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QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP (QSAR) STUDY OF THE EFFECT OF STEROIDS ON DNA REPLICATION

by

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ABSTRACT

Evidence is accumulating which demonstrates that hormonally induced cell proliferation plays a critical role in carcinogenic processes. The effect of steroids on DNA replication was examined using a mammalian HeLa cell-free, *in vitro* DNA replication assay, which uses the mammalian origin of replication DHFR oriß, to control DNA replication of a plasmid DNA.

First, replication was shown to originate preferentially within the DHFR origin. Next, the system was reproducible and found to be useful for the study of the effects of drugs and compounds on DNA replication, as tested with the two inhibitors of DNA synthesis, doxorubicin and araC (araCTP). The steroid hormone progesterone, thought to exert its effect through progesterone receptors (PR), enhanced DNA replication. However, since PR were not present in this system, the effect of progesterone must be mediated through a pathway other than PR.

Estrogens, which have been associated with breast cancer, were then studied. The compounds 17ß-estradiol, estrone, estriol, and 16α-hydroxy estrone produced an enhancement on *in vitro* DNA synthesis, whereas the *in vivo* inactive epimer of 17ß-estradiol, 17α-estradiol, had no effect. Measurement of the estrogen receptors (ER) levels in the cell extracts used, indicated that they were absent. Thus, these results reveal a new potential target of estrogens, independent of ER, which affects DNA replication.

A larger number of steroids, were subsequently examined. The results ranged from enhancement, to no effect, to decrease in the *in vitro* DNA replication. The observed effects were related to their structures through the construction of a quantitative structure-activity relationship (QSAR) analyses. The QSAR studies identified structural features in the steroid molecules, responsible for their effects on DNA replication. Using the individual steroid conformation that fits the QSAR model, several pharmacophores were built. An equation was derived from the pharmacophore model predicting the level of DNA replication.

These results demonstrated that this *in vitro* DNA replication system provides an evaluative assay for the effects of compounds and drugs on DNA synthesis. Furthermore, this system has revealed that steroids can affect DNA synthesis by a mechanism, which is

independent of a steroid receptor-mediated process. Additionally, this system can be used in combination with QSAR studies for the development of accurate pharmacophores for the design of new inhibitors or promoters of DNA synthesis.

RÉSUMÉ

De plus en plus données expérimentales suggèrent que la prolifération cellulaire induite suite à l'action d'hormones joue un rôle crucial dans la carcinogénèse. L'effet des stéroïdes quand a la replication de l'ADN a été examiné via un modèle de réplication *in vitro* de l'ADN utilisant des extraits de cellules HeLa et qui contenait l'origine de réplication mammifères DHFR oriβ.

En tout premier lieu, il a été démontré que réplication originait préférentiellement à l'intérieur de l'origine DHFR. Par la suite, le système devenu reproduisible, il fut utilisé pour l'étude de l'effet de deux inhibiteurs de la synthèse de l'ADN, la doxorubicine et l'araC sur la réplication de l'ADN. La progestérone, hormone stéroïdienne dont l'action principale se situerait au niveau de récepteurs à progestérone, a également été montré comme modulant la réplication de l'ADN. Toutefois, étant donné que les récepteurs à progestérone sont absents de ce système, cecci semble suggérer qu'un mécanisme différent soit en cause.

Les estrogènes, associées via de précédentes études avec le cancer du sein, furent également étudiées. Les hormones 17β-estradiol, estrone, estriol et la 16α-hydoxy estrone ont produites une augmentation de la synthèse *in vitro* de l'ADN alors que le épimère inactif *in vivo* du 17β-estradiol, 17α-estradiol n'eut aucun effet. La mesure des niveaux de récepteurs estrogéniques dans les extraits cellulaires utilisés indiquèrent quils étaient absents. Ainsi, ces résultats révèlent une nouvelle cible potentielle des estrogènes, indépendante des récepteurs aux estrogènes.

Une grand nombre de stéroïdes furent examinés subséquemment. Les résultats obtenus variaient entre une augmentation, une diminution ou encore aucun effet notable sur la réplication *in vitro* de l'ADN. Les effets observés furent reliés à la structure des stéroïdes étudiés via la construction d'une relation quantitative structure-activité (= QSAR). Les études QSAR ont permis l'identification d'éléments structuraux à l'intérieur de la molécule stéroïde responsible de l'effet sur la réplication de l'ADN. En utilisant la conformation individuelle stéroïdienne qui respectait le modèle QSAR, plusieurs pharmacophores furent construits. Une équation fut derivée du modèle pharmacophore permettant de prédire le niveau de réplication

de l'ADN.

Ces résultats ont démontré que ce système de réplication *in vitro* de l'ADN peut servir à évaluer l'effets de plusieurs drogues ou composés sur la synthèse de l'ADN. De plus, ce système a permis de révèler que les stéroïdes peuvent moduler la synthèse de l'ADN par un mécanisme indépendant de ceux médiés par les récepteurs stéroïdiens. Par ailleurs, ce système peut être combiné avec des études QSAR et le développement d'un pharmacophore précis pour le design de nouveaux inhibiteurs ou promoteurs de la synthèse de l'ADN. Finalement, la construction d'un pharmacophore sera utile pour une meilleure compréhension du mécanisme d'action des stéroïdes dans la carcinigénèse.

Dedicated to my parents, brother, sisters, and specially to my husband, Julian and to my daughter, Olaia for their love and constant support during these years

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ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
ANN	Artificial Neural Networks
AR	Androgen receptor
AraC	Arabinoside cytosine
Biophπ	π population on the heteroatom
BuAdATP	Butylanilino dATP
BuPdGTP	Butylphenol dGTP
CBG	Corticosteroid-binding globulin
CE	Catechol estrogens
COMT	Catechol-O-methyl-transferase
CoMFA	Comparative Molecular Field Analysis
dam+	Deoxy adenosine methylase
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DES Q	Diethylstibestrol-4',4"-quinone
DHFR	Dihydrofolate reductase
Dox	Doxorubicin
DUE	DNA unwinding element
E1	Estrone
E2	17β-estradiol
E3	Estriol
ER	Estrogen receptor
ERT	Estrogen replacement therapy
EST	Estrogen sulfotransferase
FSH	Follicle-stimulating hormone
GR	Glucocorticoid receotor
HBS	Hydrogen bond site

Hyd	Hydrophobicity index
2-OH E1	2-Hydroxy estrone
4-OH E1	4-Hydroxy estrone
16α-OH E1	16α-Hydroxy estrone
HRE	Hormone response element
HSST	Hydroxysteroid sulfotransferase
LH	Luteinizing hormone
MCTS	Multicellular tumor spheroids
MPA	Medroxyprogesterone-acetate
MR	Mineralocorticoid receptor
MRC	Multiprotein replication complex
OBR	Origin of bidirectional replication
OC	Oral contraceptives
ORE	Origin recognition element
ors	Origin enriched sequences
P	Progesterone
PCB	Polychlorinated biphenyls
PCNA	Proliferating cell nuclear antigen
PR	Progesterone receptors
PST	Thermostable form of phenol sulfotransferase
QSAR	Quantitative structure-activity relationships
RFA	Replication factor A
RFC	Replication factor C
SSB	Single-stranded DNA binding protein
SSBG	Sex steroid-binding globulin
ST	Sulfotranferase
T	Testosterone
TAE	Tris-acetate EDTA
T-Ag	SV-40 large T-Antigen

TCB	Trichlorinated biphenyls
TGFα	Transforming growth factor α
TLDU	Terminal duct lobular unit
TS	Thermostable
UDGT	Uridine diphosphate glucuronosyltransferase
Xdip	Dipole moment on the X-axis

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PREFACE

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cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

CHAPTER ONE

INTRODUCTION

Cancer is one of the major causes of mortality. The incidence of this disease, especially of breast cancer, has dramatically increased in the last 50 years. It has been estimated that one out of every ten women will develop breast cancer over the course of her life. Although its etiology remains to be elucidated, several factors have been shown to be involved in its progression. Among these factors, hormones, particularly estrogens, have been closely associated with the development of carcinogenesis. Stimulation of cell proliferation, which is a constant feature during the progress of cancer, has been proposed as one of the mechanisms for the estrogens carcinogenicity. This thesis is concerned with an investigation into the effect of several steroids on the mechanism of DNA replication. We set out to better understand the steroids' mode of action on carcinogenesis, and to determine the key elements of the steroid structure responsible for the hormones effects. Additionally, this may help in the design of new compounds, which can be used in the treatment of hormone-dependent cancers, such as breast cancer.

The general introduction describes the causative factors of breast cancer, the synthesis, metabolism and mechanism of action of steroids, and their role in carcinogenesis. The second part of the introduction deals with the mechanism of DNA replication, proteins and origins involved in eukaryotic DNA replication. As well, inhibitors of DNA synthesis and *in vitro* DNA replication systems are described. Finally, a description of QSAR studies and a pharmacophore are presented.

1. HORMONE-DEPENDENT CANCERS

The common hormonal tissues contributing to mortality due to cancer include breast, ovary and uterus in women, and prostate in men (National Cancer Institute [NCI] 1989; ACS, 1991). Cancer of hormonal tissues accounts for approximately 8% and 5% of total mortality, and 30% and 11% of cancer mortality in women and men, respectively.

1.1 Breast cancer

Carcinoma of the breast, like other cancers, is characterized by uncontrolled proliferation, invasiveness and metastasis of the tumor cells. Breast carcinomas are

thought to arise in the epithelial cells of the terminal duct lobular unit (TDLU) of the breast (Russo et al., 1990).

Today cancer of the breast is one of the major causes of morbidity and mortality among women. In the last few years, it has been estimated that the risk of developing breast cancer is 1 women in 80 in Japan, 1 in 12 in the European Community, and the value is even higher in USA, 1 in 8 (Brown and Kleiner, 1994).

Etiology and Risk factors

Even though much progress has been made in discovering the causes of cancer, the etiologies remain unknown. The causes of cancer can be divided in two groups: extrinsic and intrinsic factors. The extrinsic factors include tobacco smoke, chemicals, various occupational exposure circumstances, radiation, and viruses; the intrinsic factors include hormones, immune conditions, and inherited genes.

Environmental influences

Environmental exposures may elevate the risk of human breast cancer. Environmental factors include cigarette smoking, alcohol consumption, caffeine ingestion, exposure to electromagnetic fields and radiation, in addition to exposure to environmental chemical contaminants. Some studies have suggested an association between alcohol consumption, smoking, caffeine ingestion, and occupational exposure to electromagnetic fields and radiation with a higher risk of breast cancer development (Millikan et al., 1995). However, sufficient evidence supporting these correlations is lacking, and more studies are needed to confirm these observations. In addition, a potential contributor to an increase in breast cancer incidence could be exposure to chemical pollutants, most notably chlorinated hydrocarbons. These compounds accumulate in the adipose tissue of living organisms, such as breast tissue. Moreover, they have been detected in higher concentrations in malignant breast tissue than in adjacent normal adipose and mammary tissue (Millikan et al., 1995), thus further confirming an association to this disease. Environmental estrogens are compounds that bind to the estrogen receptor (ER), although hundreds to thousands of times more weakly

than the principal estrogen, estradiol. Some chlorinated hydrocarbons such as DDT (dichlorodiphenyltrichloroethane) and PCBs (polychlorinated biphenyls) were found to have estrogenic properties. For instance, a PCB compound called, TCB (trichlorinated biphenyl), has been shown to act as an estrogen with actions mediated through the ER (Nesaretnam, 1996). Recently, Arnold et al., (1996) showed that, singly, environmental estrogens have little effect on biological systems, in contrast to their mixture which can give rise to synergistic interactions which may have profound effects.

Genetic factors

A woman's risk of developing breast cancer increases by a factor of 1.5 to 3 when a first-degree relative has had the disease (Kelsey, 1979). Although breast cancers are heterogeneous, each cancer is characterized by the presence of multiple mutations in critical target genes, including oncogenes and tumor suppressor genes. Analysis of large families with an unusually high incidence of breast cancer has revealed the involvement of specific genes. For example, mutations in the BRCA1 gene (Friedman et al., 1994) and in the tumor suppressor gene *p53* (Malkin et al., 1990) have been associated with some breast cancers.

Recent research has also found genetically susceptible subgroups to cancer within a population. For example, there are variations in susceptibility due to genetic polymorphism in carcinogen-metabolizing enzymes, such as CYP 1A1 (Idle, 1991). The same P450 enzymes participate in the metabolism of environmental pollutants and catalyze hydroxylation of endogenous estrogens (Dannan et al., 1986). Since variations in estrogen metabolism modulate growth of human breast tissue, induction of P450 enzymes by environmental pollutants could contribute to development of breast tumors by altering estrogen levels within the breast tissue (Forrester et al., 1990).

Hormonal factors

Many of the risk factors associated with breast cancer have an endocrinological component. Approximately 30-40% of breast cancers are hormone dependent, with estrogens serving as the principal hormones supporting their growth. Estrogen hormones

are known to exert a complex influence on development and function of the female reproductive organs in vertebrates. This role is achieved by the estrogens' regulation of cell growth and differentiation, as well as being implicated in oncogenesis and maintenance of tumor growth.

The significance of ovarian hormones in the development and maintenance of breast cancer in human females was reported by Beatson in 1896, when he demonstrated that an ovariectomy ameliorated the course of this disease. This suggested that changes in the levels or metabolism of ovarian steroids may influence the etiology of this disease. For instance, it has been reported that the formation of the estrogen 16α-hydroxy estrone correlates well with breast tumor incidence and breast cancer risk factors (Bradlow et al., 1986).

Reproductive factors such as early onset of menstruation, late menopause and late age at first-term pregnancy have also been associated with a higher risk of developing breast cancer. It is also well documented that there is reduced risk of developing breast cancer in women who have an early first-term pregnancy (Ellman, 1987).

Exogenous hormones use, such as oral contraceptive agents (OC) and estrogen replacement therapy (ERT), may contribute to breast cancer. Some controversy exists as to whether young women or women who use OC late in their reproductive years may have increased risk of developing breast cancer (Thomas, 1991). Jick et al., (1980) determined that the risk ratio increased to 4.0 in women that use OC near the time of menopause. However, Thomas (1991) found that breast cancer risk was not greatly influenced when OC was used by women of all ages. There is controversy over the use of ERT as well (Swan, 1997; Brinton, 1997). Colditz et al., (1990) showed that the use of ERT did not increase the risk of breast cancer, whilst others have shown a 30% increase in risk in women who had taken ERT for many years (Steinberg et al., 1991).

Diet

Diet is considered to be a major and important factor contributing to cancer of hormonal tissues. Breast cancer was positively correlated with high fat consumption, high body weight, body fat and obesity. Obese individuals have shown elevated estrogen

production, plasma levels and urinary excretion, which is due to increased aromatization of androgen precursors in fat, especially in stromal cells and in muscle tissue of obese individuals (Adlercreutz et al., 1992). After ovarian hormone secretion ceases, (for instance, in postmenopausal women), the adipose tissue, particularly the stromal cells, appears to be the major source of extraglandular estrogen (Morabia and Wynder, 1990). However, recent evidence has been published, which did not find such a relationship between dietary fat and breast cancer, contradicting the above (Willett et al., 1992).

2. STEROIDS

Steroid hormones, either from endogenous or exogenous sources, have been demonstrated to profoundly influence both physiologic and pathologic processes.

2.1. Synthesis

Cholesterol is the source of all endogenous steroid molecules. It derives either directly from diet or via endogenous synthesis from acetate. Cholesterol can be synthesized in practically all tissues, although the principal source is the liver. The adrenal gland synthesizes and secretes variable amounts of all five classes of the steroid hormones: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestins. The testes and ovaries both synthesize and secrete androgens and estrogens in different amounts. Figure 1 summarizes the major enzymatic steps required for the formation of all of these compounds.

Estrogens can be derived from both endogenous and exogenous sources. Estrogens in women are secreted from the ovary, and peripheral conversion of adrenal derived androgens to estrogen occurs primarily in fat cells. Before menopause, these steroids are largely produced and secreted by the ovary. This process is regulated by two pituitary hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). After menopause, however, estrogens are derived almost exclusively from the aromatization of androstenedione, a C19 steroid that is secreted by both the adrenal glands and the ovaries. This metabolic transformation occurs largely in the stromal cells of adipose tissue. The immediate estrogenic product of androstenedione aromatization is

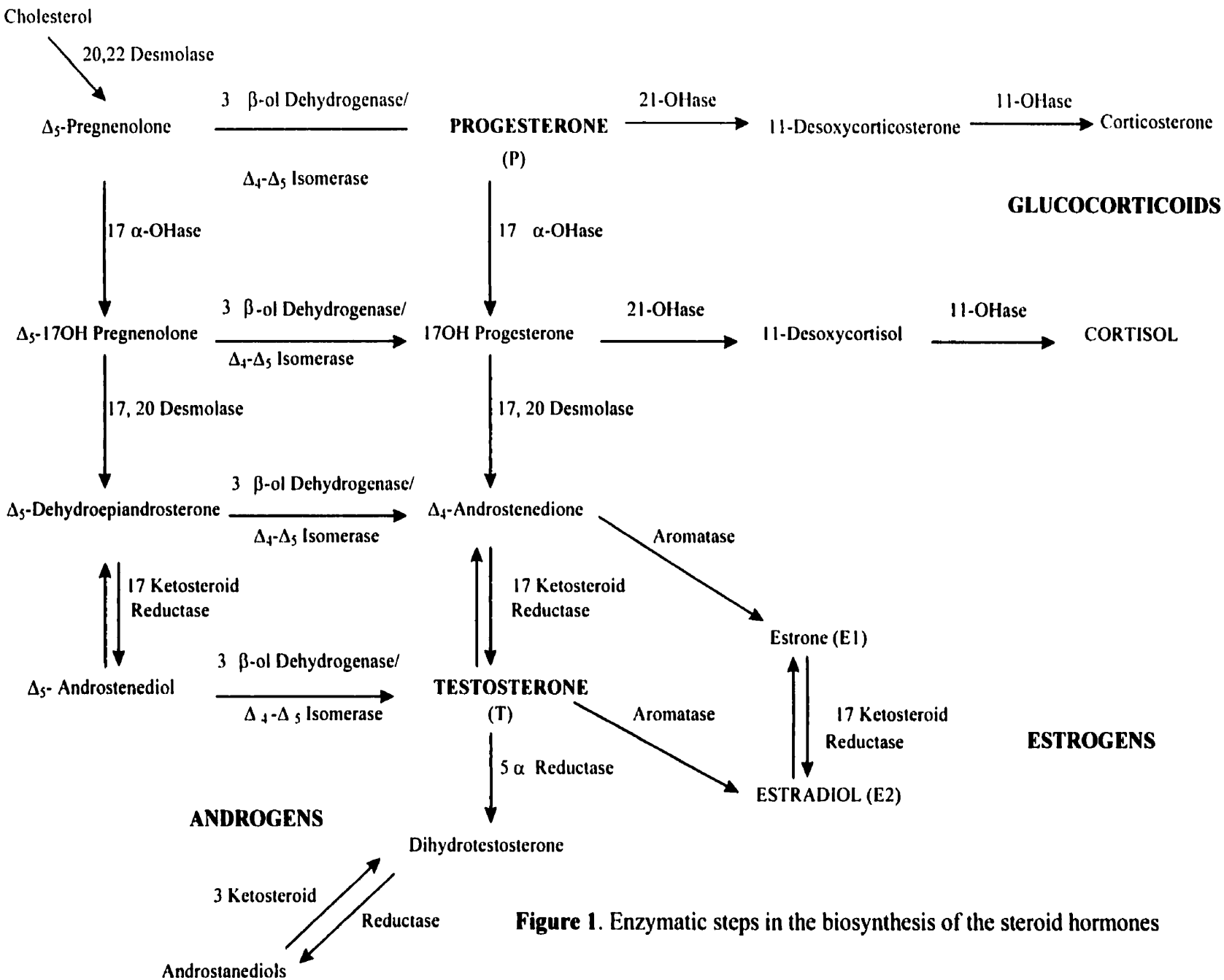

Figure 1. Enzymatic steps in the biosynthesis of the steroid hormones

estrone (E1), but this may be converted to the more biologically active and potent estradiol (E2). Estrone can also be formed from direct oxidation of E2. Further hydroxylation at the 16 position results in the formation of another biologically important estrogen in humans, estriol (E3). Estriol cannot be converted to E2. The primary exogenous source of estrogen during reproductive years is OC, while during the postmenopausal years it is ERT the primary source (Preston-Martin et al., 1990).

Progesterone (P), the most important endogenous progestin, is synthesized from cholesterol and pregnenolone in the steroid-producing tissues: the ovary, testis, adrenal cortex, and placenta. It is secreted mainly by the corpus luteum in the ovary during the second half of the menstrual cycle. This secretion is in a cyclic AMP-dependent manner, with the LH as the principal stimulator of progesterone secretion in humans (Swain, 1996). In addition to endogenous sources, there is an exposure to synthetic progestogens, which arise from OC and ERT sources.

Glucocorticoids, such as cortisol and corticosterone, are synthesized in the fasciculata zone of the adrenal cortex from cholesterol upon binding of the adrenocorticotropin hormone (ACTH), secreted by the pituitary gland, to the steroid synthesizing cells (Swain, 1996).

