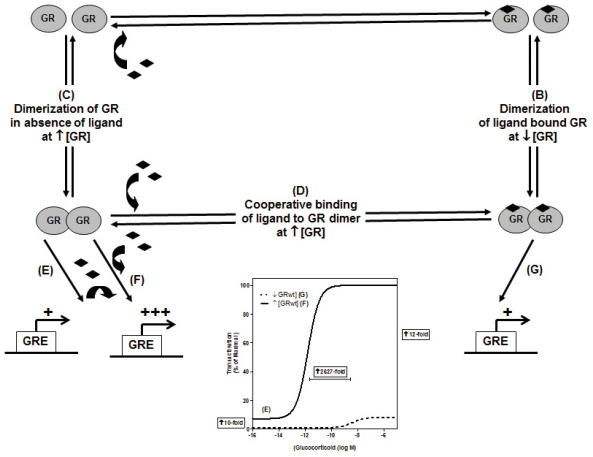

Figure 6: Model comparing downstream effects at high and low GRwt concentrations. Pathways (A), (B) and (G) denote conditions at low GR concentrations. (A) Non-cooperative ligand binding to GR monomers, followed by (B) ligand-dependent dimerisation and (G) transactivation. Pathways (C), (D), (E) and (F) denote conditions at high GR concentrations. (C) Ligand-independent dimerisation of the GR, followed by either (E) ligand-independent DNA loading and transactivation and/or (D) cooperative ligand binding and (F) transactivation with increased potency. Graph insert: Transactivation results simplified as percentage maximal transactivation. Indicated are fold increase in ligand-independent transactivation (E) and increase in transactivation efficacy and potency at higher concentrations of GR – difference between (G) and (F) (Robertson et al., 2013).

41. A ligand binding curve can provide information regarding the number of receptor molecules in the system and the affinity of the ligand for the receptor (K_d) and indicate whether ligand binding is cooperative or not. Non-cooperative binding will have a Hill slope of one while positive cooperative ligand binding will have a Hill slope > 1.

to glucocorticoids, whether endogenous or exogenous, and would exist in a suspended yet partially activated state on the promoter DNA, ready to induce a maximal transactivative response once exposed to ligand (Figure 6). In addition, tissues that contain high concentrations of GR would respond faster to glucocorticoids due to their increased rate of nuclear import and maintain nuclear localisation for longer following glucocorticoid withdrawal because of their slower rate of nuclear export, thus contributing to their enhanced sensitivity to glucocorticoids when compared to tissues with low GR concentrations.

FUTURE GROWTH: TOWARDS A QUANTITATIVE UNDERSTANDING OF THE RELEVANCE OF GLUCOCORTICOID RECEPTOR DIMERISATION

Dimerisation of the GR is an integral step in glucocorticoid signalling (Figure 1) and has been identified as a possible molecular site to target for the drug development of dissociated glucocorticoids with reduced side effects. Notwithstanding the work we have done showing profound effects of gain or loss of GR dimerisation on glucocorticoid signalling, our understanding of the broader relevance of GR dimerisation within the context of glucocorticoid signalling remains limited. In other words, we still cannot yet fully answer the seemingly trivial question, Why does the GR dimerise?

In an attempt to answer this question and in our quest to fully understand the implications of GR dimerisation for our future work, we propose to construct, with the help of Prof Johann Rohwer,[42] a realistic kinetic mathematical model of GR signal transduction that incorporates rate constants obtained under conditions that mimic gain or loss of GR dimerisation. To launch the project, an initial model of the moiety conserved cycle of the GR comprising ligand binding and dimerisation will be used (Figure 6, steps A and B versus steps C and D). We envision that the quantitative model of GR signal transduction would enable us to interpret our current and future experimental data within the context of understanding the design principles of the complex biological network of GR signalling while providing quantitative and mechanistic insights that may generate novel, experimentally testable hypotheses. Meanwhile, we will continue to accrue experimental data, both *in vitro* and *in vivo*,[43] pertaining to the gain or loss of GR dimerisation. One aspect of GR signalling currently under investigation is the implications of GR dimerisation for autologous downregulation of the GR, an important issue related to the loss of glucocorticoid sensitivity observed in about 30% of patients using long-term glucocorticoid therapy.