Androgens have been isolated from the ovaries, testes and adrenal glands. Androgens (either androstenedione or testosterone) are synthesized by ovarian thecal cells and converted to estrogens in the neighboring granulosa cells. This conversion is done by the enzyme complex, aromatase. In both the ovaries and the testes, aromatase is probably regulated by FSH. The LH seems to regulate the synthesis of testosterone in the adrenal glands. The secretion of testosterone from the testes is also regulated by the pituitary gland, through LH (Swain, 1996).

Steroids circulate in the bloodstream predominantly bound to plasma proteins, such as albumin and specific steroid binding globulins. The relative distribution of steroids is believed to govern its biological activity. Binding of steroids to proteins protects them from degradation and excretion, in addition to serving as reservoirs. Cortisol and P circulate primarily bound to corticosteroid-binding globulin (CBG), whereas androgens and estrogens are transported via sex steroid-binding globulin

(SSBG). Corticoids mostly bind to albumin, α-acid glycoprotein and CBG. During circulation in the blood, the progestogens are more or less bound to specific binding proteins with high affinity but low capacity (SSBG and corticosteroid-binding-globulin-CBG) and/or to albumin with low affinity and high capacity, which may modulate their effectiveness and elimination. Circulating testosterone (T) is more than 95% bound to SSBG and albumin (Nisula and Dunn, 1979). The proportion of E2 that is bound with a high affinity to SSBG, about 50%, is unable to undergo tissue uptake and binding to the cellular estrogen targets. The albumin-bound fraction (≈ 48%) is considered to be biologically available, as is the 1-2% of total E2, which circulates free of any protein binding (Rose, 1993).

2.2. Metabolism

Endogenous steroid hormones have a relatively short plasma half-life (less than 4 hours). Oral administration of endogenous steroids results in their inactivation due to high first-pass metabolism. The steroid molecule undergoes structural alterations, which produce dramatic changes in their biological activity. For instance, modifications of the basic structure by conjugation, sterification or alkylation (at positions 1 or 17) extend the half-life into the therapeutically useful range. Steroids undergo both Phase I and Phase II metabolism.

The cytochrome P450 system is the most important enzyme system for Phase I reactions, which includes hydroxylations, dealkylations, and oxidations. Oxygenated metabolites represent structures with newly generated hydroxyl and keto functions at specific sites in the steroid nucleus. The human cytochrome P450 is symbolized by CYP followed by letters and numbers, which indicate family, subfamily and individual enzyme (e.g. CYP1A2).

Phase II, or conjugation reactions of steroids involves enzymes known as transferases, mainly uridine diphosphate glucuronosyltransferase (UDGT) and sulfotransferase (ST) (Roy, 1970). Different forms of UDGT and ST catalyze steroid glucuronidation and sulfation, respectively. The glucuronic acid group has been found attached to 3α- and 21-hydroxyl groups of C21 steroids, and the 3-(C18) or 3α- (C19)

and 17β-hydroxyl groups of C18 and C19 steroids (Mackenzie et al., 1996). Steroid hormones are sulfated by distinct forms of the cytoplasmic ST enzyme, called estrogen ST (EST), hydroxysteroid ST (HSST), and the thermostable (TS) form of phenol ST (PST). Excretion of the metabolites is mainly in the urine as sulfates and glucuronides, but some is excreted in the bile.

Estrogen metabolism occurs primarily in the liver. Here, there is free interconversion between E1 and E2. Equilibrium slightly favors E1, which probably serves as the main precursor for the hydroxylated estrogen metabolites in the urine. Estrogens are metabolized by two main pathways: hydroxylation in the 2-position to yield 2-hydroxy E1 (2-OH E1) as the main metabolite, and hydroxylation in the 16-position with 16α-OH E1 and E3 as the main metabolites (Fig.2) (Fotsis and Adlercreutz, 1987). The phase I metabolism of E1 is irreversible. The oxidative metabolism is predominant at the C-2 position of the estrogen molecule, to form the 2-hydroxy estrogens (2-catechol estrogens). In humans, 50% of estrogen is hydroxylated at C-2, being somewhat greater in women than in men (Bradlow et al., 1986). 2-hydroxylation is catalyzed by several isoforms of P450, such as CYP 1A2, 3A3 and 3A4 in the liver (Fig.3). Due to their instability, the catechol estrogens are rapidly methylated to the methoxy derivatives. Methylation is carried out by catechol-O-methyl-transferase (COMT), which is abundant in the liver and red blood cells (Lipsett et al., 1983). The methoxy estrogens can be converted back to 2-hydroxy estrogens by a P450-dependent demethylating activity, which has been found in human intestinal microflora (Järpenpää, 1990) and rat liver (Hoffman et al., 1980). The 2-OH E1 could also be formed from 2-OH E2 by a non-P450 mediated pathway, involving the enzyme, steroid 17β-dehydrogenase (Milewich et al., 1985).

P450 enzymes mediate further oxidations (Fig.3) at C-4, C-6, C-15 and C-16 positions. The form(s) of cytochrome P450 catalyzing 4-hydroxylation of E2 may be related to CYP 1B1 (Liehr and Ricci, 1996). The 4-hydroxy E1 (4-OH E1) has been identified in human urine, although in lower amounts than 2-OH E1. It seems that members of the CYP 3A subfamily may be responsible for 6-hydroxylation of estrogens (Smith and Jones, 1992). Although not shown in humans, in the male rat, 15α-

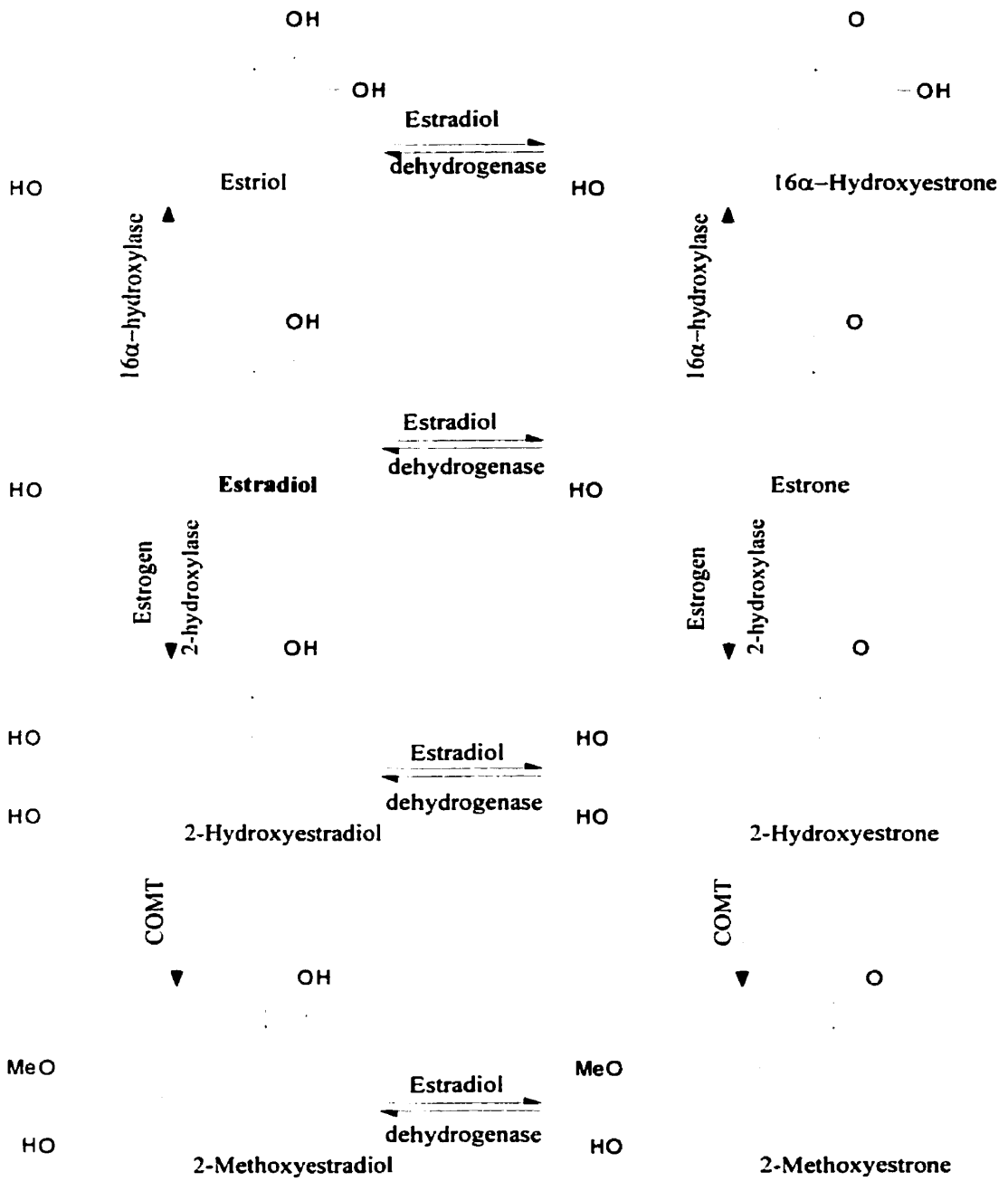

Fig.2. Main metabolic pathways of estradiol

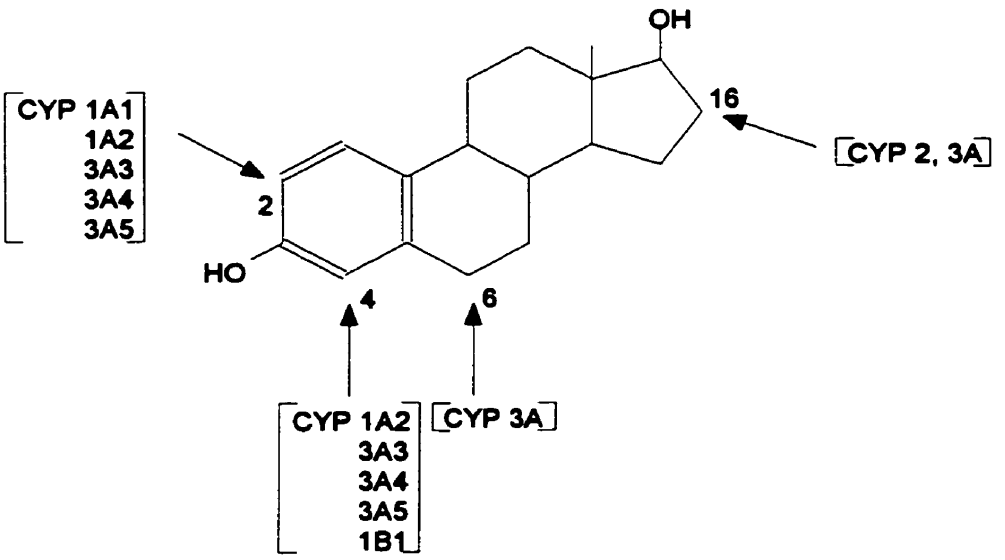

Figure 3. Estradiol oxidation by CYPs

hydroxylation has been attributed to the male-specific CYP 2C11 (Dannan et al., 1986). The hydroxylations at the C-15 position give two products, the 15α-hydroxy E1, which has been reported in human bile and feces, and 15α-hydroxy E3, which is unique in human pregnancy (Martucci and Fishman, 1993). Not as much is known about the CYP mediating C16α-hydroxylation, but it has been suggested that it may be a member of the CYP 2 or CYP 3 families (Nebert, 1993). Hydroxylation at the C-16 position produces two important products, E3 and 16α-hydroxyestrone (16α-OH E1). Hydroxylation in the epimeric position also occurs to yield 16-epiestriol and 16β-hydroxy E1, albeit in lower amounts. Reduction of 16α-OH E1 to E3 can occur in the intestine (Liehr and Roy, 1990).

Phase II metabolism involves the formation of several estrogen conjugates; they are predominantly glucuronides and sulfates, although numerous other water-soluble metabolites have been identified. The sulfates circulate in high concentrations in the blood and the glucuronides are excreted with the bile (over half of estrogen metabolites) and in the urine. These water-soluble compounds are biologically inactive metabolites. Within the lumen of the bowel, these conjugates are available for hydrolysis by enzymes of the intestinal microflora, and the resulting deconjugated estrogens are, to some extent, reabsorbed into the circulation (enterohepatic circulation) and contribute to the total body pool of estrogens that determines the level of both the blood and the urinary estrogens.

Estrogens are glucuronidated by several forms of UDGT. UGT1*1 glucuronidates E2 (Senafi et al., 1994), and UDGT1*2 glucuronidates E1 and 4-OH E1. The human UDGT2B forms also catalyze glucuronidation of estrogens. For instance, UDGT2B7 glucuronidates estrogens, such as E3, 4-OH E1, 2-OH E3, 4-OH E2, and 6α-OH E3 (Ritter et al., 1990). Other form of UDGT, UDGT2B15, slightly catalyzes the reaction towards 2-, 4-, and 16α-OH E1 (Green et al., 1994).

Sulfation of estrogens is catalyzed by STs. For example, E2 and E1 are sulfated by TS PST and EST (Luu-The et al., 1996). Estrogen-sulfates are water soluble conjugates of E1, E2 and E3 circulating in very high concentrations, which serve as a hormonally-inert reservoir from which the biologically-active estrogens are formed. The unconjugated estrogens and their respective sulfates can be interconverted in the liver and

target organs, and the sulfates can undergo the same oxidation, dehydrogenation and hydroxylation reactions as the unconjugated steroids. The unconjugated estrogens can be bound to sex-hormone binding globulin (SHBG), with more or less high affinity but limited capacity, while the estrogen sulfates are exclusively and strongly bound to albumin with high capacity (Rosenthal et al., 1972). E1 and E1 sulfate are the principal metabolites of E2, which are partly reconverted to E2. Since the half-life of the sulfate is prolonged, the levels of E1 sulfate are considerably higher and serve as a hormonally inactive estrogen reservoir. There is a reversible equilibrium between E2, E1, and E1 sulfate, which are interconverted by 17β-estradiol dehydrogenase, sulphotransferase and aryl sulphatase (Hawkins and Oakey, 1974). The extent and directions of the transformations, which take place not only in the liver but also in the endometrial and breast tissues, are governed by the respective concentrations and the influence of P, which stimulates enzyme activity (Tseng and Gurpide, 1975).

P is predominantly metabolized in the liver. The principal metabolic steps are the reduction of the double bond at C4 and the oxo groups at C3 and C20, and hydroxylation at C16 and C21 (Kuhl, 1990). The isomers of pregnanediol are the principal metabolites. Several CYPs are involved in the Phase I metabolism of progestogens; CYP P17 by a steroid 17α-hydroxylation metabolizes P and pregnenolone, and CYP P21A2 by a steroid 21-hydroxylation reaction metabolizes P and 17α-hydroxy P . The metabolites are conjugated, in the liver to sulfates (via the HSST) and glucuronides, and the main metabolite excreted in the urine is 5β-pregnanediol-glucuronide. The enterohepatic circulation plays virtually no role in the pharmacokinetics of P, since conjugation reactions mainly concern the reduced and hydroxylated metabolites (Kuhl, 1990).

Cortisol is extensively metabolized (98-99%) to inactive glucuronides, sulfates, and other forms in several tissues, mainly in the liver. In the liver certain synthetic 11-keto glucuronides (e.g. cortisone) are also activated by a 11-keto reductase system, to the active 11-hydroxy metabolites. Close to half of the total urinary metabolites of cortisol are formed by a tetrahydroreduction of the A ring, which occurs predominantly in the liver (Peterson, 1971). HSST catalyzes the sulfate conjugation of cortisol.

The three major metabolic pathways for clearance of testosterone (T) are: 1) conversion of T back to androstenedione via the reversible 17-keto reductase step, 2) reduction of the double bond at the 5 position to form the androgen dihydrotestosterone, and 3) reduction of the 3-ketone to an alcohol (Katzung, 1995). Hydroxylations also occur at the 6β (CYP 3A4), 11β, 16 (CYP 2C9 and CYP 2C10), and 18 positions. Small amounts of T are converted to E2 by aromatase (CYP P19) in muscle and adipose tissue, as well. Aromatase is a unique cytochrome P450 enzyme complex which catalyzes the synthesis of E1 and E2 from androstenedione and T (Mendelson and Simpson, 1987). T is glucuronidated by UDGT2B7 and UDGT2B11 (Mackenzie et al., 1996). Sulfate conjugation of T is carried out by HSST.

2.3. Mechanism of action

Steroids can act through two basic mechanisms: receptor-mediated and non-receptor mediated.

Receptor-mediated

The receptor-mediated mechanism, also known as the classical genomic mechanism, is the mechanism by which the actions of steroids are mediated by hormone specific nuclear receptors. There exist similarities in the general mechanism by which ligands activate steroid hormone receptors. In the absence of hormone, receptors form an inactive oligomeric complex with heat shock proteins (hsp 70 and hsp 90), immunophilins and other unidentified proteins (Smith and Toft, 1993). In response to a binding hormone, receptors, which are trans-acting transcriptional enhancer factors, are dissociated from the oligomeric complex. Subsequently, they acquire the ability to dimerize and bind to specific, cis-acting enhancer elements, i.e. hormone response elements (HRE), usually located within the 5'-flanking regions of target genes. Then, the receptor is activated by several phosphorylations. The active receptor-DNA complex leads to gene activation (gene transcription or DNA replication). This complex activates recruitment and stabilization of transcriptional factors of the target gene promoter and activation of RNA polymerase II. Consequently, this leads to the regulation of

transcription of hormone-sensitive genes.

The nuclear receptors comprise a superfamily of transcription factors which can be grouped into three subfamilies: a) thyroid hormone and retinoic acid receptors, b) orphan receptors, and c) steroid hormone and vitamin D receptors. The last subfamily includes glucocorticoid (GR), mineralocorticoid (MR), androgen (AR), progestin (PR), and estrogen (ER) receptors. A novel estrogen receptor (ER_β) has been recently discovered (Gustafsson, 1997). Steroid receptors are the product of single genes. Some of the steroid receptors are expressed as two or more isoforms (e.g. PR), which are different post-translational or post-transcriptional products of a single gene. Steroid hormone receptors, which are steroid- and tissue-specific, may be located in the cytoplasm (glucocorticoids) or within the cell nucleus (estrogens and progesterone).

Hormone responsive elements are DNA sequences that bind to the DNA binding domain of the steroid receptor. The HRE are generally arranged in short repetitive sequences, containing two half sites that can bind receptor homodimers or heterodimers. The same 15 base pair HRE sequence can mediate progestin, glucocorticoid, mineralocorticoid and androgen induction of gene expression, which differentiates it from HRE of the ER. There is evidence that the same HRE may behave differently in different cells with regard to its ability to modulate promoter function, and presumably cell-specific factors, as yet undefined, may play a part in directing steroid modulation of gene transcription in hormone-sensitive cells.

The targets of steroids in the cellular genome remain to be fully characterized. Estrogen regulates gene expression by influencing mRNA stability or via an interaction of the estrogen receptor with transcription regulatory factors. Most effects of estrogens and progestins are mediated via intracellular receptor proteins, and the classical model for the interaction of these two classes of hormones is that estrogens increase both ER and PR and thereby increase sensitivity to progestins. Progestins down-regulate both PR and ER. Additionally, estrogen stimulates the synthesis and secretion of transforming-growth factor α (TGFα) (Gullick, 1990), while progesterone activates transcription of the prolactin receptor and down regulates retinoic acid receptor (Mackenzie and Sucumar, 1996).

Non-receptor mediated

The relatively poor correlation between receptor binding and biological activity made it necessary to look for other mechanism(s) of action of steroids. For instance, among the estrogens, there are compounds, which are very active, but with very low affinity to the estrogen receptor, e.g. 11β-acetoxyestradiol (Rosser et al., 1991); and conversely other compounds with high affinity to the estrogen receptor and display poor estrogenic activity (Korach et al., 1989). There is evidence that steroid hormones can exert non-classical actions, which probably contribute along with the classical mechanism to the final biological effects of steroid hormones (Brann et al., 1995).

Some lines of evidence supporting the non-classical mechanism of action of steroid hormones are: the observation of rapid effects of short duration, which occur even if the steroid hormone can not enter the cell or occur in the presence of protein synthesis inhibitors; and the high biological activity of some steroid hormones that have little or no affinity for the steroid hormone receptor. The rapid effects of steroid hormones, appears to be mediated at the plasma membrane level through either: a) a non-specific effect on membrane fluidity, b) binding to sites present in the cell membrane, or c) binding to and modulation of neurotransmitter membrane receptors such as the $GABA_A$ receptor. The lipophilic properties of the steroid hormones allow them to be inserted in the phospholipid bilayers of the membranes and modulate membrane fluidity. For instances, E2 and P have been reported to influence membrane fluidity in several tissues including breast cancer cells (Clarke et al., 1990) and vaginal epithelial cells (Reddy et al., 1989). Both hormones increase cytosolic free calcium due to calcium influx from extracellular sources to induce the acrosome reaction (Foresta et al., 1993). There is also evidence for steroid binding sites in the plasma membrane of a variety of tissues, e.g. breast cancer cells (Berthois et al., 1986). Steroid hormones usually bind to the membrane receptors with specificity and low affinity. Steroids, particularly 3α-hydroxy ring A reduced steroids, are potent regulators of the $GABA_A$ receptors in the brain; for example, androsterone potentiates GABA effects and enhances the binding of GABA ligands *in vitro* (Simmonds et al., 1984). The 3α, 5α- P metabolite inhibits the uterine smooth muscle contractility through the $GABA_A$ receptor system (Putnam et al., 1991).