My journey with CpdA has indeed brought us unimagined possibilities and glorious prospects; starting with *grootlamsiekt*e and the gannabos meandering via contraception in rats, cytochrome P450 and CBG to the GR and dissociated glucocorticoids, it is now poised on the brink of 'a Great Perhaps', embarking on the quest for obtaining a deep understanding of the relevance and significance of varying GR levels, which by implication encompasses GR dimerisation, for predicting changed responses in relation to health, disease and drug administration.

42. Prof Rohwer has many years of experience in the mathematical modelling of complex biological systems and will direct the mathematical modelling aspects of the project.

43. We have recently received funding to establish a collaboration with Holger Reichart from the University of Göttingen in Germany and aim to investigate gain and loss of GR dimerisation by examining tissue samples obtained from wtGR and GRdim mice.

Cyclopia: More than a cup of tea

About 10 years into my research on CpdA and GR signalling, I was approached by Prof Lizette Joubert[44] regarding *Cyclopia*, better known as honeybush (Figure 7). She was on her own quest of 'seeking a Great Perhaps' and kindly asked me to join this quest.

Figure 7: Cyclopia or honeybush. Shoots of C. subternata (left) and C. genistoides (right) with distinctive yellow flowers, characteristic of Cyclopia species (Louw, Joubert and Visser 2013).

Cyclopia, in contrast to the other established herbal tea, rooibos tea, was lagging behind in popularity and commercial success and was considered to be in need of development. Both teas were initially used for medicinal purposes; over time, however, this evolved into non-medicinal use and they are currently consumed as a beverage (Joubert, Gelderblom, Louw and de Beer, 2008). In a quest to expand the potential use of *Cyclopia*, Lizette was characterising the phenolic content of the tea and exploring its health-promoting properties for possible non-beverage applications. Scrutiny of the phenolic composition of *Cyclopia* coupled with anecdotal claims of use in alleviating menopausal symptoms led her to the notion that *perhaps Cyclopia* could be a source of phyto-oestrogens.

Phyto-oestrogens are secondary metabolites from plants with a structural and functional similarity to the endogenous human hormone oestrogen, which suggests that they may potentially modulate oestrogen signalling. The first step in oestrogen signalling involves binding to the ER (Figure 1, Step 1). Two of the ER subtypes, namely ER-alpha (ERα) and ER-beta (ERβ), are of clinical interest: ERα, as it is the main driver of cell proliferation in the breast and endometrium, which could cause or enhance the spread of cancer, and ERβ, as it acts as a brake, limiting ERα-mediated proliferation. Thus compounds that prefer ERβ over ERα are of great interest pharmacologically.

44. Lizette Joubert, from the Agricultural Research Council, works on the indigenous South African herbal teas such as rooibos (*Aspalathus linearis*), honeybush (*Cyclopia* spp.) and bush tea (*Athrixia phylicoides*).

In 2007 (Verhoog, Joubert and Louw, 2007b) we established for the first time that two *Cyclopia* species, *C. genistoides* and *C. subternata*, displayed significant phyto-oestrogenic activity, with one extract from *C. genestoides*, P104, emerging as the most active extract although it did not bind more strongly to ERβ than to ERα. We had screened both aqueous and methanol extracts from each of the four commercially available *Cyclopia* species, *C. intermedia, C. subternata, C. genistoides and C. sessiliflora*, using both fermented and unfermented *Cyclopia* and independent harvestings.[45] Generally, the methanol extracts from unfermented *Cyclopia* were more active, probably due to the fact that fermentation had been shown to reduce polyphenolic content. Of note was the great variation in ER-binding capacity found even within a species, which led us to conclude that no blanket claims regarding the phyto-oestrogenicity of honeybush tea could be made.

Further in-depth studies with the unfermented *C. genistoides* methanol extracts established that despite the fact that the P104 extract had an affinity for ERα that was about 450-fold higher than its affinity for ERβ, the extract was only able to induce an oestrogen response element (ERE[46])-containing promoter reporter construct via ERβ, not via ERα (Verhoog, Joubert and Louw, 2007a). In addition, neither the potency nor the efficacy was significantly different from that obtained with genistein, a well-known phyto-oestrogen obtained from soy. Furthermore, although the extracts could induce cell proliferation in the well-known breast cancer cell line MCF7, the potency of the extract was around 10 000-fold lower than that obtained for oestrogen and besides, the extract could very effectively antagonise the proliferation induced by oestrogen (Verhoog, Joubert and Louw, 2007a). The binding of extracts to SHBG was also evaluated and found to be as effective as genistein in displacing oestrogen from SHBG. At this stage, we were cautiously optimistic regarding the phyto-oestrogenic potential of *Cyclopia*, especially the disproportionably effective activation of the ERβ, an attribute thought to be most desirable for the preparation of a nutraceutical[47] for use in HRT.