It has also been proposed there may be an insertion of steroid hormones between base pairs in partially unwound double stranded DNA (Hendry and Mahesh, 1995). Many of the natural steroids exhibit a remarkable physicochemical complementarity with DNA, forming stereospecific bonds with adjacent groups in the DNA and fitting the hydrophobic core of the steroids with the hydrophobic space between the base pairs of the DNA. Kendrew skeletal models and silastic polymer models also revealed stereochemical relationships between steroids and DNA, as well as structure-activity relationships (Uberoi et al., 1985; Hendry and Mahesh, 1991). These studies, in addition to energy calculation studies, found that the degree of fit of steroid hormones into the DNA correlates quite precisely with the degree of hormonal activity. For instances, various compounds correlated uterotropic activity much better with fitting into the DNA than with binding to the estrogen receptor.

Recent reports have demonstrated that certain steroids can come in direct contact with DNA. Therefore, another possible mechanism of action could be due to the chemical interactions of steroid or their metabolites with proteins and/or DNA. Certain estrogen oxygenated metabolites possess a higher degree of chemical reactivity that can cause damage to cellular macromolecules. The 2- and 4-hydroxylated metabolites of both E2 and E1 can directly or indirectly damage DNA, proteins and lipids through the generation of reactive free radicals by the reductive-oxidative cycling of these catechol estrogens between the semiquinone and quinone forms. Liehr et al., (1991) have found *in vivo* covalent linkages between estrogens and DNA. They showed that chronic administration of estrogens, e.g. diethylstilbestrol (DES), produced kidney tumors in Syrian hamsters. The oxidation of DES is catalyzed by cytochrome P450 (peroxidases activity) leading to diethylstilbestrol-4',4"-quinone (DES Q), which is the reactive metabolite intermediate in DES-DNA adduct formation. The reduction of DES Q to DES is catalyzed by NADPH-dependent P450 reductase. Others (Telang et al., 1992) found that an estrogen metabolite, (16α-OH E1), by a direct interaction with the DNA, produced genotoxic damage and tumor promoter activity. Interactions of several steroids with targets different than the DNA molecule, i.e. proteins and peptides, have been reported as well. The covalent steroid-protein adducts are formed by a nonenzymatic process. This reaction occurs via

the Heyns rearrangement of an initial Schiff base adduct. Steroid molecules with a carbonyl function adjacent to a hydroxyl group possess reactivity. Then, the steroid carbonyl moiety would permit formation of a freely reversible Schiff base intermediate with the amino group of proteins, whereas the adjacent hydroxyl group would allow a Heynes rearrangement to occur and form a stable Schiff base adduct with the protein. The estrogen metabolite, 2-OH E1 was found to attach covalently to proteins and peptides, particularly glutathione, which binds and inactivates 2-OH E1 under *in vitro* conditions (Liehr and Roy, 1990). In addition, albumin lysine #199 adducts with 16α-hydroxy estrone and cortisol have been reported. Interestingly, 16α-OH E1 is highly estrogenic *in vivo*, while possessing a low binding affinity for the estrogen receptor. Steroids can also react with erythrocyte membrane proteins and hemoglobin (Bucala et al., 1982). Some of these hormone adducts with DNA and proteins have been suggested to be associated with several types of pathologies. For instance, 16α-OH E1 protein adduct products have been found in systemic lupus erythematosus (Bucala et al., 1986), and it has been suggested these can participate in the oncogenic process (Yu and Fishman, 1985). In addition, glucocorticoid-protein adducts have been detected in the lenses of patients with cataracts produced as a result of long-term exposure to therapeutic levels of glucocorticoids (Manabe et al., 1984).

2.4. Proliferative activity

Cellular proliferation is controlled by the balance between the effects of different regulatory molecules, including steroid hormones. The molecular mechanisms by which steroid hormones control proliferation are not well understood. Nevertheless, most of the information comes from studies with estrogens.

The regulation of growth and development of most female sex organs involves a balance between the actions of the hormones, E2 and P. Two major functions of these hormones are exerted at the uterus and mammary gland. The epithelium of the normal breast undergoes synchronous proliferation during each menstrual cycle, peaking during the luteal phase, under the influence of both E2 and P (Longacre and Bartow, 1986; Henderson et al., 1988). In the mammary gland, E2 and P stimulate proliferation, but in

different morphological elements. During normal development, estrogens promote ductal growth whilst additional progestin is required for lobular development (King, 1993). The potential role of E2 and/or P in mediating breast epithelial DNA synthesis has been investigated using *in vitro* techniques such as organ culture, transplantation of normal human breast into nude mice and primary cell culture.

In contrast to the situation in the breast, peak uterine mitosis follows the rise in serum estrogen levels observed in the follicular phase of the menstrual cycle (Longacre and Bartow, 1986). P acts on the uterus to induce endometrial changes, characteristic of pregnancy and responsible for maintaining pregnancy in animals. In humans, the uterine stroma proliferates during the secretory, P-dominated phase of the cycle in preparation for blastocyst implantation. The estrogen metabolite E3 acts as a weak estrogen, because the duration of nuclear receptor binding is relatively short. When the concentration of E3 in the target cells is high, its hormonal action is enhanced, and it has proliferative effects on the endometrium.

3. STEROIDS AND BREAST CANCER

The concept that hormones can cause, i.e. increase the incidence of neoplasia, was first developed by Bittner (1948). Bittner's findings were based on experimental studies of estrogens in mammary cancer in mice. Today, it is accepted that steroid hormones can stimulate proliferation of both normal and transformed target cells.

During the progression of cells from a normal to a neoplastic state, hormone sensitivity changes (King, 1991). In normal breast epithelium, progestins are mitogenic. However, changes during cell progression cannot be assessed because the present data on breast cancer cells are confusing. On the other hand, estrogen has a poor mitogenic action on normal epithelium, which contrasts with its strong effect on established cancers (King, 1991).

Several steroid hormones (such as progesterone, androgens, and estrogens) have been reported to play a role in the development of breast cancer. However, among the steroid hormones, estrogens have been the most extensively investigated. The high estrogen levels that have been associated with an elevated incidence of breast cancer can

be derived from endogenous (estrogen secretion from the ovary and conversion of adrenal-derived androgens) or exogenous sources (OC, HRT and environmental estrogens). Estrogens clearly stimulate the proliferation of established breast cancer cells, as determined by both clinical (Santen et al., 1990) and laboratory studies (Darbre and King, 1988).

There are four main lines of evidence supporting the concept that estrogens are a major adverse factor in human breast cancers: (1) the ability of estrogens to generate mammary tumors in rodents (Welsch Leung BS, 1992); (2) epidemiologically-derived risk factors such as the protective effect of ovariectomy and increased risk of breast cancer in young women given DES to prevent abortion (Harlap, 1991); (3) the mitogenic effects of estrogens on established breast cancer cell lines (Darbre and King, 1988); and (4) efficacy of antiestrogens in treating established breast cancer (Santen et al., 1990).

Three possible mechanisms have been proposed for the carcinogenic activity of estrogens: 1) hormonal stimulation of cell proliferation (Preston-Martin et al., 1990); 2) inheritable reprogramming of cellular differentiation; and 3) induction of genetic changes in target cells. It is well known that the risk of cancer increases when cell division, induced by external or internal stimulation, increases. Cell division enhances the risk of genetic errors, and is necessary for the conversion of adducts to gaps or mutations. Since estrogens produce cell proliferation, increased levels of estrogens can enhance cell proliferation and the risk of breast cancer.

The carcinogenicity of estrogens has been primarily attributed to their action as ER agonists. Estrogens, acting through their nuclear receptors (ER), stimulate growth of hormone-responsive breast cancer cells. This in turn induces cell cycle progression and 'immediate-early' and 'delayed' gene responses (Weisz and Bresciani, 1993). However, similar responses to estrogen in target cells that do not contain the ER have also been reported (Newbold et al., 1990).

There is increasing evidence suggesting that other mechanisms mediate steroid carcinogenicity, such as cellular DNA or protein damage induced by chemicals. Liehr et al., (1991) found that chronic administration of estrogens produce kidney tumors in Syrian hamsters. The data suggested that the tumors were caused by covalent DNA

alterations by estrogens. For instances, stilbene and steroid estrogens (endogenous electrophiles) lead to kidney covalent DNA adducts, while DES produces DNA adducts in kidney, liver and other hamster organs including, testicles.

Two principal lines of research are focused on the estrogen metabolic pathway. The first one claims there is an elevation in catechol estrogen formation, which is associated with a relative enhancement in breast cancer risk. The second one associates a shift in metabolism away from E2 2-hydroxylation and into increased 16α-hydroxylation with increased breast cancer risk.

Several investigators have focused on the catechol estrogen metabolites. Catechol estrogens (CE) have been proposed to mediate physiological processes such as blastocyst implantation and regulation of ovarian function, but biochemical mechanisms of catechol estrogens not involving the ER remain elusive (Hayes et al., 1996). In contrast, the importance of CE (the 2- and 4-hydroxylated metabolites of both E2 and E1) in toxicity and tumorigenesis has been described in several cell types and tissues including MCF-7 human breast cancer cells (Spink et al., 1994) and hamster kidney (Liehr et al., 1986). The mechanism of these toxic effects appears to involve potentially mutagenic free-radicals, generated from the oxidative-reductive cycling of the CE with their respective semiquinone and quinone forms, which cause cellular damage. Evidence for free radical damage by redox cycling of estrogens includes single strand breaks of DNA in MCF-7 cells induced by estrone-3,4-quinone (Nutter et al., 1991). Moreover, 4-OH E2 induces single strand breaks of DNA in hamster kidney and hydroxy radical-mediated 8-hydroxylation of guanine bases of DNA *in vitro* and in hamster *in vivo* (Liehr and Ricci, 1996).

Several studies in animal and human samples have shown a predominant hydroxylation at the 2 or 4 position of the estrogen, depending on the tissue. In rodent models, there is a differential formation of the two catechol metabolites, 4-OH E2 predominates in organs which are susceptible to estrogen-induced carcinogenesis (kidney, uterus, etc.), while 2-OH E2 predominates in organs, which are resistant to estrogen-induced carcinogenesis (e.g. liver). A specific E2 4-hydroxylase activity has been

identified in animals, which serve as models of estrogen-induced tumorigenesis, such as hamster kidney, mouse uterus, and rat pituitary.

Liehr and Ricci (1996) found that microsomal preparations from human liver or placenta have mainly 2-OH E2 formation, but from normal mammary tissue have similar levels of both 2- and 4-OH E2. In addition, 4-hydroxylation of estrogen was the predominant catechol in human mammary fibroadenoma and adenocarcinoma, higher than in normal mammary tissue. In humans, an E2 4-hydroxylase has been identified in the breast cancer cell line MCF-7 and in benign and malignant neoplastic mammary tissue. In human uterine tissue, 4-hydroxylation of E2 is increased in myomas compared with surrounding myometrium. Here, E2 4-hydroxylation is catalyzed by CYP 1B1. Expression of CYP 1B1 has been detected in 73% of human breast tumor samples (Hayes et al., 1996).

Much interest has also focused on 16α-hydroxylated metabolites as potent estrogens. An increased 16α-hydroxylation has been suggested as a risk factor for breast cancer development (Schneider et al., 1982). However, the P450 isoenzyme responsible for 16α-hydroxylation has not been found yet. Clinical and animal studies *in vivo* have shown a positive correlation of upregulation of E2 C16α-hydroxylation with either the presence or the risk of breast cancer, suggesting that this metabolic alteration may represent an early-occurring event in the multistep process of tumorigenesis. In subjects at risk (with a family history of the disease) and in patients with identifiable breast cancer, E2 metabolism via the C17-oxidation and C2-hydroxylation was unaltered. However, E2 metabolism via C16α-hydroxylation was selectively and specifically upregulated (Fishman et al., 1995). Bradlow et al. (1986) found that 16α-hydroxylation is 50% higher in patients with breast cancer compared with controls. Furthermore, they noticed that there is a remarkable correlation between the activity along this pathway and the incidence of mammary carcinoma in mice. They emphasize that 16α-OH E1 is a potent estrogen, and that it is capable of binding to amino acids and nucleotides covalently as well as to the estrogen receptor. The reaction of 16α-OH E1 with ER is unique in that it can be irreversible and leads to aberrant gene expression. The mammary tissue exhibits cancer risk-dependent alteration in E2 C16α-hydroxylation in the human

mammary TLDU, which is the presumptive site for human mammary carcinogenesis, and in the rodent mammary explant culture model, indicating that E2 metabolites may directly influence the mammary epithelium (Fishman et al., 1995). Cell culture studies showed that 16α-OH E1 could induce unscheduled DNA synthesis and anchorage-independent growth in mammary epithelial cells (Telang et al., 1992). The genotoxic DNA damage and aberrant hyperproliferation induced by the 16α-hydroxylated metabolite of E2, 16α-OH E1, was similar to that induced by chemical carcinogens in the rodent cell culture model. Additionally, in preinitiated or fully transformed rodent or human cells, 16α-OH E1 promotes the expression of a transformed phenotype (Fishman et al., 1995). Hayes et al., (1996) supported these results by showing that 16α-OH E1 was uterotropic in mice and that it increased unscheduled DNA synthesis and anchorage-independent growth of mammary cells in culture. In contrast, they found that 2-OH E1 was much less active than 16α-OH E1 in the uterotropic assay, did not increase unscheduled DNA synthesis, and suppressed growth of breast cancer cells in culture. Except for the importance in the excretion of E1 and E2, they concluded that a physiological role for hydroxyestrogens remains unclear. The observed results suggested that the effects of E2 on mammary tumorigenesis may be due in part to the generation of 16α-OH E1, which functions as a weak initiator or a potent promoter of tumorigenic transformation in mammary epithelial cells (Fishman et al., 1995). Therefore, 16α-OH E1 may contribute, in conjunction with other factors (i.e. molecular, metabolic, and cellular events), to the initiation (appearance) or the promotion (progression of tumor via increased cell proliferation) of the preneoplastic transformation of normal target tissue.

In vivo and *in vitro* studies indicated that not all estrogens are active in inducing tumors, suggesting that not all estrogens are equal in contribution to the multistep process of carcinogenesis.

Progesterone, another prominent hormone in the development of normal breast, is also implicated in the initiation and progression of breast cancer. Data on progestin effects on neoplastic human breast epithelium are both sparse and confusing. Clinically, high doses of progestins induce regression of some breast cancers (Santen et al., 1990), but interpretation of such data in the context of physiological concentrations is difficult.

High doses of estrogen can induce regressions (Santen et al., 1990), despite their mitogenic effect at lower concentrations; such a biphasic effect might also occur with progestins. (King, 1993). It was found that with multicellular tumor spheroids (MCTS) of BT 20 (steroid receptor negative cell line) cells, MPA (medroxyprogesterone-acetate) may exert weak growth stimulation in certain breast cancer cells at a concentration of 1μM, which can nearly be achieved in clinical application. (Muller-Holzner et al., 1996).

In summary, hormones affect carcinogenesis by epigenetic mechanisms such as stimulation of cell proliferation of estrogen-dependent cancer cells and reprogramming of cellular differentiation and gene expression. In addition, significant evidence exists that certain estrogens can also cause genetic alterations by mechanisms not involving the classical estrogen receptor. These findings indicate that hormonal carcinogenesis is most likely a result of both genetic and epigenetic factors.

4. DNA REPLICATION

DNA replication is one of the most crucial processes in the cell. The disposition of replication is careful and precise, and its control is the most important step for cell proliferation. The regulatory mechanisms that govern mammalian DNA replication must be well understood in order to develop a complete knowledge of cell growth. Disturbances in the regulation of DNA replication inevitably result in uncontrolled cell proliferation, which can contribute, to cancer and developmental disorders.

4.1. Mechanism of DNA replication

Research suggests that the mechanism of DNA replication is quite similar in the majority of organisms investigated thus far. However, since the nuclear DNA of eukaryotes, and particularly that of mammalian cells, is very complex, studies with prokaryotes (Fuller et al., 1981) and other eukaryotic systems (viruses) (Li and Kelly, 1984) have been the major systems used to better understand the replication machinery in higher eukaryotes.

There are two principal steps comprising DNA replication, initiation and elongation. Replication in eukaryotes is semiconservative and bidirectional. The process

is always 5'→ 3' direction due to the catalytic properties of the DNA polymerases. Replication proceeds from specific DNA sequences, called origins of replication (Kornberg and Baker, 1992). The 5'→ 3' direction from the origin on either strand is referred to as leading strand synthesis, which is synthesized continuously, and the 3'→ 5' direction from the origin on either strand is referred to as lagging strand synthesis, which is synthesized discontinuously in small pieces (5'→ 3') called Okazaki fragments. The size of these DNA fragments is ~ 200 bp (1000 bp in bacteria). The process of DNA replication (Fig. 4) begins by recognition of the origins and binding by a specific initiator protein or complex of proteins, followed by the unwinding of the double stranded DNA by enzymes called DNA helicases; the superhelical tension is relieved by topoisomerases. The unwound single stranded DNA that is very labile, is protected and stabilized by single-stranded DNA binding proteins (SSB's), and DNA primase initiates the nascent replicating chains, synthesizing short RNA primers of ~ 10 bases. Subsequently, DNA polymerases add deoxynucleotides to the free 3'-OH ends of the RNA initiator chain. In addition to the DNA synthesis function, DNA polymerases have a proofreading function carried on by a second enzymatic activity, namely 3'→ 5' exonuclease. This correction mechanism allows for an accurate synthesis. The process is completed with the help of other enzymes, such as RNase H, which removes the RNA primers, DNA ligase, which seals the Okazaki fragments, and finally topoisomerases, which separate the daughter strands.

4.2. Eukaryotic origins

Several initiation sites for DNA replication have been mapped in mammalian chromosomes, indicating that DNA synthesis initiates at specific DNA sites rather than randomly along the chromosome (DePamphilis, 1993a, 1996). Mammalian cells contain greater than 25,000 origins of replication. This is in contrast to simpler organisms, such as bacteria and viruses, that contain only one or very few origins (Kornberg and Baker, 1992). The origin region in higher eukaryotes is composed of two basic components: a core and an auxiliary component. The core origin is the minimal sequence required to initiate DNA replication under all conditions. The auxiliary component seems to

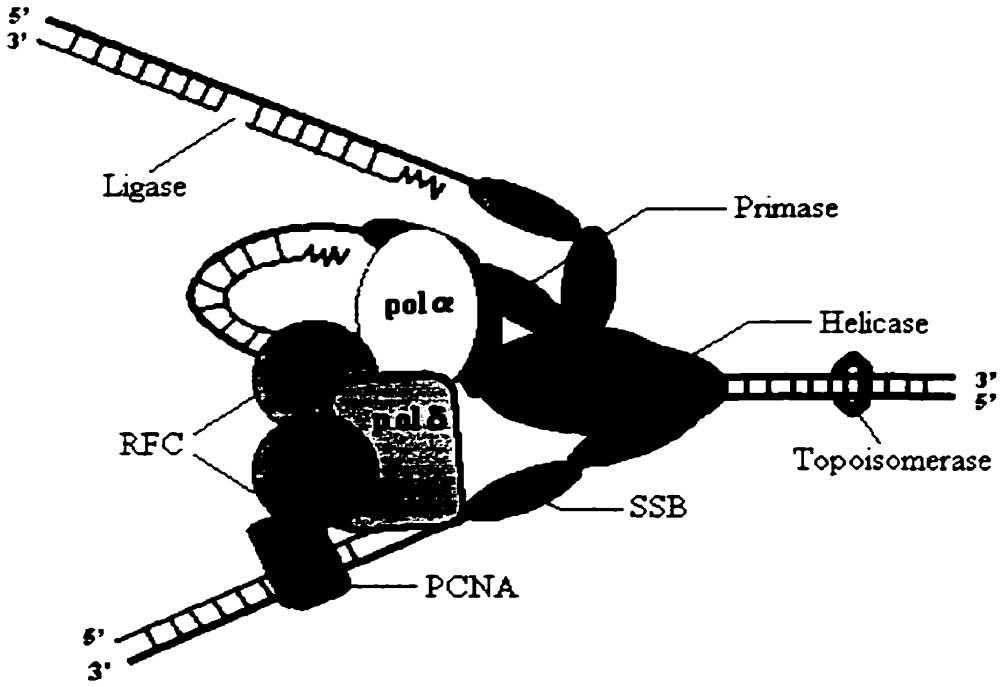

Fig. 4. DNA replication fork and replication proteins

stimulate or modulate the activity of the origin (DePamphilis, 1993b). The core consists of an origin recognition element (ORE) which is the binding site of initiator proteins, an AT-rich region, a DUE (DNA unwinding element) region, and perhaps a DNA secondary structure. Auxiliary sequences, near the core, bind transcription factors or enhancers (DePamphilis, 1993b).