Several factors have contributed to the renewed interest in alternatives for HRT and the identification of plants as a possible source of new oestrogen analogues. Firstly, some studies on HRT use have identified several associated risks, with breast cancer identified as a primary adverse outcome, resulting in a concomitant reluctance of usage by consumers. Secondly, epidemiological studies suggest that an Asian diet rich in soy (which contains genistein, a phyto-oestrogen) is protective against hot flashes, coronary artery disease and oestrogen-dependent cancers such as breast and prostate cancer. Finally, two attributes of phyto-oestrogens, weak oestrogenicity and preference for ERβ, have been linked to their beneficial health effects.

Our previous work had been conducted using small harvestings obtained for research purposes. Now that we were considering *Cyclopia* extracts for nutraceutical applications coupled with the fact that we had established that harvestings may differ considerably in terms of oestrogenic potential, we focussed our attention on new *C. genistoides* (M7-9) and *C. subternata* (M6) harvestings for which material was available in bulk. In addition, we investigated the possibility of enhancing the phyto-oestrogenicity of our extracts by using a two-dimensional approach that explored the use of five solvents of differing polarities and two extraction methods while, in addition, also investigating extracts that mimicked the preparation of a cup of tea, which is how *Cyclopia* is generally consumed (this yielded 22 extracts to be examined). Furthermore, we benchmarked our extracts against four commercially available preparations.[48] The study yielded an extract, the sequentially extracted M6 methanol extract (SM6Met[49]), which was 7.2 to 10.5 times more potent than the closest contender (Mfenyana, Joubert and Louw, 2008). In addition, 'cup-of-tea' extracts, prepared by steeping the plant material in boiling water, did show appreciable phyto-oestrogenic activity. Furthermore, the study established that *Cyclopia* extracts generally had comparable potency and efficacy to the commercial extracts and thus had potential as marketable phyto-oestrogenic nutraceuticals (Mfenyana, Joubert and Louw, 2008).

45. Traditionally, only 'fermented' (oxidised to release distinct aroma and flavour) honeybush tea was available to the public, but 'unfermented' (green) tea has recently been introduced to local and international markets. Independent harvestings suggest that the plants were harvested from different locations and at different times.

46. Similar to the NRE of Figure 1 but specific for the ER.

47. Non-toxic food extract supplements with health benefits.

48. Phytopause Forte® , a soy isoflavone extract, Promensil® , a red clover isoflavone extract, Remifemin® , a black cohosh extract, and Femolene Ultra® , a combination of extracts from several plants including soy, black cohosh, Mexican wild yam and maidenhair tree.

49. SM6Met was produced by sequential extraction of *C. subternata* (harvesting M6) with dichloromethane, followed by ethyl acetate, ethanol and methanol.

The previous study had not investigated extracts for ER isoform specificity, an attribute that is currently a hot topic in the search for oestrogenic analogues with reduced side effects. Current oestrogens in HRT activate both subtypes of ER in all tissues, which is beneficial in bone (prevention of osteoporosis) and for hot flashes but detrimental in the breast where it promotes cell proliferation that increases the risk of cancer. SERMs, such as raloxifene and tamoxifen, although not ER subtype specific, act as agonists in certain tissues, such as bone, increasing bone mineral density, and as antagonists in others, such as breast tissue, decreasing the risk of breast cancer. However, they are not considered as suitable alternatives for HRT as they have been linked to an increased risk of venous thromboembolism and occurrence of hot flashes and can stimulate endometrial growth. It has been suggested that the development of ER subtype-specific modulators (SERSMs) could herald a new era of HRT in which replacement therapy not only alleviates menopausal symptoms but also protects against breast and endometrial cancer. On a molecular level, such an ideal SERSM[50] would have the following attributes: it would act as an ERα-selective antagonist and an ERβ-selective agonist, selectively downregulate ERα but not ERβ protein levels and display anti-inflammatory properties (important in preventing postmenopausal osteoporosis) (Maximov, Lee and Jordan, 2013).