Among the identified mammalian origins of DNA replication are those located within or near the ribosomal protein S14 (Tasheva and Roufa, 1994), CAD (Kelly et al., 1995), rhodopsin (Gale et al., 1992), and dihydrofolate reductase (DHFR) (Hamlin et al., 1993) loci in hamster cell lines, mouse origins mapped in the immunoglobulin heavy chain enhancer (Ariizumi et al., 1993) and at both ends of the adenosine deaminase gene (Carroll et al., 1993; Virta-Pearlman et al., 1993), the chicken histone H5 (Dobbs et al., 1994) and α-globin (Krajewske and Razin, 1992). Other origins include the monkey ors clones (Zannis-Hadjopoulos et al., 1992), the human β-globin gene (Kitsberg et al., 1993), the c-myc gene (Vassilev and Johnson, 1990), the human 343 gene (Wu et al., 1993a,b) and the lamin B2 gene (Giacca et al., 1994). The DHFR domain gene is the most thoroughly investigated mammalian origin of DNA replication. The results from several laboratories, using different mapping techniques, suggested that replication is initiated within a 4,300-bp *Xba* I fragment. Moreover, Burhans et al., (1990) showed an origin of bidirectional replication (OBR), which is the physical switch point for initiation of leading- and lagging-strands, located within a 450-bp region of the 4,300-bp early-replicating *Xba I* fragment that contains *oriβ*. For instance, the application of competitive PCR amplification (Pelizon et al., 1996) to the single-copy DHFR locus of CHO KI cells showed that replication starts at a defined region of ~ 800 bp, located approximately 17 kb downstream of the gene. This site is coincident with the one detected by the analysis of Okazaki fragment polarity (Burhans et al., 1990), which is in agreement with most of the other studies analyzing the DNA replication pattern of the amplified domain, such as, earliest labeled fragment (Leu and Hamlin, 1989), nascent DNA lengths (Vassilev and Johnson, 1990), and earliest replicating DNA (Burhans et al., 1986); this fragment also displays autonomous replicating activity in *in vivo* and *in vitro* DNA replicating assays (Zannis-Hadjopoulos et al., 1994). Moreover, common features which are present in the

origin structure (eukaryotic and prokaryotic) also have been found in the DHFR origin, e.g. AT-rich DUE regions, cruciform structures and bent sequences (Caddle et al., 1990), as well as transcription factor binding elements (DePamphilis, 1993b).

4.3. Replication proteins

DNA synthesis requires the coordinated action of different types of enzymes. The basic enzymatic activities required in DNA replication are the ones that are described below.

Initiator protein or multiprotein complex

The initiator protein(s) recognize and alter specific DNA sequences at the origin of replication. In general, the binding requires energy from ATP. The initiator protein has been identified in some eukaryotic models of replication, such as in simian virus 40 (T antigen is the initiator protein) (Stillman, 1989) and in *Saccharomyces cerevisiae* (ORC is the origin recognition complex) (Bell and Stillman, 1992); the initiator protein in mammalian cells remains to be fully characterized.

DNA helicases

DNA helicases transiently melt double-stranded DNA by using the energy of ATP hydrolysis. There is an entire set of helicases in higher eukaryotes. In human cells, six helicases have been characterized, most of them from HeLa cells. The function of the different helicases are not completely defined. For example, the RP-A dependent DNA helicase from human cells (Seo et al., 1991) is stimulated by RP-A to unwind long stretches of DNA. However, DNA helicase III from human cells prefers forklike DNA as a substrate (Tuteja et al., 1992).

DNA polymerases

Five different DNA polymerases have been identified in eukaryotic cells. These include polymerase α, β, γ, δ, and ε (Linn, 1991). Polymerase α forms a complex with primase and initiates leading and lagging strand synthesis. Recently, it has been reported

that polymerase δ continues the polymerization initiated by polymerase α in both the leading and lagging DNA strands (Waga and Stillman, 1994). In contrast to polymerase α, polymerase δ (So and Downey, 1992) contains a 3' → 5' exonuclease activity, which aids in maintaining the accuracy of DNA replication. This polymerase requires auxiliary proteins such as proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) for processive synthesis, and seems to be regulated by the single-stranded binding protein (SSB) RP-A. Polymerase γ (Fry and Loeb, 1986) is responsible for DNA replication in the mitochondria. Polymerase β (Wilson and Abbotts, 1988) has been suggested to be the main repair DNA polymerase in the nucleus, and might act in base excision repair. DNA polymerase ε is involved in gap-filling reactions such as Okazaki fragment DNA synthesis on the lagging strand and nucleotide excision repair DNA synthesis. Polymerase ε, is similar to polymerase δ and γ, in that it contains 3' → 5' exonuclease activity.

Primase

DNA polymerases are unable to initiate a DNA chain *de novo*. Consequently, polymerase α forms a complex with primase. The primase synthesizes ribonucleotide primers of ~ 10 bases that DNA polymerase α subsequently elongate resulting in DNA strand synthesis (Harrington and Perrino, 1995).

Single-stranded DNA binding proteins (SSB's)

SSB's bind, with high affinity, to single stranded DNA. However, they lack sequence specificity. Moreover, the 32 kDa subunit of RP-A is phosphorylated during S phase of the cell cycle, suggesting that this subunit may play a role in the regulation of DNA replication (Din et al., 1990). In mammalian cells this protein has received different names, such as RP-A, replication factor A (RF-A), and SSB. Replication protein A was found to have a preference for binding to single-stranded pyrimidine stretches, and has direct effects on polymerases α and δ. It stimulates polymerase α by interacting with the polymerase α/primase complex. To stimulate polymerase δ, RPA requires the previous

presence of the auxiliary proteins, PCNA and RF-C. Finally, RPA also seems to be involved in cellular DNA repair. (Coverley et al., 1992).

Proliferating cell nuclear antigen (PCNA)

PCNA is a cell-cycle regulated replication protein. It specifically enhances the processivity of DNA polymerase δ and its binding to a template/primer. PCNA forms a complex with RF-C and ATP, which is only recognized by polymerase δ and ε, and not by polymerase α/primase complex. A role of PCNA in DNA excision repair has also been suggested (Shivji et at., 1992).

Replication factor C (RF-C)

RF-C's function involves the recognition of a primer terminus. Through its DNA-stimulated ATPase activity, RF-C binds PCNA resulting in the formation of a primer recognition complex (Podust et al., 1995). This latter is the substrate used only by polymerase δ and ε, and not by polymerase α/primase. Reports have been published suggesting that, *in vitro*, RF-C also stimulates the activity of both polymerase α and δ Podust and Hubscher, 1993).

Topoisomerases

Topoisomerases regulate the topological state of the DNA. There are two classes of DNA topoisomerases: type I and II. DNA topoisomerase I catalyzes the relaxation of both positive and negative supercoiled domains. It makes a transient break at one of the DNA strands and allows the other strand to pass through the nick before resealing it. This process does not require energy (Hubscher and Spadari, 1994). DNA topoisomerase II breaks the double-stranded DNA in an ATP-dependent manner, allowing the separation of the two daughter DNA molecules in replication. It has been shown that the catalytic activity of this enzyme is stimulated by phosphorylation (Hubscher and Spadari, 1994).

Ligases

DNA ligases catalyze the formation of a phosphodiester bond between adjacent 3'-OH and 5'-POH termini of polynucleotides hydrogen-bonded to a complementary strand. Its main role in DNA replication is the ligation of Okazaki fragments on the lagging strand. In mammalian cells, multiple forms of this enzyme have been identified (Lindahl and Barnes, 1992). For instance, ligase I increases its catalytic activity by phosphorylation and acts on DNA replication. Unlike ligase I, forms II and III, whose physiological roles are unknown, seem to be involved in DNA repair (Jessberger et al., 1993).

4.4. Inhibitors of DNA replication

Drugs that inhibit DNA synthesis continue to be the primary resource for control of proliferative diseases, such as cancer (for general review see Goodman Gilman, 1990). Although there are compounds that can act directly or indirectly on DNA synthesis, my focus will be on drugs that act directly on the DNA replication machinery. It should be noted that there is a general lack of drug specificity, and that the majority of drugs have multiple targets.

DNA inhibitors

Within this group of DNA inhibitors there exist compounds that inhibit DNA synthesis by one of two mechanisms, either by inhibiting nucleotide biosynthesis or by a direct interaction with the DNA molecule. I will focus solely on the second group.

Nucleotide analogs are compounds modified either in the sugar or base group, which can be incorporated into DNA by polymerases. The incorporation of these modified nucleotides causes a block in DNA polymerization or an interference with DNA functions. A commonly used anti-cancer drug is the nucleoside analog, arabinoside cytosine (araC), which inhibits DNA chain growth (Kutcha et al., 1992). Defective nucleic acids, such as α-aminopurine, inhibit DNA synthesis and are mutagenic (Langen, 1975).

There are also inhibitors that modify DNA by binding to it, either noncovalently or covalently. The noncovalent DNA binders interact with DNA by intercalation, like doxorubicin (Pratt et al., 1994), or interact with DNA grooves by hydrogen-bonding and/or electrostatic interactions, like netropsin (binds to the minor groove) (Coll et al., 1987). Covalent DNA binders interact with DNA by different mechanisms: monoalkylation (e.g. alkylating agents); cross-linking of DNA strands (e.g. alkylating agents, psoralen, cisplatin); chain breakage (e.g. bleomycins); and/or intercalation (e.g. psoralen, benzo[a]pyrene 4,5-epoxide). These interactions with the DNA can cause inhibition of DNA synthesis and functions.

Protein inhibitors

Protein inhibitors are drugs that inhibit replication proteins either by binding to the DNA or by binding directly to the replication proteins, leading to inhibition of DNA synthesis. Inhibitors of several replication proteins have been found; however, I will concentrate on the inhibitors of three replication proteins, which are the most studied thus far.

i) DNA polymerases inhibitors

Relatively few compounds are known to bind and inhibit DNA polymerases. The most potent and selective nucleotide analog inhibitors of eukaryotic DNA polymerases (mostly polymerase α) are N(2)-(butylphenyl) dGTP (BuPdGTP) and N(2)-(butylanilino) dATP (BuAdATP). It is thought that these modified nucleotides form a ternary complex with DNA polymerase and DNA (Wright and Brown, 1990). Another important inhibitor of polymerase is aphidicolin. This antibiotic inhibits DNA polymerases α, δ, and ε, by competitive binding with pyrimidinic dNTP, mostly dCTP (Spadari et al., 1984).

ii) DNA topoisomerases inhibitors

In contrast to the reduced number of DNA polymerase inhibitors, there is a great number of DNA topoisomerase inhibitors. Among them are found compounds that can inhibit topoisomerase I or II in eukaryotes, although most of the compounds found thus far are inhibitors of topoisomerase II. The alkaloid, camptothecin seems to have a specific and unique interaction with the eukaryotic topoisomerase I. It stabilizes the

topoisomerase I-DNA complex, causing inhibition of DNA synthesis (Slichenmyer et al., 1993). Regarding topoisomerase II inhibitors, there are different modes of action of different agents to induce topoisomerase II-mediated double-strand breaks. For instance, there are compounds that stimulate the production of a cleavage complex between the topoisomerase II and DNA, such as doxorubicin, VP-16, and amsacrine (Liu, 1989). Another group of compounds inhibits DNA topoisomerase II in a step prior to the formation of a cleavage complex, e.g. merbarone (Drake et al., 1989).

iii) DNA ligases inhibitors

Mammalian DNA ligases have two intermediate steps in DNA ligation. In the first, DNA ligase interacts with ATP, and in the second DNA interacts with the ligase-AMP complex. Therefore, the inhibitory effect of these agents can be either mediated by the interaction with ligase (e.g. F-ara-ATP) or by the interaction with the DNA substrate (e.g. doxorubicin, ethidium bromide) (Ciarrocchi et al., 1991). F-ara-ATP acts at two different levels, it interacts with DNA ligase I and it is also incorporated into the 3'-terminus of the DNA (Yang et al., 1992).

4.5. *In vitro* DNA replication studies

To study the effects of drugs or their mechanism of action, several *in vitro* systems have been developed and used. There are various *in vitro* cell-free systems to study the action of drugs on DNA replication; some of them use purified proteins (reconstituted systems) while others use whole cell extracts. The second system mimics more closely the conditions required for replication of nuclear DNA in eukaryotic cells. One of these systems is the SV40 replication system (Li and Kelly, 1984). It consists of extracts from human cells, SV40 origin-containing plasmid templates, SV40 large T antigen, and nucleotides. The extracts contain all the necessary enzymes to support DNA synthesis with the exception of large T-antigen, a virally encoded protein supplied exogenously. This system has been used to study the effects of drugs, such as cisplatin and platinum complexes (Hollis et al., 1991; Heiger-Bernays et al., 1990), the alkylating agent adozelesin (Cobuzzi et al., 1996), and mimosin (Gilbert et al., 1995). Subsequent to this SV40 system, others have been developed, also using human cell extracts and

mammalian origins of replication (Pearson et al., 1991; and Berberich et al., 1995). Systems using mammalian origins of replication have the advantage of allowing for DNA replication without the exogenous addition of SV40 large T-antigen. Therefore, they more closely resemble the nuclear DNA replication in mammalian cells. These systems require mammalian origins of replication carrying vectors (e.g. ors, DHFR, c-myc, etc.) as DNA templates to allow for the initiation of DNA. Similar to the SV40 system, these systems consist of nuclear and cytoplasmic extracts from human cells which contain the replication proteins, a mixture of nucleotides including radiolabel nucleotides to visualize the replication products, an ATP regenerating system, and a plasmid containing a specific mammalian origin of replication. Replication is assayed by digestion of the replication products with the restriction endonuclease *Dpn* I (Peden et al., 1980), which discriminates between bacterial methylation of the plasmid and the loss of methylation after replication in the human system. *Dpn* I cleaves only fully methylated input DNA, at the sequence G ^{m}ATC. The plasmids are propagated in deoxyadenosine methylase bacteria; thus, they are fully methylated. Since the extracts are from mammalian cells, they lack this enzyme, and the products of DNA replication are hemimethylated or unmethylated and, therefore, resistant to *Dpn* I cleavage. Recently, another *in vitro* system based on the SV40 system has been developed, which uses a multiprotein replication complex (MRC) from human cells instead of the cell extracts (Coll et al., 1996).

Additionally, there are other *in vitro* systems (reconstituted systems) that use purified protein(s). For example, the Malkas group (Bachur et al., 1992, 1993) used helicases purified from HeLa cells to study the effects of different anthracyclines. Their results indicate that these drugs modified the DNA duplex and made it an unsuitable substrate for DNA helicases. In a similar system, but using DNA ligase obtained from HeLa cells, Ciarrochi et al., (1991) found that several anthracyclines inhibited DNA-ligase joining activity. Reconstituted systems for topoisomerases have also been designed. ICRF-193 inhibited topoisomerase II, but did not have any effect on topoisomerase I (Ishimi et al., 1992) while doxorubicin inhibited topoisomerase II (Bodley et al., 1989).

5. QSAR STUDIES

Quantitative Structure-Activity Relationships (QSAR) attempts to model the "activity" of a series of compounds using measured or computed properties, or descriptors of the compounds (Hahn and Rogers, 1995). Specifically, the aim of QSAR is to find an empirical equation relating experimental biological activity and structural features of the compounds: this equation can then be used to predict the activity of new active molecules, and to better understand drug-receptor interactions.

The physicochemical descriptors include parameters for lipophilicity (e.g. log P-partition coefficient in octanol/water system, molecular lipophilic potential), electronic properties (e.g. atomic charges, molecular electrostatic potential, dipole moment), and steric effects (e.g. molecular volume, molecular surface, and topological indeces). The molecular descriptors can be determined by computational methods (quantum chemistry, molecular mechanics) or empirical methods.

QSAR analyses are based on regression models, where the biological activity of a compound is considered to be dependent on the structure of the compound. Therefore, the biological activity is the dependent variable, and the molecular descriptors are the independent variables or explanatory variables. The measurement of the biological activity (dependent variable) is assumed to have an associated error. However, the molecular descriptors are assumed to be accurate. The method of least squares, which is applied to many independent variables, is used to determine the best fit for the explanatory variable to the observed activity. Once regression coefficients have been estimated, statistics are generated to determine the best fit and the best predictive power of the model.

6. PHARMACOPHORE

A pharmacophore model can be described as the essential three-dimensional arrangement of functional groups that a molecule must possess to be recognized by the receptor, i.e. the component required for biological activity (Blaney and Dixon, 1991). An important issue in constructing a pharmacophore model is whether to use a few of very active rigid structures as templates, hoping to capture the most important structural

requirements for the biological activity, or whether to use ligands with a range of activities, hoping to identify features that discriminate between active and inactive compounds. The traditional approach applied to determine the pharmacophoric groups is a comparative structural analysis of a set of drugs in relation to their biological activity. Once the essential features have been identified, new compounds are synthesized and assayed to test the correlations. Once a plausible pharmacophore has been proposed then the conformational properties of all active compounds can be analyzed to investigate their ability to assume this pharmacophore (Humblet and Marshall, 1980).

There are a number of methods, which have been successfully used in constructing practical receptor models of structure-activity results for a series of ligands. The methods are the following:

1. Models based on a surface. A Van Der Waals surface or Connolly surface (Connolly,1983) (accessible solvent surface) are generated over the set of compounds (after superimposition). Such a surface can visually convey the steric requirement of the receptor binding site (Culberson and Walters, 1991). With computer graphics, it is possible to map electrostatic or lipophilic potentials (Heiden et al., 1990) onto these surfaces to provide information about the electronic or hydrophobic pocket.

2. Models based on a grid of points surrounding the set of compounds. Another way to construct a model around a series of superimposed analogs, is to place the structures in a 3D grid and to look at those grid points near to the surface of the ligand set. This is the starting point for the Comparative Molecular Field Analysis (CoMFA) approach developed by Cramer (Cramer et al., 1988). Field properties (e.g. steric, electrostatic) are calculated with respect to the ligands at each of the grid points. QSAR methods are then used to find relationships between field interaction energies and activity.

3. Models based on a set of atoms, pseudo-atoms or molecular fragments.

Pharmacophore models can be built by placing atoms, pseudo-atoms (e.g. hydrogen bond site acceptor or donor, center of ring) or group of atoms (e.g. amino acids side chains) around the active ligands (Hahn, 1995). The APEX-3D (Apex-3D 95.0, *Molecular Simulation Inc.*, San Diego,CA) expert system for biological activity prediction, uses this approach. APEX-3D is based on principles that model the thinking process in the analysis

of structure-activity relationships. In many cases, the analysis is attempting to identify biophoric (e.g. pharmacophoric, toxicophoric) structural patterns responsible for manifesting certain types of biological activity. Such patterns are usually identified by visual inspection of structural formulas and/or 3D models of chemical compounds. Once a biophoric pattern is identified, structural modifications in distal regions of the structures are analyzed to understand the effect on activity. The pharmacophore representation in APEX-3D is comprised of two parts, the descriptor centers and the distance matrices. Descriptor centers represent parts of hypothetical moieties capable of interacting with a receptor. On the other hand, distance matrices describe the mutual orientation of descriptor centers using topology (number of bonds) or distances (angstrons). Descriptor centers can be either atoms or pseudo-atoms that can participate in the ligand-receptor interactions based on several types of physical properties, i.e. electrostatic interactions (charges, electron acceptor or donor), hydrogen bonds (presence), charge transfer complexes (Lumo, Homo), hydrophobic interactions, Van Der Waals (London) dispersion forces (π-electron density on atoms). Descriptor centers are composed of a combination of atoms type and an atomic property. An atom type can be a set of atoms (e.g. all hydrogen-bond donating groups), or pseudo-atoms (e.g. aromatic ring centers).

CHAPTER TWO

RESEARCH OBJECTIVES

RESEARCH OBJECTIVES

Steroids, and particularly estrogens, are involved in the development of breast cancer. Their effect on carcinogenesis is usually attributed to their capacity to stimulate the replication of malignant and preneoplastic cells. Although the effect of these compounds on breast cancer has been well established, little is known about the molecular mechanism(s), which implicate them in carcinogenesis. It is generally believed that steroids mediate their actions through steroid receptors. However, due to some biological actions of steroids that cannot be explained exclusively from binding to the steroid receptors, other possible mechanisms of action have emerged. A better understanding of the mode of action of these compounds in carcinogenesis may lead to the development of new therapeutic agents for the treatment of breast cancer. Thus, the objectives of this research were:

1. To validate a mammalian *in vitro* DNA replication system for the study of the effects of drugs and compounds on DNA replication.

2. To investigate the effect of several steroids, mostly estrogens and presumably involved in carcinogenesis, on the process of DNA synthesis.

3. To develop QSAR studies and to design a pharmacophore, aiding in determination of key steroid structural elements responsible for the DNA replication effects of a series of steroids.

CHAPTER THREE

APPLICATION OF AN IN VITRO SYSTEM IN THE STUDY OF THE CHEMOTHERAPEUTIC DRUG EFFECTS ON DNA REPLICATION

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Application of an *in vitro* system in the study of chemotherapeutic drug effects on DNA replication

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ABSTRACT

DNA replication machinery is an important target for chemotherapeutic drugs. We have used an *in vitro* system to study the effect of drugs on mammalian DNA replication, either by direct interaction with the DNA structure or with replication proteins and machinery. The anthracycline, doxorubicin (Dox), showed a dose-dependent inhibitory effect on DNA replication, whether incubated with HeLa cell extracts or with DNA and nucleotides. Earliest-labeled fragment analysis revealed that inhibition of replication began within the origin containing fragment in both control and Dox-containing reactions *in vitro*. AraC, a nucleoside analog, had no significant effect on DNA synthesis. In contrast, araCTP was able to inhibit DNA replication *in vitro*. Since metabolism is diminished in this *in vitro* system, the degree of phosphorylation of araC was apparently low. Progesterone showed an increase in nucleotide incorporation (sensitive to BuPdGTP inhibition of replication specific polymerases α and δ) after preincubation with HeLa cell extracts, although progesterone receptors were not detectable in the HeLa cell extracts. In addition, we observed an inhibition in DNA replication when progesterone was preincubated with DNA and nucleotides. These results suggest that progesterone may have a mechanism of action that is different from any known to be mediated through progesterone receptors. In conclusion, these results indicate that this mammalian *in vitro* replication system will be useful for the study of mechanisms and design of therapeutic drugs that inhibit mammalian DNA replication.

DNA replication is one of the most fundamental biological processes in living cells. Alterations in DNA replication may result in uncontrolled cell proliferation, which can contribute to cancer and other abnormal growth disorders, or block propagation and maintenance of cells. There is an intense search for new antitumor agents with more favorable therapeutical profiles; among these, is an important group of drugs that target the machinery of DNA replication. However, in the evaluation of drug-efficacy of new compounds, there are many biological factors, such as transport of the drug into the cell and into the cell nucleus, which make it difficult to determine the efficacy of these drugs as related to structure. In order to overcome these problems, investigators have used *in vitro* systems (Bachur et al., 1992; Ciarroechi et al., 1991; Frosina and Rossi, 1992; Bigioni et al., 1994). There are several *in vitro* cell-free systems which seem to mimic closely eukaryotic nuclear DNA replication *in vivo*, where the efficacy of drugs can be studied in the absence of confounding factors (Li and Kelly, 1984; Pearson et al., 1991; Berberich et al., 1995). One of the current models of eukaryotic DNA replication, is based on SV40 (Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe et al., 1985), and although it is very well characterized, it uses a viral origin of replication requiring a specific viral initiator protein, the SV-40 large T-Antigen (T-Ag). Specific protein-protein interactions, DNA-protein interactions and drug interactions with complexes essential for DNA replication in the SV40 *in vitro* system only mimic certain eukaryotic DNA replication traits, which may not be entirely appropriate models for the evaluation of the sensitivity of mammalian cellular DNA replication systems to drugs. We and others have used an *in vitro* DNA replication system which uses a mammalian origin of replication and extracts of human cells, HeLa (Pearson et al., 1991, Pearson et al., 1994) and 293S (Berberich et al., 1995). Plasmids containing monkey origin enriched sequences (*ors*) which replicate in extracts from HeLa cells were used by Pearson et al. Berberich et al. reported that a plasmid containing the c-*myc* insert can be recognized by the mammalian replication machinery from 293S cells to initiate semiconservative DNA synthesis. Since proteins involved in DNA replication and the DNA molecule, itself, are potential targets for chemotherapy of cancer, we have designed the *in vitro* assay protocol to better discriminate between the effect of drugs on DNA and the effect on the replication

proteins that are present in the HeLa cell extracts, as well as to assess the overall efficacy of drugs on DNA replication. In this initial study we have investigated compounds belonging to three different families. We have studied the effects of the anthracycline, doxorubicin; the nucleoside analog, araC; and the steroid, progesterone. The results demonstrate that we can use this mammalian *in vitro* replication system to study the effect of drugs on DNA or on proteins involved in the process of DNA replication. In particular, we found that the steroid, progesterone, could have an effect or mechanism of action apparently unrelated to receptor-ligand nuclear interactions.

METHODS

Plasmids and cell extracts

Plasmid pX24 of Dr. J. Hamlin (University of Virginia) was provided by Dr. M.L. DePamphilis (Roche Institute of Molecular Biology). pX24 is able to replicate autonomously in a cell-free system that uses HeLa cell extracts (M.Zannis-Hadjopoulos et al., 1994). Plasmid pX24 contains a 4.8 kb *Xba* I fragment of the DHFR origin of replication region, from the *Xba* I site at 14.0 kb to the *Xba* I site at 18.8 kb, and inserted into the *Xba* I site of pUC13 (Burhans et al., 1986). Plasmid 30.4 carries a randomly selected sequence of 0.7 kb inserted into the pBluescript vector, obtained from human breast tumor DNA, and without autonomously replication activity.

HeLa S3 extracts (nuclear and cytoplasmic) were prepared as described previously by Pearson et al., 1991 .

Replication reactions

The cell-free replication assay was adapted from the method described previously by Pearson et al., 1991, with slight modifications. The system essentially consists of nuclear and cytoplasmic extracts from HeLa cells, a mixture of nucleotides (ATP, CTP, GTP, UTP, dATP, dGTP, dTTP and dCTP), 10 μCi of [α-^{32}P] dCTP and 10 μCi of [α-^{32}P] dTTP, an ATP regenerating system, and equimolar amounts of a plasmid containing either a specific mammalian origin of replication (pX24) or a random human breast tumor DNA sequence (30.4). The drugs have been tested using concentrations which include reference values within the pharmacological margin, i.e. IC_{50} in HeLa cells for doxorubicin (0.13 μM) and araC (4.5 μM), and C_{max} in serum for progesterone (200 nM). The IC_{50} for araC was taken as the reference value for araCTP. The experiments were performed by preincubation of the different drugs with either HeLa cell extracts or plasmid DNA and nucleotides at 30°C. After 15 min, the remaining components, plasmid DNA and nucleotides (preincubations with HeLa cell extracts) or HeLa cell extracts (preincubations with DNA and nucleotides), were added and the samples were incubated at 30°C for 1 h. The reactions were terminated by the addition of a stop mix (30 mM

EDTA, 1% SDS) and proteinase K, and the samples were incubated at 37°C for 1 h. DNA was purified by standard procedures (Pearson et al., 1991; M. Zannis-Hadjopoulos et al., 1994), and the reaction products were divided in three portions. One of the three aliquots was digested with 1 U *Dpn* I (New England Biolabs) at 37°C for 1.5 h. The undigested and *Dpn* I-digested products were resolved on a 1% agarose gel in 1x TAE (Tris-acetate, EDTA) buffer (16 h, 2.5 V/cm). The gels were fixed, dried and exposed to NEF-496 film (Dupont). Quantification was performed on *Dpn* I-digested products by densitometric measurements using a Phosphoimager analyzer (Fuji BAS 2000). Quantification involved the measurement of the density of bands corresponding to forms II and III of pX24 plasmid DNA, and normalized by subtracting the background and for the amount of DNA in ethidium bromide gels. The amount of [α-^{32}P] dCTP and [α-^{32}P] dTTP incorporated into DNA was expressed as a percent of a control pX24 containing no drug. The total incorporation into DNA was 0.033 pmols for pX24 and 0.009 pmols for the control plasmid 30.4; *Dpn* I resistant incorporation was approximately one-third of the total incorporation for pX24, plasmid 30.4 did not show *Dpn* I resistance.

Stock solutions of drugs were: 0.1 M araC (SIGMA) in water, 1 mM araCTP (SIGMA) in water, 3.5 mM doxorubicin (Adria laboratories) in DMSO, 1 mM progesterone (SIGMA) in ethanol. Dilutions were made in water to the desired concentration. The final concentrations of DMSO and ethanol never exceeded 0.1%. Solutions were stored at -20°C.

Earliest labeled DNA fragment

In vitro DNA replication of pX24 in the absence or presence of 0.33 μM concentration of doxorubicin was performed, and the reactions were stopped at 4, 8, or 12 min of incubation. The DNA products were digested overnight with 1 U of each of the restriction enzymes, *BamH* I, *Bgl* I and *Xba* I. The digestion products were electrophoresed on a 1.5% agarose gel in 1x TAE buffer (16 h, 2.5V/cm). Incorporation of [α-^{32}P] dCTP and [α-^{32}P] dTTP into each fragment was quantitated by densitometry of a phosphoimager screen using the Fuji BAS 2000 analyzer, and expressed as incorporation/kb of DNA.

RESULTS AND DISCUSSION

The purpose of this study was to demonstrate the effect of different drugs, particularly chemotherapeutic drugs, on DNA replication. To address this, we chose a mammalian *in vitro* DNA replication system, dependent upon the presence of a mammalian origin of DNA replication, which is thought to closely mimic nuclear mammalian DNA replication (Pearson et al., 1991; Pearson et al., 1994). This system uses soluble extracts from cytoplasm and nuclei of HeLa cells, which provide the replication proteins to support replication of exogenous plasmid templates containing a mammalian origin of replication. The plasmid pX24 was chosen because it has been shown by several autonomous replication assay techniques, *in vivo* and *in vitro,* to contain a mammalian origin of replication from the DHFR locus (Zannis-Hadjopoulos et al., 1994). Several mapping techniques, such as nascent DNA PCR mapping (Vassilev et al., 1990) and Okazaki fragment distribution mapping (Burhans et al., 1990) have also shown that pX24 contains a bidirectional origin of replication. In this initial study, we have investigated the effects of three compounds belonging to different families of drugs, i.e. having different mechanisms of action. Specifically, we describe results for ß-cytosine arabinoside (araC), doxorubicin and progesterone. The first two agents are thought to interact with the DNA replication machinery, such as, DNA polymerase, topoisomerase II, DNA ligase and helicase (Kuchta et al., 1992; Bodley et al., 1989; Ciarroacchi et al., 1991; Bachur et al., 1992), and the third one is thought to exert an indirect effect on DNA replication by binding to the progesterone receptor (Spelsberg and Toft, 1976). The drugs have been tested using concentrations which include reference values within the pharmacological margin. Different drug concentrations were either preincubated with DNA or cell extracts, in an attempt to differentiate direct effects of drugs on the DNA molecule or on the proteins involved in the process of DNA synthesis.

Both, preincubation of doxorubicin with DNA and with cell extracts, which contain the proteins involved in DNA replication, resulted in a concentration-dependent inhibition of DNA synthesis (0.13-13 μM) (Fig. 1 and 5A). However, at low concentrations of doxorubicin (0-0.33 μM), the inhibitory effect was higher when the

drug was preincubated with the HeLa cell extracts (Fig. 1, lanes 2,3 and 12,13) than with the DNA and nucleotides (Fig. 1, lanes 6,7 and 16,17). The results obtained with this *in vitro* assay showed that the method is reproducible, and an example is shown in Fig. 2. Moreover, we observed that the increase of doxorubicin concentration produced an alteration in topoisomeric forms of DNA, increasing the amount of form I (supercoiled) plasmid (Fig. 2, lanes 7-10). The maximal amount of form I DNA was reached with 1.3 μM doxorubicin (Fig. 2, lanes 7,8)(100%); the relative amounts of supercoiled plasmid with respect to form I DNA were, 9.2% (Fig. 2, lanes 1,2), 11.6%_(0.13 μM, lanes 3,4), 28% (0.33 μM, lanes 5,6), and 32.4% (13 μM, lanes 9,10). There was similar electrophoretic mobility of DNA in the 13 μM doxorubicin sample for both the autoradiogram of the gel and in the ethidium bromide-stained gel (Fig. 3, lane 3). This effect is probably due to the intercalation of doxorubicin in the duplex of DNA, as it has been reported previously (Bodley et al., 1989). To determine whether replication of pX24 initiated within the DHFR fragment, pX24 was incubated in the *in vitro* replication system for 4, 8 or 12 min and subsequently digested with *BamH* I, *Bgl* I and *Xba* I, and fractionated on a 1.5% agarose gel. The digestion yielded 8 fragments ranging from 6 to 1750 bp. The incorporation of [α-^{32}P] dCTP and [α-^{32}P] dTTP into each restriction fragment was quantitated by densitometry using a phosphoimager analyzer, and normalized as indicated in the methods and for the size of the fragment. The results showed that the DHFR-containing fragment 1.6 kb has the highest incorporation/kb, and is the earliest labeled fragment. Moreover, the same experiment in the presence of 0.33 μM doxorubicin showed that the inhibitory effect of the drug did not change the apparent initiation site on overall profile of incorporation (Fig. 4).

The nucleoside analog, araC, only showed a slight inhibition of nucleotide incorporation ($\leq$ 20%, at concentrations up to 13 μM), when it was preincubated with DNA or with cell extracts, which contain the proteins involved in the mechanism of DNA replication (Fig. 5B). Since araC needs to be phosphorylated to araCTP to interact with DNA or DNA replication proteins, the suboptimal metabolism afforded by these HeLa cell extracts probably failed to adequately duplicate the phosphorylation of araC that can be obtained in intact cells. This was confirmed by using araCTP, which showed a

concentration-dependent inhibition of DNA synthesis after preincubation with DNA or with HeLa cell extracts (Fig. 5C).

In addition, we observed new activities for the steroid, progesterone. Cells are generally considered to be positive for progesterone receptor if they contain greater than 20 fmol/mg total protein (Leclercq et al., 1977); however, measurements of receptor levels in the HeLa nuclear and cytoplasmic extracts that were used in the *in vitro* replication system did not reach this threshold (data not shown). The progesterone receptors were measured in the cytoplasmic and nuclear extracts by the standard dextran-coated charcoal adsorption method using frozen cell extract pellets (RIANEN [^{3}H] Progestin Receptor Assay Kit, Dupont, Billerica, MA, USA 01862) (King et al., 1979; Bloom et al., 1980). Preincubation of progesterone with the HeLa cell extracts resulted in an enhancement of DNA synthesis (Fig. 5D). The maximal stimulation was reached at a concentration of 8 nM, and the same level of DNA replication was maintained up to 200 nM progesterone. At concentrations between 200 nM and 500 nM the stimulatory effect decreased towards no drug control levels. To determine whether this effect of progesterone was due to DNA replication or repair, the ability of progesterone to increase the incorporation of precursor nucleotides into DNA was tested in the presence of the DNA synthesis inhibitor, butylphenyl deoxyguanosine triphosphate (BuPdGTP) (Fig. 6). BuPdGTP inhibits polymerases α and δ (Byrnes, 1985), both of which participate in the process of nuclear DNA replication. Preincubation of HeLa cell extracts with two different concentrations of BuPdGTP (100 and 200 μM) in the presence and absence of 150 nM progesterone showed an inhibition of incorporation of approximately 65% with 100 μM and 75% with 200μM BuPdGTP (Fig. 6, lanes 3-6 and 3'-6'). These data indicate that the enhancement of nucleotide incorporation observed in the presence of progesterone is due to DNA replication. The stimulatory effect of progesterone on *in vitro* DNA replication may be due to the interaction of progesterone with one or more proteins involved in the process of DNA replication, a mechanism likely different from any known to be mediated through progesterone receptors. Furthermore, preincubation of progesterone with DNA and nucleotides indicated an inhibition of DNA synthesis (Fig. 5D), which was maximal at 8 nM concentration, and was maintained between 8 nM and

500 nM progesterone. The observed effects of progesterone are specific since prednisolone (C_{max} approximately 2.8 μM) (Hill et al., 1990), a structural analog of progesterone, showed no effects in this *in vitro* DNA replication system (data not shown). These studies indicate that this *in vitro* DNA replication assay will be useful for determining the multiple effects of compounds on mammalian DNA replication, whether directly interacting with DNA or replication proteins and machinery. The greater flexibility of this *in vitro* mammalian system offers unique opportunities to uncover new mechanisms of drug function, to optimize drug efficacy and provide for greater selectivity in design and development of new anticancer agents.

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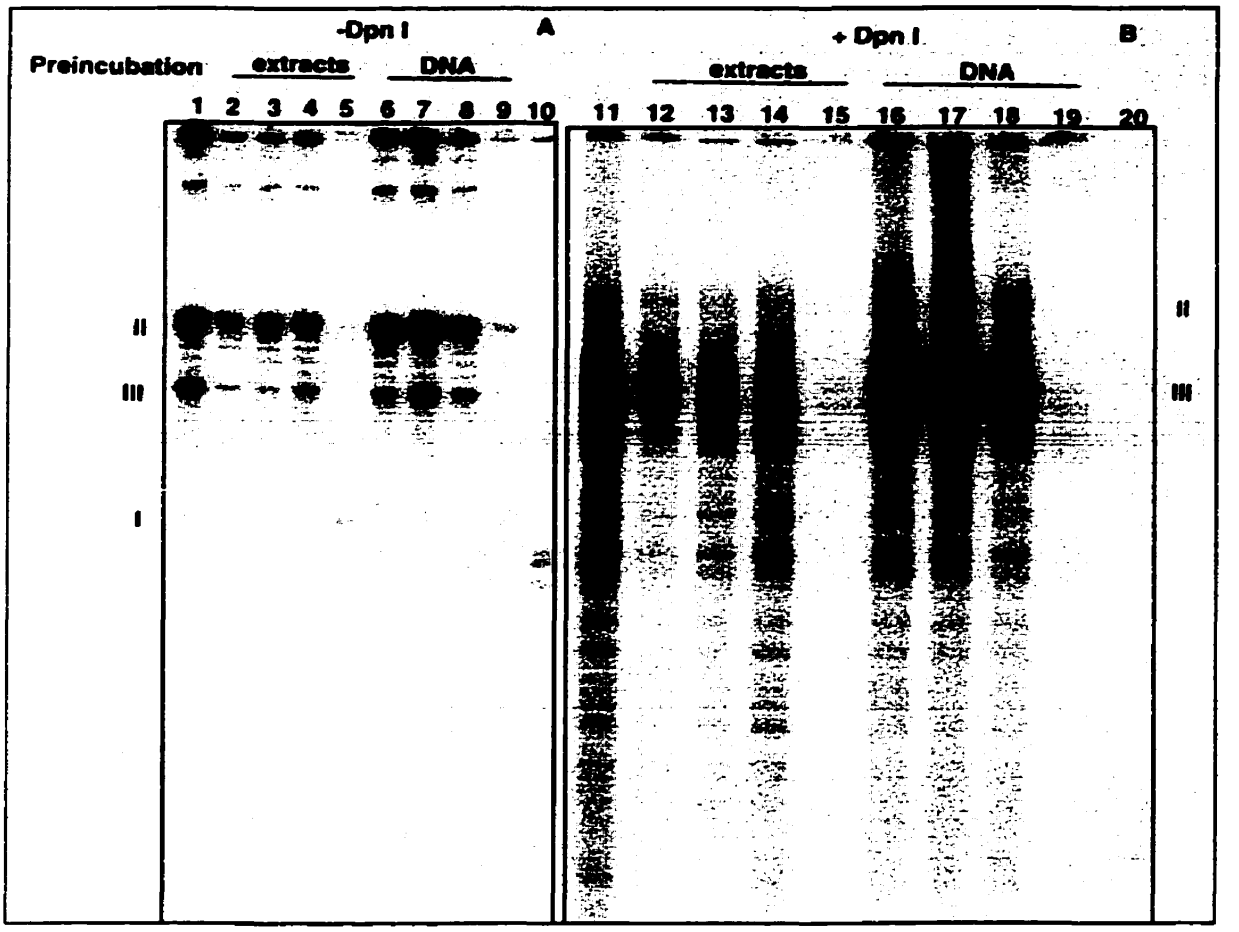

Fig. 1. pX24 replication *in vitro* in the presence of increasing concentrations of doxorubicin. Reactions (50 µM) contained 105ng of pX24. Different drug concentrations were preincubated with HeLa cell extracts or with DNA and nucleotides. Reaction mixtures were completed as described in the Methods.The products were divided in three aliquots: two of them (one untreated, A, and the other digested with 1 U *Dpn* I at 37°C for 1.5 hr, B) were electrophoresed on 1% agarose and analyzed by a phosphoimager analyzer. Electrophoresis of replication products without *Dpn* I digestion (A) and with *Dpn* I digestion (B) was performed in the same manner, excepting the comb size and the running time. Lanes 1, 11: positive control, no drug; lanes 2, 6, 12, 16: doxorubicin (0.13µM); lanes 3, 7, 13, 17: doxorubicin (0.33µM); lanes 4, 8, 14, 18: doxorubicin (1.3µM); lanes 5, 9, 15, 19: doxorubicin (13µM); lanes 10, 20: negative control (30.4). The mobilities of supercoiled (form I), relaxed (form II), and linear (form III) pX24 are indicated.