We thus set out to investigate the ER subtype-specific activity of three *Cyclopia* extracts, P104 (Verhoog, Joubert and Louw, 2007a and 2007b), SM6Met (Mfenyana *et al*., 2008) and 'cup-of-tea' (Mfenyana *et al*., 2008), from two species, *C. genistoides* and *C. subternata*. All three extracts displayed ERα antagonism while two extracts (P104 and SM6Met) displayed robust ERβ agonism (similar to that of 1nM E_2) (Visser, Mortimer and Louw, 2013). In addition, in breast cancer cells, all *Cyclopia* extracts displayed anti-inflammatory action and although they weakly induced proliferation, they antagonised E_2-induced breast cancer cell proliferation. We also for the first time evaluated the *in vivo* effects of SM6Met (the only extract available in sufficient quantities) in an immature rat uterotrophic model[51] and found that SM6Met alone did not stimulate the growth of rat uteri (a negative side effect of the SERM tamoxifen), antagonised E_2-induced uterine proliferation and delayed vaginal opening (Figure 8). This was an important milestone as it was for the first time established that the phyto-oestrogenic extracts of *Cyclopia* had *in vivo* biological activity. The combination of ERα antagonism and ERβ agonism observed with SM6Met might be advantageous for the treatment of menopausal symptoms as ERβ agonist activity has recently been shown in clinical trials to reduce hot flashes and to diminish the postmenopausal surge in inflammatory disorders (anti-inflammatory activity was also observed with SM6Met in a promoter reporter assay) while SM6Met's ERα antagonist activity has been shown to prevent hyperplasia of the uterus, commonly associated with unopposed HRT.[52] Furthermore, this combination of ERα antagonism and ERβ agonism observed with SM6Met may be especially relevant for the chemoprevention of breast cancer, both with respect to the known roles of ER subtypes in breast cancer and as ERα antagonism serves as the basis of current chemopreventative agents while ERβ-specific agonists have recently been identified as having potential for the chemoprevention of breast cancer.

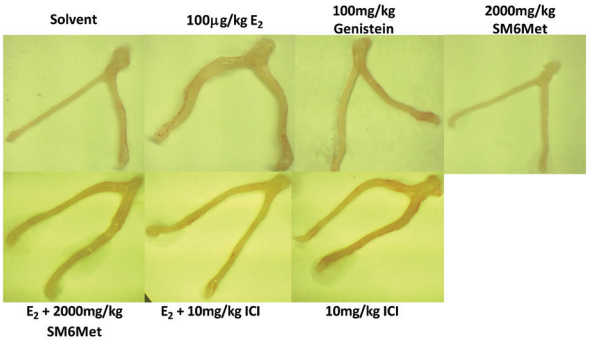

Figure 8: Immature rat uterotrophic model. Immature female Wistar rats were treated with E_2, in the presence and absence of SM6Met or ICI 182,780 (an ER antagonist), genistein (a well-known phyto-oestrogen), SM6Met and ICI 182,780 (Visser, Mortimer and Louw, 2013).

50. On a systemic level, an ideal SERSM would decrease menopausal symptoms such as hot flashes while protecting against breast and endometrial cancer as well as menopause-induced osteoporosis, Alzheimer's disease, stroke and deep vein thrombosis.

51. This is common *in vivo* assay to evaluate estrogenic activity. As the rat matures, endogenous oestrogen will result in growth of the uterus and vaginal opening. In the immature rat, any estrogenic compound will accelerate these features. As the uterus contains mainly ERα, the effect is thought to evaluate ERα-specific effects.

52. In women with an intact uterus, unopposed HRT (i.e. replacement of only oestrogen) would result in uterine hyperplasia. Thus progestogens, which oppose the proliferative effect of oestrogens in the uterus, are commonly prescribed in conjunction with oestrogen for women with an intact uterus.

Having provided several lines of evidence suggesting that the *Cyclopia* extract SM6Met displays many of the attributes of an ideal SERSM and thus has potential as a chemopreventive and/or chemotherapeutic agent for breast cancer, the *Cyclopia* project is now on the brink of its own 'Great Perhaps': that *perhaps* extracts of *Cyclopia* may provide women with a uniquely South African alternative to HRT that could moreover reduce the risk of breast cancer. This is most pertinent as although breast cancer can affect women of all ages, it occurs most commonly in women over the age of 50 and thus postmenopausal women are not only the group most at risk for breast cancer but also the group that would be interested in using HRT, whether traditional or in the form of phyto-oestrogen supplements.