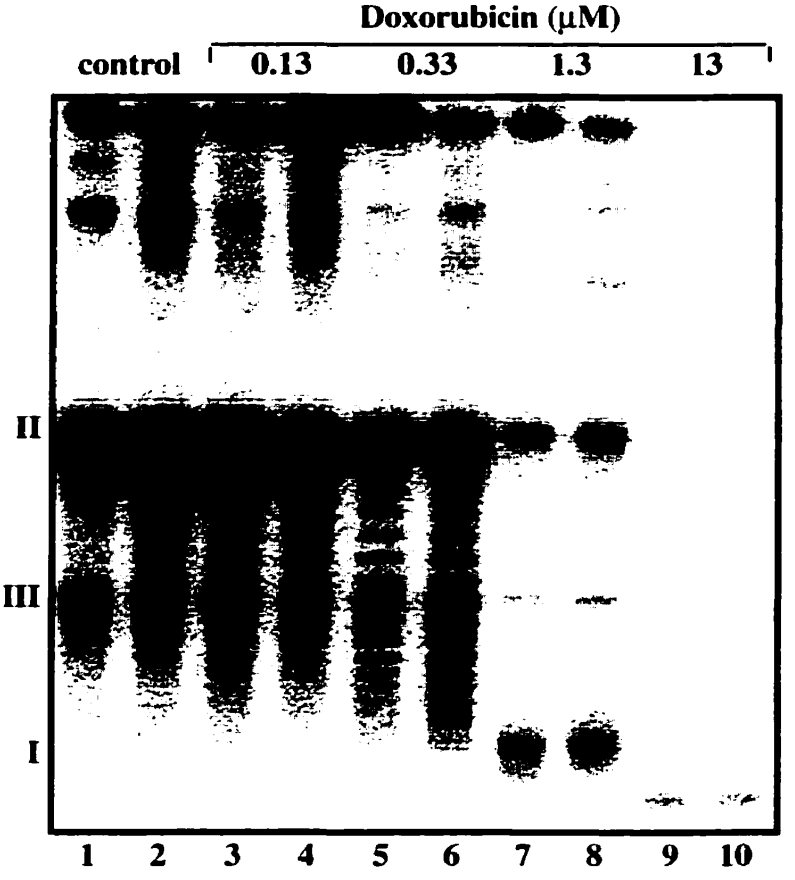

Fig. 2. Reproducibility of the *in vitro* DNA replication assay, and effect of doxorubicin on DNA structure and DNA replication. Replication was carried out as previously indicated in the Methods.This gel represents the undigested replication products, after template DNA and nucleotides were preincubated with different concentrations of doxorubicin. The samples are in duplicate. Lanes 1, 2: positive control, no drug; lanes 3, 4: doxorubicin (0.13 μM); lanes 5, 6: doxorubicin (0.33 μM); lanes 7, 8: doxorubicin (1.3 μM); lanes 9, 10: doxorubicin (13 μM). The position of pX24 form I, II, and III DNA are indicated. The ladder of bands migrating between forms II, III and I indicate the presence of a series of topoisomeric molecules with supercoils.

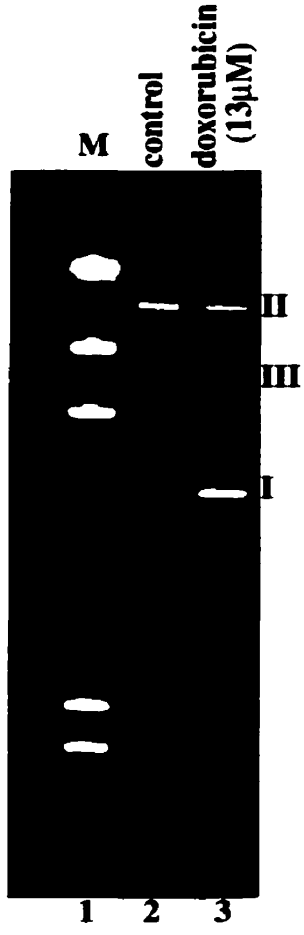

Fig. 3. Increase in the amount of supercoiled DNA in the presence of 13 μM doxorubicin. Electrophoretic mobility of DNA in the absence (lane 2) and presence (lane 3) of 13 μM doxorubicin in the ethidium bromide-stained gel. Marker (M) in lane 1. The mobilities of form I, II, and III DNA are indicated.

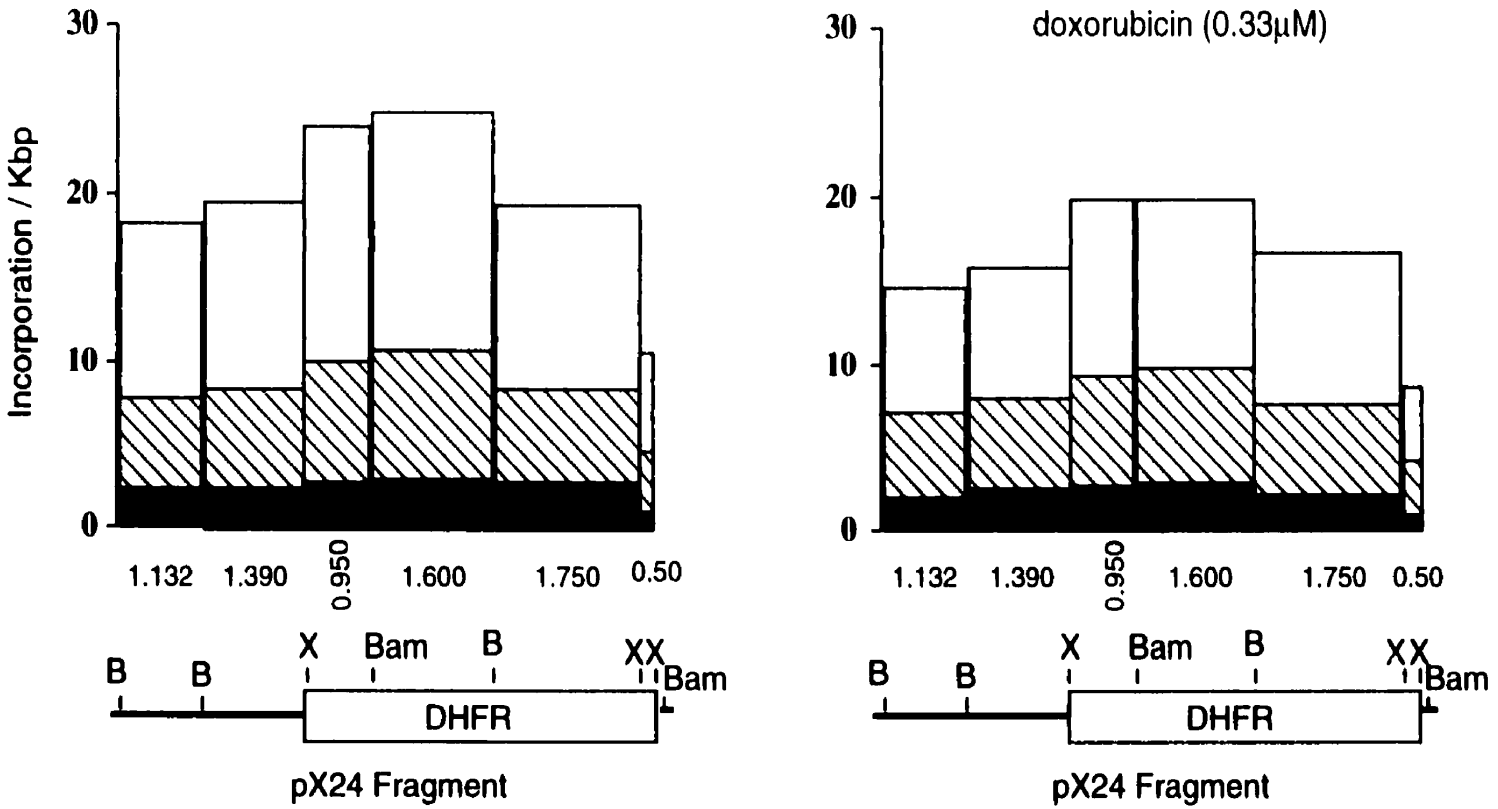

Fig. 4. Preferential labeling in the DHFR insert of pX24 at early times of *in vitro* replication. pX24 was assayed in an *in vitro* replication reaction in the absence (left) or presence (right) of 0.33 µM doxorubicin, and the reactions were stopped at 4 (black bars), 8 (striped bars), or 12 (white bars) min. After extraction and purification of DNA, the products were digested with *BamH* I (Bam), *Bgl* I (B), and *Xba* I (X) before analysis by agarose gel electrophoresis and quantitative densitometry. The results were expressed as incorporation per kb for each fragment. At the bottom of the graphic is shown the linearized map of pX24, where the box represent the DHFR insert.

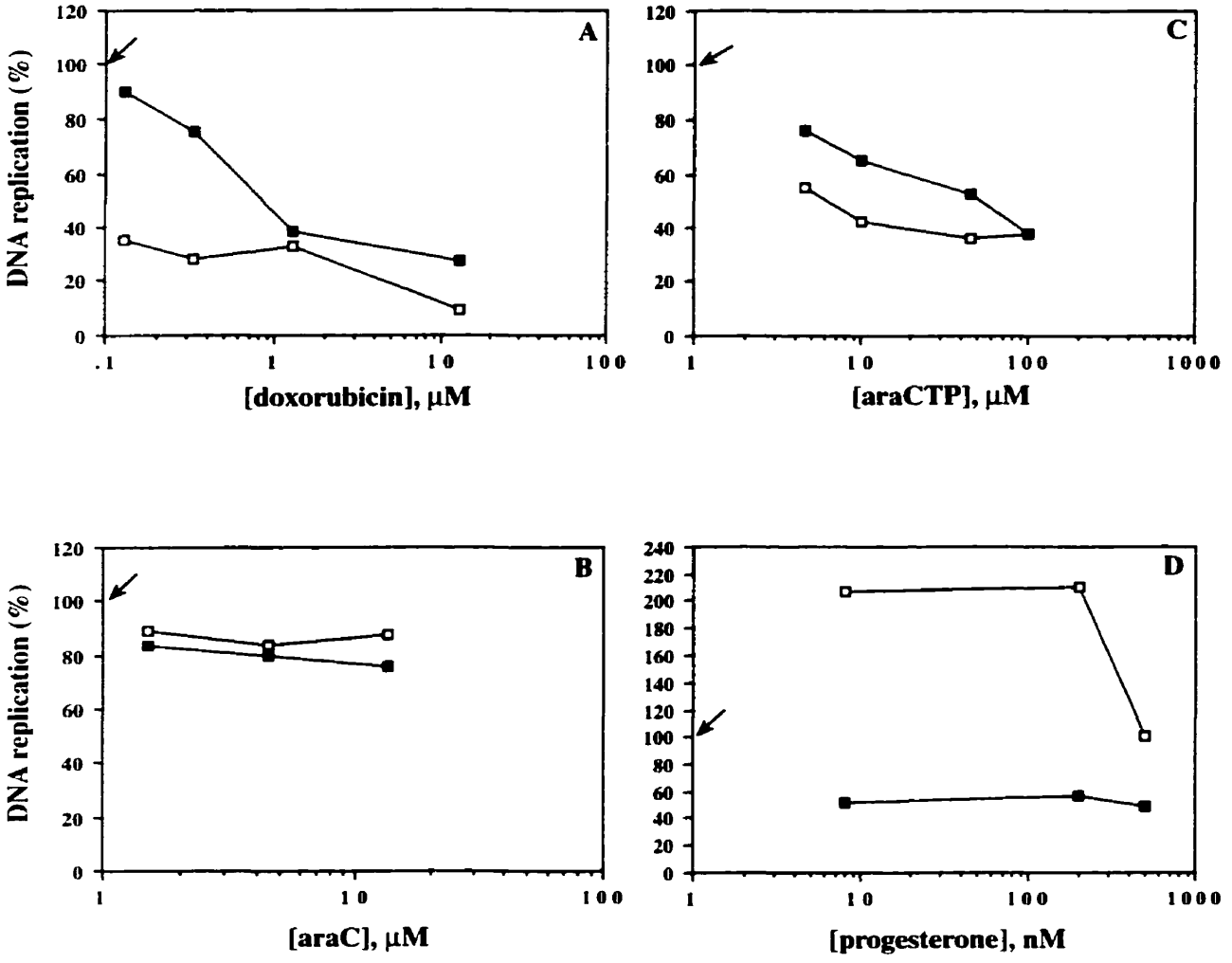

Fig. 5. Effect of different drugs on DNA replication. pX24 replication was assayed in HeLa cell extracts in the presence of increasing concentrations of doxorubicin (**A**), araC (**B**), araCTP (**C**), or progesterone (**D**). Incorporation of radioactive nucleotide precursor was determine for each sample by autoradiography and quantitative densitometry scanning. DNA replication (%) was plotted against the drug concentration. Each point represents the mean of duplicate samples, with a maximal variation of 15%. Open squares, preincubation with HeLa cell extracts; solid squares, preincubation with DNA and nucleotides. Arrows denote control values.

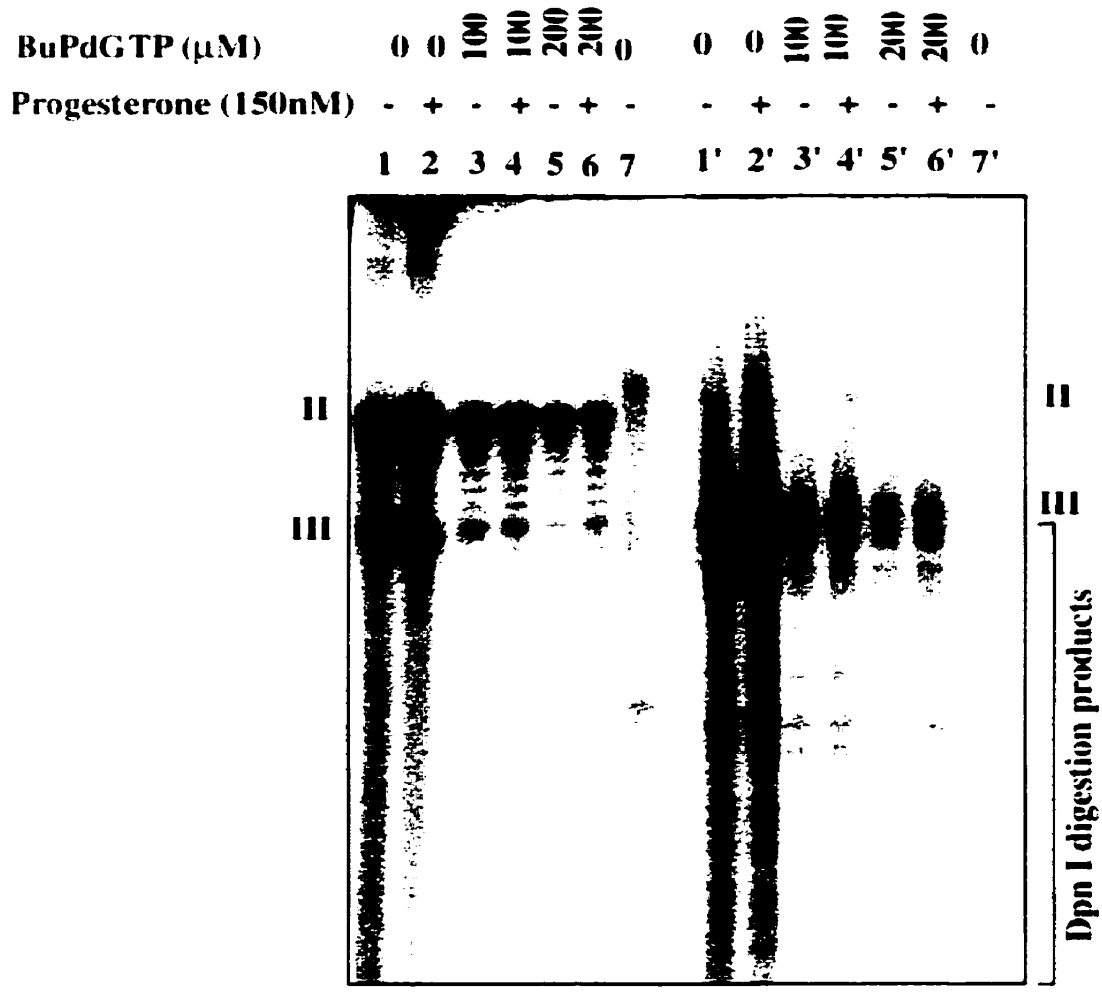

Fig. 6. Effect of BuPdGTP on *in vitro* reactions. pX24 was incubated with HeLa cell extracts and different concentrations of BuPdGTP (100 and 200 μM), in the absence (-) or presence (+) of 150 nM progesterone, as described in Methods. Lanes 1-7 represent total incorporation, and lanes 1'-7', Dpn I-digested samples. Lanes 7 and 7' contain the reactions done with the negative control, p30.4. Relaxed circular (II) and linear (III) forms of plasmidDNA are indicated, as well as the area corresponding to Dpn I digestion products.

CHAPTER FOUR

RECEPTOR INDEPENDENT ENHANCEMENT OF DNA REPLICATION BY ESTROGENS

Maria J. Diaz-Perez, Maria Zannis-Hadjopoulos, Gerald B. Price, and Irving W. Wainer

Chapter Three demonstrated the reproducibility and usefulness of the *in vitro* DNA replication assay to study the effect of drugs and compounds on DNA synthesis. Additionally, we found that the steroid progesterone seems to have a receptor-independent effect on DNA synthesis. Since we found an effect of steroids upon DNA replication and there has been a great interest in their role in carcinogenesis, we have used this method in Chapter Four to study the effects of several estrogens on DNA synthesis, in an effort to understand better the mechanism of action of estrogens in carcinogenesis.

This Chapter has been accepted in the *Journal of Cellular Biochemistry* for publication. I performed all experiments, assembled the results and wrote all portions of the paper. Gerald B. Price, Irving W. Wainer and Maria Zannis-Hadjopoulos made editorial revisions. Gerald B. Price and Irving W. Wainer provided supervision in the planning of the experiments.

Receptor independent enhancement of DNA replication by estrogens

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ABSTRACT

There is now convincing evidence associating estrogens with an increased risk of some cancers. However, the absence of a complete correlation between estrogen receptor binding and their biological activity, has suggested the possibility of other mechanism(s) of action. The effect on DNA replication of several hormones that are putatively involved in breast cancer, was tested at a physiological concentration. The studies were conducted in a HeLa cell-free system using a plasmid containing a specific mammalian origin of replication (DHFR *oriβ*) as template DNA. 17ß-estradiol, estrone, estriol, and 16α-hydroxy-estrone produced an enhancement of *in vitro* DNA synthesis. These studies indicate a new possible target, which may help to better understand the effect of these hormones in breast cancer. Furthermore, the results show that this *in vitro* DNA replication system provides an evaluative assay for the effects of compounds on hormone responsive cancers, independent of some hormone receptors.

INTRODUCTION

Steroid hormones, in particular estrogens, induce cell proliferation and regulate the human mammary epithelial morphogenesis. Additionally, these hormones have been associated with neoplastic changes. Estrogens have been shown to be promoters of mammary carcinogenesis, in both human and experimental models. The therapeutic use of estrogens (e.g. hormone replacement therapy, oral contraceptives) has been associated with an enhancement in the risk of breast cancer (1, 2). Other evidence supporting the link of estrogens and breast cancer is their ability to generate mammary tumors in rodents (3), and their mitogenic effects on mammary human tissue (4, 5) and established breast cancer cell lines (6).

Despite intensive research, the molecular and cellular mechanism through which estrogens influence carcinogenesis is not completely understood. The carcinogenicity of estrogens has been primarily attributed to their principal mechanism of action, which is mediated through the estrogen receptor. For some estrogen compounds there is no correlation between binding of them to the estrogen receptor and their biological activity, suggesting that estrogen action may not be exclusively mediated through the receptors.

Several non-receptor mediated mechanisms have been suggested. These include direct chemical interactions of certain estrogens with DNA and/or proteins (7-10). In addition, estrogens may interact with the DNA structure, by insertion between base pairs into partially unwound double stranded DNA (11).

A common feature during the development of carcinogenesis is cell proliferation. Therefore, since estrogens affect cell proliferation, the DNA replication apparatus is one of the potential targets. In order to help elucidate the effect of estrogens on DNA replication, we employed a mammalian *in vitro* DNA replication system which is both progesterone- and estrogen-receptor negative (12, 13). This Hela cell-free system is used to replicate a bacterial plasmid containing a specific mammalian origin of DNA replication, in this case the origin from the hamster dihydrofolate reductase (DHFR) locus. The DHFR origin, oriß, is one of the most thoroughly characterized origins of bidirectional replication (OBR), and is located approximately 17-kb downstream of the *dhfr* locus (14). It has been shown to be an origin of replication by various mapping

techniques, including the nascent DNA PCR-based assay (15) and by Okazaki fragment distribution (14). Zannis-Hadjopoulos and colleagues (16) showed by the *Dpn* I resistance assay (17) that pX24 plasmid containing the DHFR oriß was able to replicate autonomously in human cells and in an *in vitro* cell free-system, which used human HeLa cell extracts (18). This system was used in this study. Moreover, we recently demonstrated by 'earliest-labeled DNA fragment labeling', that initiation of replication in this cell free-system begins preferentially within pX24 (12).

This *in vitro* DNA replication assay allows the study of the possible interactions between various compounds with either DNA or the protein(s) that are involved in DNA synthesis. The data reported here suggest a direct non-receptor mediated effect of several estrogens on DNA replication.

MATERIALS AND METHODS

Cell extracts and plasmids. HeLa S3 nuclei and cytosol were purchased from Cellex Biosciences, Inc. (Minneapolis, MN). The extracts were prepared as previously described by Pearson and colleagues (18). The protein concentrations of the nuclear and cytosolic extracts were 3.8 mg/ml and 11 mg/ml, respectively.