Current and future work on the molecular front includes evaluation of the effects of SM6Met on ERα and ERβ protein levels (one of the attributes of an ideal SERSM that is unresolved) and on SHBG (both on SHBG levels and its potential to displace E_2) while confirmation of the hypothesis that the ERα antagonism and ERβ agonism of SM6Met would be superior to current SERM treatment in breast cancer cell models is also in the pipeline. In addition, future development of a phyto-oestrogenic nutraceutical necessitates fulfilling important prerequisites for the marketing of health claims, such as formulation standardisation, which comprises identification of the active compound(s) and elucidation of the molecular mechanism of action, including the ADME[53] of the active compound(s). The identification of the active compounds in *Cyclopia* extracts conferring the desirable oestrogenic attributes is proving quite challenging, and currently we are considering the possibility that more than one compound may be involved, possibly in a synergistic manner. Finally, as *in vivo* work is the 'gold standard' of biological research, we, in collaboration with Günter Vollmer,[54] have embarked on an evaluation of the ability of SM6Met to prevent and/or treat breast cancer in a rat model of breast cancer. The preliminary results of this study are very promising in that they suggest that SM6Met may be delaying tumorigenesis of the mammary gland by increasing the latency phase.

53. ADME is an abbreviation for 'absorption, distribution, metabolism and excretion'. All four criteria influence the levels and kinetics of exposure of the extract to tissues and thus determine the pharmacological activity of the extract.

54. Prof Günter Vollmer from the Technische Universität Dresden in Germany is an authority on rat breast cancer models.

55. We were appointed at the same time and as the newest members of the department, and at the time the only female academics, often commiserated with each other about the difficulty of balancing our teaching loads with our research.

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I have been enormously privileged to conduct my research in a vibrant and supportive environment, that of the Department of Biochemistry at Stellenbosch University. My mentors were all members of this department: Prof Kirsten van der Merwe introduced me to *Salsola tuberculatiformis* and CpdA, Pieter Swart shared his knowledge of cytochrome P450 and was the first to suggest that perhaps CpdA was being stabilised in plasma, and Janet Hapgood was instrumental in guiding my quest in the direction of signal transduction. Although my appreciation extends to all my colleagues in the department, I would like to single out a few: Marina Rautenbach,[55] who was my comrade in arms in developing the second-year biochemistry curriculum, Donita Africander, who shares my passion for steroid receptor signalling and with whom my laboratory works closely, Johann Rohwer, who is as excited as I am about the new direction that we are taking with GR dimerisation, and my friend Jannie Hofmeyr, who has an infectious appreciation for 'the Great Perhaps'.

I salute all my fellow searchers in the quest to obtain an approximate understanding of steroid hormone signalling. A special mention is due to my collaborators, Kathy Myburgh, Carine Smith, Janet Hapgood, Guy Hageman, Wim vanden Berghe, Karolien de Bosscher, Holger Reichardt, Gunter Vollmer and Lizette Joubert. Seeking 'the Great Perhaps' requires endless curiosity and restless enquiry, and for their boundless endeavours, I commend all my postgraduate students: Fatima Allie, Craig Andrews, Chris Barry, Herzelle Claasen, Ciko Mfenyana, Morne Mortimer, Brendon Reikert, Steven Robertson, Tamzin Tanner, Lorinda van Dyk, Nicky Verhoog, Michael Vismer, Koch Visser, Legh Wilkinson and Nathan Wilson. It has been an honour to share in the enormous satisfaction of a hypothesis confirmed, in the aesthetic pleasure of a beautifully executed experiment and in the gratification of curiosity satisfied while overcoming troubles and tribulations together.

My natural inclination is to probe, to seek out the truth, to want to understand how things work: not always the most comfortable attributes to live with. For their loving support and indulgence with my quest, I thank my mother, Una Ramsay, my husband, Albé, and my children, Niel, Ramsay and Una. It has been a quest that has awed me with its uncertain but glorious possibilities and that has produced many promised marvels, wonders and delights.

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