Plasmid pX24 of Dr. J. Hamlin (University of Virginia, Charlottesville, VA) was provided by Dr. M. L. DePamphilis (NIH, Bethesda, MD). pX24 contains a 4.8 kb *Xba* I fragment of the *oriß* region of DHFR, inserted into the *Xba* I site of pUC13 (14). Plasmid 30.4 carries a randomly selected sequence of 0.7 kb inserted into the pBluescript vector, obtained from human breast tumor DNA, and without autonomously replication activity (19).

Biochemicals. 17β-estradiol, estrone, estriol, 17α-estradiol, and 16α-hydroxy-estrone were purchased from Sigma (Mississauga, Ontario, Canada); [α-^{32}P]dCTP and [α-^{32}P]dTTP from NEN-DuPont (Boston, MA); *Dpn* I from New England Biolabs; 6,7 17β-[^{3}H]estradiol from Armesham (Oakville, Ontario, Canada). Stock solutions of hormones were in ethanol, and dilutions in water, never exceeded 0.1% of ethanol.

Estrogen receptor binding assay. The level of estrogen receptors in the nuclear and cytoplasmic extracts was determined by using the steroid-binding assay. Aliquots of nuclear or cytoplasmic HeLa extracts (50 μg of protein) were incubated with 20 nM 17β-[^{3}H]estradiol for 2 hr at room temperature in the presence or absence of unlabelled estradiol (4 μM). Non-specific binding was defined as binding remaining in the presence of 4 μM 17β-estradiol. Bound ligand was separated from free by the dextran-coated charcoal method, and determined by liquid scintillation counting. A value higher than 10 fmol/mg cytosol or nuclear protein indicated detectable ER and is designated as positive (20).

***In vitro* replication assay.** *In vitro* replication was carried out as previously described (12). Standard *in vitro* reactions were basically composed of equimolar

amounts of a supercoiled plasmid, either pX24 or plasmid 30.4 (150 ng and 74 ng, respectively), HeLa nuclear and cytoplasmic extracts, an ATP regenerating system, PEG, a mixture of nucleotides (ATP, CTP, GTP, UTP, dATP, dGTP, dTTP and dCTP), and 10 μCi of [α-^{32}P]dCTP and [α-^{32}P]dTTP. The experiments were conducted in two different ways: 1) preincubation of the experimental compounds with HeLa cell extracts, followed by addition of the DNA template and precursor nucleotides to allow replication to occur; 2) preincubation of the experimental compounds with the template DNA and the precursor nucleotides, followed by addition of the remaining components of the *in vitro* reaction to initiate DNA replication.

Following DNA isolation and purification, DNA synthesis was measured using the *Dpn* I resistance assay (17). *Dpn* I cleaves only fully methylated input DNA, at the sequence G^{m}ATC. Both plasmids (pX24 and 30.4) were propagated in dam^{+} (deoxy adenosine methylase) bacteria and therefore, they are fully methylated. Since HeLa cell extracts lack this enzyme, the products of one round of DNA replication that occur in this *in vitro* system (18) are hemimethylated and, therefore, resistant to *Dpn* I cleavage. Forms II (circular) and III (linear) DNA were quantitated as previously described (12) by densitometry of a phosphoimager screen using the Fuji BAS 2000 analyzer. We have used in this assay physiologically relevant concentrations (10 nM) of the steroids (21).

Dose-response experiments. HeLa cell extracts or DNA and precursor nucleotides were preincubated with various concentrations (0.1 nM, 1 nM, 10 nM, or 100 nM) of the estrogens, 17β-estradiol, estrone, and estriol. DNA replication proceeded as above.

Data analyses. The data was expressed as the percentage of the non-drug-treated control (100%), and represents the average of at least two separate experiments (mean ± s.d.).

RESULTS

Measurement of estrogen receptors in HeLa S3 cells. HeLa S3 cells have been previously reported to be estrogen receptor negative (13). Since our *in vitro* replication system uses HeLa cell extracts, we decided to verify the estrogen receptor levels in nuclear and cytoplasmic extracts from HeLa cells, in order to eliminate the possibility of receptor-mediated mechanism as a mode of action of these estrogens in this system. The estrogen receptors were measured by a ligand binding assay. Recently, a potential role for the ER_β has been suggested. Since the assay employed to measure the ER level in the HeLa cell extracts can detect significant activity for all ERs (as determine by a value of ≥ 10 fmol/mg protein) and since a value lower than 8 fmol/mg protein was obtained, it was concluded that both the nuclear and cytoplasmic extracts were estrogen receptor negative. Therefore, these extracts were suitable for use in our *in vitro* DNA replication system

Determining an effective hormone concentration. To determine an effective hormone concentration we analyzed a range of estrogen concentrations, from 0.1 to 100 nM. This range represents intracellular concentrations, which can be reached *in vivo*. Replication was carried out as explained in **Materials and Methods**. Different concentrations (0.1-100 nM) of the parent estrogen, 17β-estradiol, along with two of its principal metabolites, estrone and estriol, were preincubated with either HeLa cell extracts (Fig.1 and 2A) or DNA and nucleotide precursors (Fig.2B), in order to determine an effective hormone concentration. A typical autoradiograph of *in vitro* replication products, following the *in vitro* assay, is shown in Figure 1. The results demonstrated a concentration dependent effect of 17β-estradiol, estrone and estriol, on DNA replication. *Dpn* I resistant bands corresponding to form II and III of DNA were quantitated by subtracting the background and normalized for the amount of DNA in ethidium bromide gels, as previously described (12, 19). The results are plotted in Figure 2A.

Preincubation of the estrogens with HeLa cell extracts (Fig. 2A) showed a small and similar effect at 0.1 nM for the three estrogens; however, at 10 nM we observed an important and distinct enhancement on DNA synthesis for all of the estrogens mentioned above, i.e. approximately 1.7-fold for estrone and 17β-estradiol, and 3.6-fold for estriol.

At 100 nM estriol and estrone exhibited a decline in DNA synthesis, while 17β-estradiol increased by approximately 2.5-fold by comparison to the 0.1 nM level. Preincubation of estrogens with DNA (Fig.2B), at 0.1 nM, resulted in similar effects as above. At 10 nM concentration the estrogens showed different effects. 17β-estradiol showed an increase of approximately 1.9-fold, estriol showed no different effect, and estrone showed a decrease of DNA synthesis, by comparison to the 0.1 nM level. Consequently, the dose-response experiments showed that 10 nM was an effective hormone concentration in order to compare one compound to another.

Effects of steroids on DNA replication. In order to analyze the possible interaction of the hormones with the replication proteins or DNA, they were preincubated with either HeLa cell extracts or DNA and nucleotide precursors, respectively. Pretreatment of HeLa cell extracts with the hormones (10 nM) resulted in an enhancement of DNA synthesis (Fig. 3A). In this system, estriol increased replication by 3.6-fold relative to control, estrone by 1.7-fold, 16α-hydroxy-estrone by 1.6-fold, and 17β-estradiol by 1.7-fold. In contrast, the weak estrogen, 17α-estradiol, did not alter DNA synthesis levels significantly above control in this system.

On the other hand, when input DNA was pretreated with the hormones (10 nM) (Fig. 3B), 17β-estradiol, 16α-hydroxy-estrone and estriol resulted in an enhancement of DNA replication by 1.9-fold, 1.5-fold and 1.6-fold, respectively, while pretreatment with 17α-estradiol and estrone had no significant effect by comparison to control.

DISCUSSION

Previous studies (12) have demonstrated that this mammalian *in vitro* DNA replication system can be used to identify enhancers and inhibitors of DNA replication. In the present study, four of the five steroids produced an increase of DNA synthesis, 17β-estradiol, estrone, estriol, and 16α-hydroxy-estrone. These enhancements were consistent with previous *in vivo* studies. 17β-estradiol, estriol and 16α-hyroxy-estrone enhanced DNA synthesis in mouse mammary epithelial cells (8, 22) and in the human mammary carcinoma cell line MCF-7 (23). Moreover, Li and collaborators (24) found that 17β-estradiol, estrone and 16α-hydroxy-estrone increased *in vitro* cell proliferation of primary renal epithelial hamster cells in culture. Additionally, Fishman and coworkers (5) observed that 16α-hydroxy-estrone promotes the expression of a transformed phenotype in human cells.

In contrast, 17α-estradiol had no effect on DNA replication, in this system. This result is also consistent with previous studies, which have shown that 17α-estradiol did not promote tumor development in hamster, and treatment corresponded with low cell proliferation activity (24).

The HeLa cell extracts used in this study lack estrogen receptors; therefore, these hormones, at physiological concentrations, enhance DNA synthesis by a process different from receptor mediated mechanisms. Thus, this *in vitro* DNA replication assay has allowed the identification of a possible additional mode of action for these estrogens, the direct interaction of estrogens with the replication machinery, independently of receptors. These results may help the understanding of the role of these agents in the development and treatment of breast cancer.

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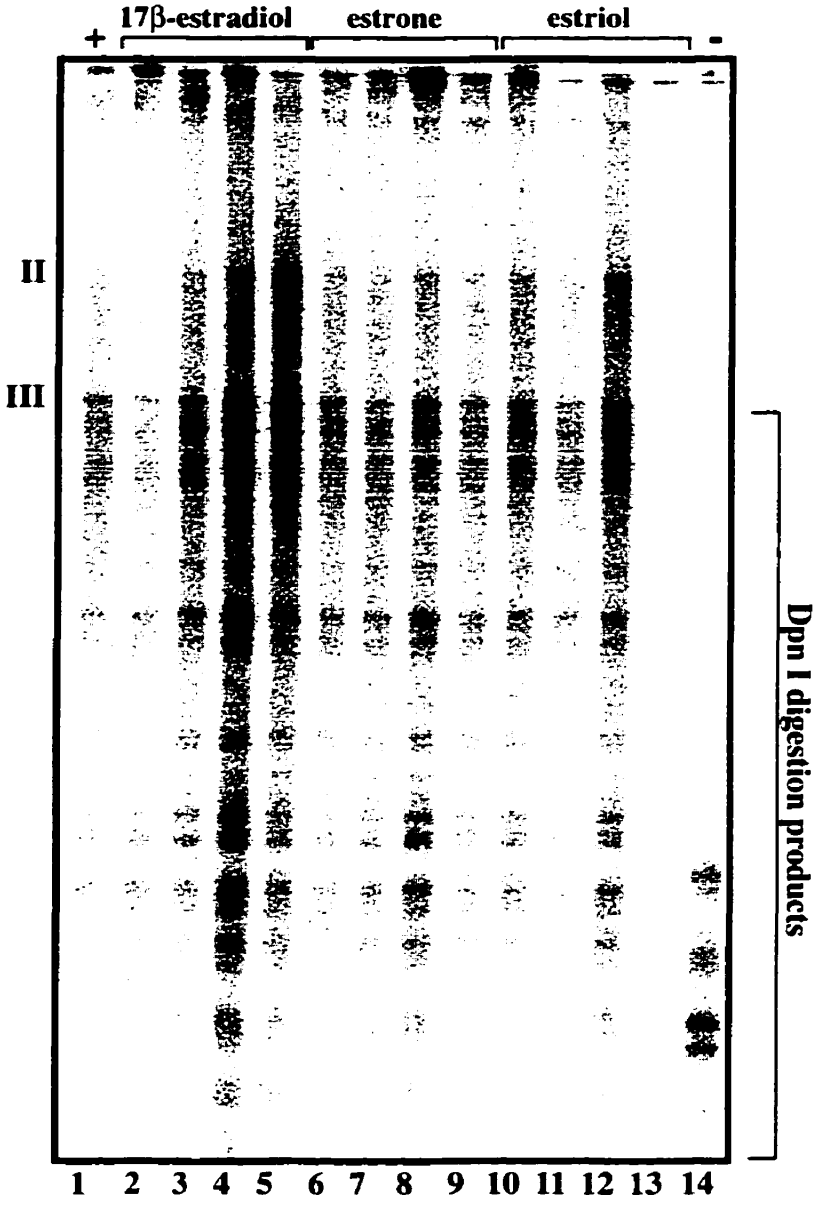

Fig. 1. *In vitro* DNA replication in the presence of varying concentrations (0.1 - 100 nM) of estrogens. HeLa cell extracts were preincubated for 15 min at 30° C with H_2O (lane 1, positive control), 17β-estradiol (lanes: 2 {0.1 nM}, 3 {1 nM}, 4 {10 nM}, 5 {100 nM}), estrone (lanes: 6 {0.1 nM}, 7 {1 nM}, 8 {10 nM}, 9 {100 nM}) or estriol (lanes: 10 {0.1 nM}, 11 {1 nM}, 12 {10 nM}, 13 {100 nM}). *In vitro* DNA replication reactions were carried out as explained in the **Materials and Methods**. Dpn I-resistant bands corresponding to forms II and III of DNA (indicated in the figure) were quantified, by subtracting background, and normalized for the amount of DNA in ethidium bromide gels. lane 14 denotes the negative control, plasmid 30.4, which did not replicate.

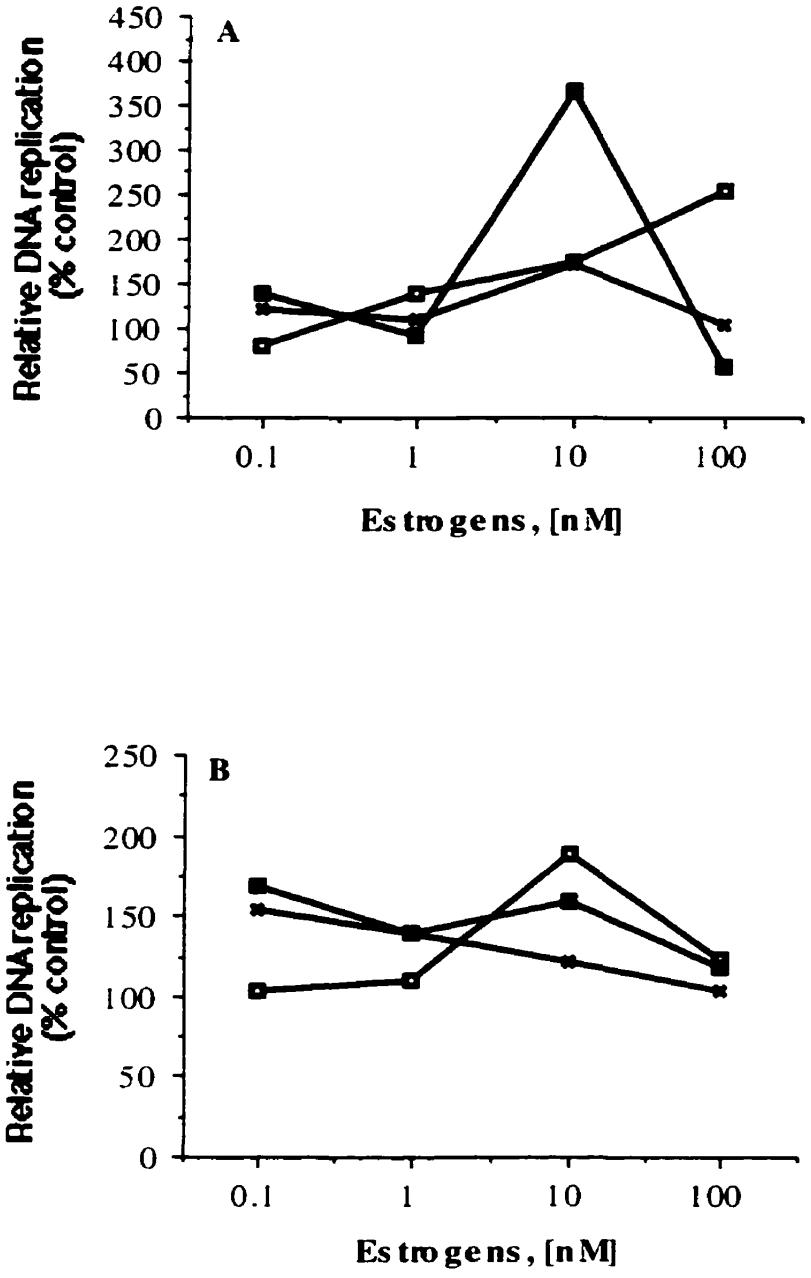

Fig. 2. Determining an effective hormone concentration of estrogens. Estrogens (17β-estradiol, estrone and estriol) were preincubated either with HeLa cell extracts (A) or DNA and precursor nucleotides (B). All *in vitro* conditions are the same as described in **Materials and Methods.** The data are expressed as the average percent of DNA replication activity (relative to untreated controls, 100%) for two separate experiments versus different concentrations (0.1 - 100 nM) of estrogens. 17β-estradiol (□), estrone (x), estriol(■).

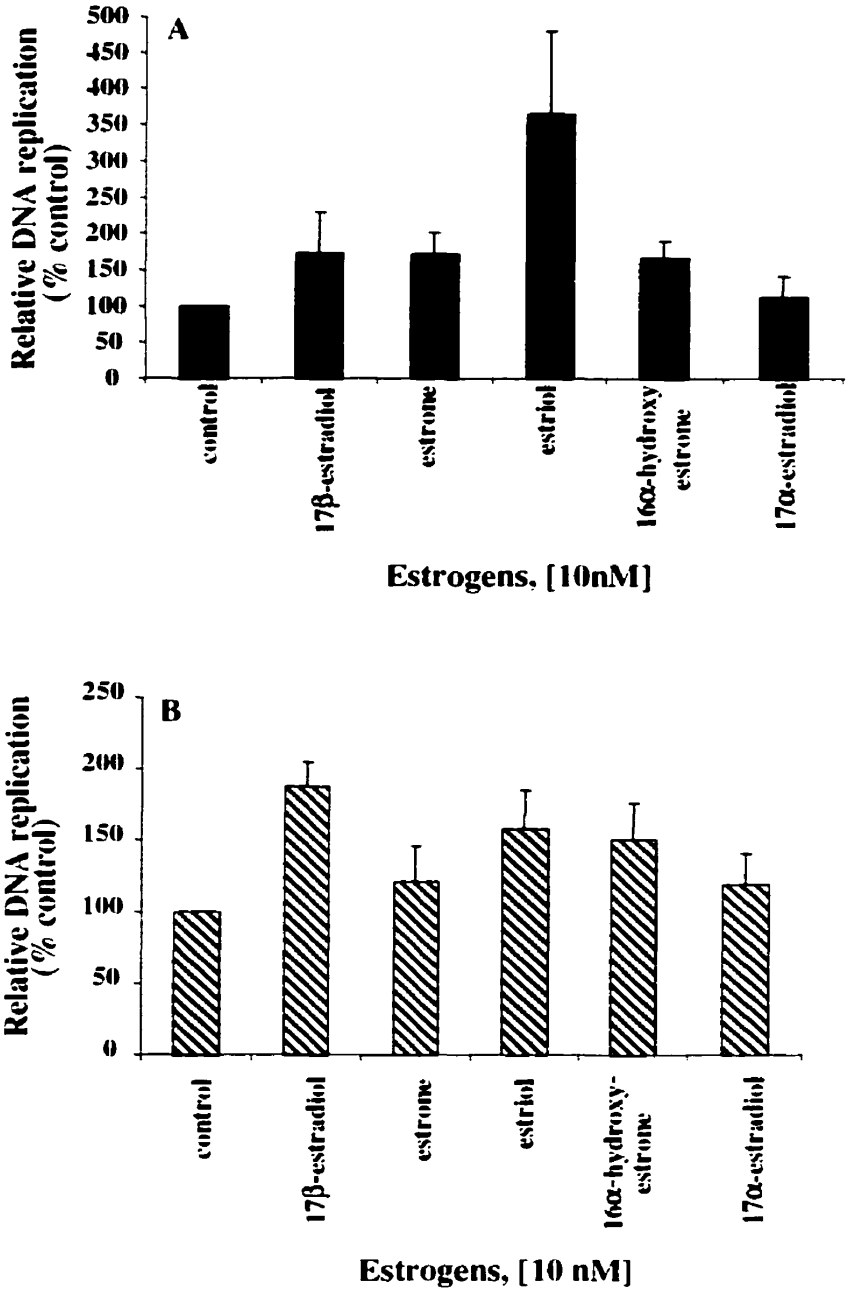

Fig. 3. Effect of hormones on *in vitro* DNA replication. The *in vitro* DNA replication assay was done as described in Figure 1. The compounds were either preincubated with HeLa cell extracts (black bars) or with DNA (stripped bars). The effect was plotted as the percent of DNA replication activity (relative to untreated controls, 100%) versus the concentration (10 nM) of hormones. The data are representative of 2 to 5 separate experiments. Bars indicate mean ± s.d. All hormones, except 17α-estradiol, produced an enhancement on DNA synthesis that was maximal when estriol was preincubated with the HeLa cell extracts.

CHAPTER FIVE

THE EFFECT OF STEROIDS ON DNA SYNTHESIS IN AN *IN VITRO* REPLICATION SYSTEM: INITIAL QSAR STUDIES AND CONSTRUCTION OF A NON-ESTROGEN RECEPTOR PHARMACOPHORE

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Chapter Four showed the effect of several estrogens, presumably involved in breast cancer, on *in vitro* DNA synthesis. To better understand their mode of action, we did a QSAR study, using a set of related steroids (Chapter Five). The effect of these steroids on DNA replication was correlated to electrostatic and hydrophobic interactions between the ligands and the target.

This chapter has been accepted in the *Journal of Medicinal Chemistry* for publication. All planing and performing of the *in vitro* experiments was done by myself. I also participate in the molecular modeling studies. Additionally, I wrote the parts of the manuscript concerning the biological data, in the Introduction, Results and Discussion. Kamal Azzaoui performed the QSAR study and designed the pharmacophore. As well, he wrote the rest of the manuscript. The paper was edited by Drs. Wainer, Price, and Zannis-Hadjopoulos.

The effect of steroids on DNA synthesis in an *in vitro* replication system: initial QSAR studies and construction of a non-estrogen receptor pharmacophore

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ABSTRACT

The molecular mechanism(s) by which steroids affect carcinogenesis is an active area of investigation. Recent studies with a series of related steroids in an *in vitro* DNA replication system produced a wide range of effects including enhancement and inhibition of DNA synthesis. The majority of hormone effects on cellular replication have been attributed to interactions with estrogen receptors; however, the HeLa cell-free system used in these studies did not contain these receptors. Thus, an alternative description of the results was required. Quantitative Structure-Activity Relationships were used to relate the observed bio-activity of these steroids with their structure. The results indicated that the percentage of DNA replication could be related to three parameters according to the following equation:

$$\%DNA = 23.9\ (\pm 3.8)\ Xdipact + 57.8\ (\pm 22.4)\ Hyd - 19.4\ (\pm 10.4)\ Biophπ + 128.9$$

where: Xdipact is the dipole moment on the X axis; Hyd is the atomic hydrophobicity index; and Biophπ is the atomic π population on the heteroatom found in the pharmacophore. For each molecule, the orientation of the functional groups changed the dipole moment value and this descriptor was used as a selector of active conformations. A 3D-QSAR model was then constructed combining pharmacophoric features and global properties, and the active and inactive space were defined using a Boolean volumetric operation.

INTRODUCTION

Steroid hormones, in particular estrogens, have been related to the increased risk of some types of cancers, such as breast and ovarian cancers. Indeed several *in vitro* and *in vivo* studies indicate a correlation between estrogens and carcinogenesis[1]; in particular, estrogens have been associated with increased cellular proliferation[2].

The effects of estrogens in carcinogenesis have been primarily attributed to their action on estrogen receptors. Several groups have explored the interaction of steroids with estrogen, progesterone, corticoid and androgen receptors using Quantitative Structure-Activity Relationships (QSAR)[3,4] and X-ray crystallographic[5] approaches. However, an absolute relationship between receptor binding and the activity has not been established and other mechanisms of carcinogenesis have been proposed. For example, it has been postulated that estrogens and other steroids directly interact with DNA, through intercalation into the DNA structure[6].

Since cellular proliferation, and therefore DNA replication, is a common feature associated with the carcinogenic effects of steroids, the effect of a series of steroids on DNA synthesis[7] has been recently studied in an *in vitro* DNA replication system[8]. The effect of seventeen structurally related steroids on the *in vitro* replication of a bacterial plasmid containing a specific mammalian origin of DNA replication from the hamster dihydrofolate reductase (DHFR) locus was analyzed. On the seventeen test compounds, four increased DNA replication, six decreased replication while seven had no effect, by comparison to the *in vitro* replication of the same plasmid in the absence of any steroid treatment.

Unlike other systems, the DNA replication system used in this study did not contain progesterone or estrogen receptors[7,9]. Thus, an alternative interpretation of the data was required and a chemometric approach including a 3D-QSAR analysis was undertaken. QSAR are a useful tool in medicinal chemistry in the explanation of the forces governing the pharmacological activities of a particular class of compounds. In addition, when the crystallographic structure of the pharmacological target is unknown, it is possible to use molecular modeling techniques to construct models of receptor sites or a graphical pharmacophore[10], and use these models to improve description and prediction

of activity. There are successful methods that have been used in constructing practical receptor models[11], which are mainly based on a surface or grid point surrounding the ligand[12,13].

In this study, the *Apex-3D* expert system was used to find a pharmacophore for the test set of steroids. Based on the logico-structural approach[14], *Apex-3D* identifies biophoric (i.e., pharmacophoric, toxicophoric) structural patterns responsible for manifesting certain types of biological activity. Descriptor centers can be either atoms or pseudo-atoms that can participate in the ligand-receptor interactions based on a number of physical properties such as electrostatic interactions (charges, electron acceptor or donor), hydrogen bonds (presence), charge transfer complexes (Homo, Lumo), hydrophobic interactions and Van Der Waals (London) dispersion forces (π-electron density on atoms).

The results of these studies indicated that the observed *in vitro* biological activity could be correlated to electrostatic interactions and hydrophobic interactions between the ligands and the target. The X component value of the dipole moment vector was identified as the most significant descriptor and its absolute value is directly related to DNA replication. The magnitude of this variable for each ligand was dependent upon the compounds conformation and could be changed by rotating the substituents on the D- or A-ring. Since the crystallographic structure of the receptor was not available, the X dipole moments of the test compounds were compared by assuming that a common mode of binding existed for all of the ligands. This descriptor was then used as an active conformation selector. The results produced by this approach are presented below.

RESULTS

Conformational analysis

The molecular structures of the seventeen steroids used in this study are shown in Scheme 1 and the nature of functional groups for each compound are presented in Table 1. For thirteen of the seventeen steroids used, the substitution on the D-ring included a hydroxyl group at the position 16 and/or at the position 17 and 12/15 had an hydroxyl function at position 3 on the A-ring. Three compounds contained an acetyl moiety at the position 16 (2 compounds) or position 17 (1 compound).

All the compounds were subjected to a conformational search using the *Search/Comp* module (MSI), containing an algorithm to eliminate high energy due to the steric effects. This effort produced a number of acceptable conformations which were dependent upon the shape of the rotatable group. The estimated and accepted conformations after optimization are reported in Table 2. Only non-duplicate conformations with values of less than 10 Kcal/mol were allowed. The number of acceptable conformations after the energy optimization varied between 2 and 25. These conformations were used to build the QSAR models.

1D QSAR Results

The results from the DNA replication studies are presented in Table 3 as the percentage of DNA replication with the standard deviation and the number of assays. The experimental control was set at 100% and the average of the standard deviation for all the compounds is approximately 20%. Thus, compounds producing a DNA replication >120% {compounds 4, 7, 11 and 12; Table 2} were considered promoters, compounds producing DNA replication of <80% {compounds 1, 9, 10, 13, 16 and 17} were considered inhibitors, and compounds producing DNA replication of 80%-120% {compounds 2, 3, 5, 6, 8, 14 and 15} were deemed inactive.

The position and the number of the hydroxyl groups on the D-ring seemed to affect the observed activity, since all the inhibitors were unsubstituted at the position 16 (Scheme 1) on the D-ring, as were four inactive analogs (**Cpds. 2, 3, 14** and **15**). The

inhibitors testosterone, 2-OH-estradiol and 4-OH-estradiol contained one β-hydroxyl group in the position 17 of the D-ring.

All the promoters had two oxygens on the D-ring {as well as a one oxygen moiety on the A-ring}. When two hydroxyl groups were present on the D-ring, the molecules containing 16α-17β or 17α-16β configurations promoted the DNA replication (**Cpds. 4 and 7**), while compounds with 16α-17α or 16β-17β configurations were inactive (**Cpds. 5** and **6**).

When the observed activity in the DNA replication system was correlated to the calculated descriptors for all seventeen test steroids, no significant multi-parameters relationship was found. However, the dipole moment on the X-axis of the minimum energy conformation (Xdip) was significantly correlated to activity (R=0.77) for twelve compounds (**Cpds. 1, 2, 3, 5, 6, 10, 11, 13, 14, 15, 16 and 17, Set 1**), Eqn. 1:

Eqn.1

%DNA = 28.9 Xdipmin + 128.8

n=12, R = 0.77

While the steroid backbone is rigid, the substituents on the D-ring have a great deal of conformational mobility. Since the magnitude and orientation of the X-axis dipole moment can be changed by small alterations in the orientation of these substituents, the assignment of an "active" conformation is not a trivial operation. In this study, it was assumed that for **Set 1**, the minimum energy conformation produced the optimum ligand-receptor interaction. While for the other five compounds (**Cpds. 4, 7, 8, 9, 12, Set 2**), the minimum energy conformation did not approximate the "active" conformation. Optimum binding conformations for the five compounds in **Set 2** were chosen using Apex 3D and these "active" conformations were included in the total data set used to produce the best superimposition for all of the compounds. Table 4 contains the values of the X dipole moment for the minimum energy conformations and for the low-energy "active" conformations. The energetic cost required to adopt the active

conformations for **Set 2** compounds ranged from 0 to 3.6 Kcal/mol which is generally acceptable in QSAR studies.

3D QSAR Results

Using Apex 3D software, over 50 pharmacophores were found with different sizes and arrangements (center of aromatic ring, center of non-aromatic ring, hydrogen bond donor, hydrogen bond acceptor, hydrogen bond site, methyl group). We selected the ones with the best superimposition (match > 0.7) and with the best active conformation which fit the 1D-QSAR model for the **Set 2** compounds (see Table 4). Then we performed a 3D quantitative analysis including the X dipole moment for the active conformations.

The best 3D pharmacophore selected (Figure 2) had 6 key structural features: 4 ring centers, 1 hydrogen bond site (HBS) and 1 heteroatom site. The quality of match for molecules having this common pharmacophore was 0.80. The distance between the heteroatom and the hydrogen bond site when all the compounds were superimposed was 13.59 Å. This distance was 13.61 Å when only inhibitors were superimposed. When only promoters were superimposed, the distance decreased to 13.31 Å and when only inactive analogs were superimposed, the distance increased to 13.75 Å.

An interaction mechanism involving A-ring binding/D-ring activation is generally accepted as the source of the high affinity of steroids for estrogen, progestin and corticoid receptors[15]. Androgen receptor binding data and molecular modeling studies suggest an opposite mechanism of D-ring binding/A-ring activation[16]. Both mechanisms are possible with the pharmacophore identified in this study where: 1) D-rings of inhibitors are situated in the hydrogen bonding region of the receptor (HBS) except for the β-estradiol-17-acetate and testosterone which had this ring situated in the heteroatom region; 2) A- rings of promoters are near the HBS; 3) for the inactive analogues, 3 had the D-ring (**Cpds. 3, 14** and **15**) and 4 the A-ring (**Cpds. 2, 5, 6** and **8**) near to HBS.

In the HBS region of the pharmacophore, the orientation of the hydroxyl group is different for each class of compound. For promoters (**Cpds. 4, 7** and **11**) the hydrogen of the hydroxyl is oriented in the direction of HBS while for inactive analogues (**Cpds. 5, 6** and **8**) and inhibitors (**Cpds. 9, 10** and **13**) the lone pairs of the oxygen are oriented in the direction of HBS. The value of the angle C-O-HBS is around 120 degrees for inactive

compounds and promoters. This value is 127 degrees for β-estradiol-17-acetate and 107 degrees for 2-OH-estradiol and 4-OH-estradiol. These results suggest that the HBS on the pharmacophore contains both a heteroatom and an acidic hydrogen.

In order to derive a 3D multi-parameters equation, the pharmacophore was used as a superimposition model. We found that the percentage of DNA replication was related to 3 parameters: the X dipole moment value for the active conformation, the atomic hydrophobicity index at the hydrophobic site (situated at 5.93 Å from the HBS and 7.93 Å from the heteroatom) and the atomic π population on the heteroatom.

Eqn. 2

%DNA = 23.9 (±3.8) Xdipact + 57.8 (±22.4) Hyd -19.4 (±10.4) Biophπ + 128.9

n=17, R=0.93, F = 26

According to the Eqn. 2, electronic and hydrophobic interactions are primarily responsible for binding and activity. The electronic effects are represented by the X dipole moment (Xdipact) and the π population on the heteroatom (Biophπ) while the hydrophobic effect is represented by the hydrophobicity index (Hyd). The predicted *versus* observed values of DNA replication (expressed as percentage of control) are presented in Figure 1 .

The X component value of the dipole moment vector is the most significant descriptor in Eqn. 2; and its absolute value is directly related to DNA replication. This parameter is a quantitative measurement of separation of charges along the X axis as defined by the average plane of the four steroidal rings. Since the magnitude of this variable for each ligand was dependent upon the compound's conformation and could be changed by rotating the substituents on the D- or A-ring, it was assumed that a common mode of binding existed for all of the ligands. The derived descriptors are presented in Table 4.

Unlike the X dipole moment an increase in the π population on the heteroatom decreased DNA replication. This parameter characterizes the total electron population of the atomic orbitals with π-symmetry on the atom, and it reflects a local interaction with

the receptor involving π electrons such as dispersion interactions. The π population of the oxygen decreases in this order: O of a ketone group > O in a hydroxyl group attached to the A-ring > O attached to the D-ring (Table 4). The hydrophobic index (Hyd) found in Eqn. 2 is the carbon atomic increment calculated according to Ghose and Crippen[17]. Most of the inactive compounds and inhibitors had a methyl group at C-13 near to the HBS while promoters had an aromatic carbon in this region. These results suggest the presence of a hydrophobic pocket on the receptor which is involved in the binding of the steroids and the resultant activity.

Receptor mapping

The model discussed above defined only electrostatic, hydrophobic and dispersive interactions; no steric parameter appeared in the equation. Since differences in activity may be related to access to the binding site of the target, "inactive", "promotion" and "inhibition" spaces were determined. An "inactive" space, or exclusion volume, was defined as the difference in Van Der Waals volume between inactive ligands (449 $Å^3$) and the active ligands (540 $Å^3$) according to the pharmacophore model. The derived "inactive" space is presented in Figure 3 and contained a steric region with the total resultant volume around 38 $Å^3$. This region is situated near to the heteroatom site and covers the four ring centers in the opposite side of the methyl groups at position 13 or position 10 (progesterone and testosterone). However steric constraints were absent around the hydrogen bond site.

The "promotion" space was defined as the difference in volume between promoters (358 $Å^3$) and the union volume of inhibitors and inactive analogues (530 $Å^3$), while the "inhibition" space was the difference in volume between inhibitors (464 $Å^3$) and the union volume of promoters and inactive analogues (511 $Å^3$). The value of the inhibition space and promotion space are respectively 67 $Å^3$ and 49 $Å^3$. As shown in Figure 4 the inhibitors occupy a large space around the heteroatom site while promoters have a preferred region near to hydrogen bond site.

CONCLUSIONS

The 3D-QSAR methodology has been applied in a set of steroids which affected DNA replication in an *in vitro* test system. The active conformations of these compounds were identified using the dipole moment on the X-axis as a molecular descriptor, and an equation relating the percentage of DNA replication to electrostatic and hydrophobic parameters was developed. Since no crystallographic data of the target exist, the receptor mapping approach was used to define the binding and active regions for this set of compounds. These results may aid in the design of new compounds for use in the treatment of cancer (inhibitors of DNA synthesis) or wound healing (promoters of DNA synthesis).

MATERIALS AND METHODS

Compounds

The set of 17 test compounds belonged to different subfamilies of steroids, i.e. estrogen, androgen, glucocorticoid, and progestin. The steroids were purchased from Sigma (Mississauga, Ontario, Canada) and Steraloids INC. (Wilton, NH, U.S.A.) The compounds were dissolved in 100% ethanol, and diluted in water. The amount of ethanol added to the biological system never exceeded 0.1%. Physiologically relevant steroid concentrations (10nM) were used in assay.

Biological data

The biological data is the relative effect of these steroids on DNA replication. An *in vitro* replication assay has been used to obtain this parameter. *In vitro* replication was carried out as described in Diaz-Perez et al., 1996. Standard *in vitro* reactions are basically composed of equimolar amounts of a supercoiled plasmid, either pX24 or plasmid 30.4, HeLa nuclear and cytoplasmic extracts, an ATP regenerating system, PEG, a mixture of nucleotides (ATP,CTP, GTP, UTP, dATP, dGTP, dTTP, and dCTP), and 10μCi of [α^{32}P]-dCTP and [α^{32}P]-dTTP. Plasmid pX24 contains a specific origin of replication from the hamster dihydrofolate reductase locus; and plasmid 30.4 contains a fragment of cDNA randomly selected from a human cDNA library[7]. The HeLa cell extracts have been tested for receptors, and are estrogen and progesterone receptor negative[7,9]. The compounds were preincubated with the template DNA and the precursor nucleotides, followed by addition of the remaining components of the *in vitro* reaction to initiate DNA replication. DNA was purified and DNA synthesis was measured using the *Dpn I* resistance assay. DNA replication products were quantitated by densitometry of a phosphoimager screen using the Fuji BAS 2000 analyzer.

The data was expressed as the percentage of the non-drug-treated control (100%), and represents the average of at least two separate experiments (mean ± s.d.).

Molecular modeling

The compounds used for our data set (testosterone[18], progesterone[19], estrone[20], estriol[21], 2-hydroxy-estradiol[22], 4-hydroxy-estradiol[23], 2-hydroxy-estrone[24]) were retrieved from *Cambridge Data Base* [25] available via *Quest* program (ICOA, University of Orléans, France). The compounds for which the crystallographic data did not exist were built using a fragments library on *InsightII 95.0* program (MSI, San Diego, CA.) running on IBM Risc6000.

Conformational analysis was done with *Search/Comp* module. The rotatable bonds were defined in the range of 0 to 360° using 10° to 180° as an increment angle. For each compound, a systematic search and energy optimization was performed. Several optimization were used in sequence: steepest descents, followed by conjugate gradients, followed by a quasi-Newton-Raphson method with a maximum number of iteration set at 500. A cff force field with charge and cross terms was used and the thresholds for removing duplicate conformers were specified using the Dupl_E_threshold at 0.01 Kcal/mol and Dupl_RMS_threshold at 0.01 Å. The Dupl_E_threshold parameter specifies the maximum energy difference in Kcal/mol, and Dupl_RMS_threshold parameter specifies the maximum Root Mean Square difference in Å for which two conformers are considered to be the same.

Tsar V2.41 software (Oxford Molecular Ltd. Oxford. UK) was used to calculate molecular descriptors (electronic, steric or lipophilic descriptors), *Tsar* was also used for statistical analysis of the data. All charges and dipoles moments were calculated using *Mopac V6* [26] program. The origin of the dipole moment vector was set to center of mass for each compound.

A routine written in C-shell was useful for extracting the coordinates of each conformation from the *Search/Comp* archive file. The routine splits the .arc file into .car files and converts the .car format to .dat (Mopac's internal coordinates format) and then print and sort the Mopac results (heat of formation and dipole moment components) to a result file.

The pharmacophores were built using *Apex-3D 95.0* software (MSI, San Diego, CA.) running on Silicon Graphics Indy workstation. The pharmacophores selected included all of the compounds in the set with a match superimposition greater than 0.7.

The 3D-QSAR equation was derived with the site radius set at 1.2, the occupancy at 5, the sensitivity at 2.5 and the randomize at 500. The total hydrophobicity and X dipole moment values were selected as global properties. The biophoric centers and secondary sites combined to global properties (total hydrophobicity, molecular refractivity and the components of the dipole moment) were used to obtain an equation to predict the percentage of DNA replication. The biophoric sites were set to charges, π-population, Homo, Lumo, hydrogen acceptor, hydrogen donor and hydrophobic site. The secondary sites were set to: hydrogen acceptor: presence, hydrogen donor: presence, heteroatom: charge, hydrophobic: hydrophobic, steric: presence, ring: π-sum.

Volumes were generated using *Volume/Create* option of *InsightII*, the Van Der Waals scale was set to 1 Å and Van Der Waals increment to 0.

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No.	Compounds	R_2	R_3	R_4	R_{16}	R_{17}
1	testosterone	H	O	H	H	OH (β)
2	progesterone	H	O	H	H	CH3-CO (β)
3	estrone	H	OH	H	H	O
4	estriol	H	OH	H	OH (α)	OH (β)
5	16-epiestriol	H	OH	H	OH (β)	OH(β)
6	17-epiestriol	H	OH	H	OH (α)	OH(α)
7	16,17-epiestriol	H	OH	H	OH (β)	OH(α)
8	16-keto-β-estradiol	H	OH	H	O	OH(β)
9	2-OH-estradiol	OH	OH	H	H	OH(β)
10	4-OH-estradiol	H	OH	OH	H	OH(β)
11	16α-OH-estrone	H	OH	H	OH(α)	O
12	16β-OH-estrone diacetate	H	CH3-CO	H	CH3-CO (β)	O
13	β-estradiol-17-acetate	H	OH	H	H	CH3-CO (β)
14	estrone acetate	H	CH3-CO	H	H	O
15	2-OH-estrone	OH	OH	H	H	O
16	4-methoxy-estrone	H	OH	CH3-O	H	O
17	2,3-methoxy-estrone	CH3-O	CH3-O	H	H	O

Table 1: The compounds studied

Compounds	IncD	IncA	Estimated	accepted
1	10	-	22	3
2	10	-	14	3
3	-	10	36	2
4	20	180	144	18
5	20	180	144	16
6	20	180	144	16
7	20	180	144	12
8	10	180	52	6
9	10	90	352	25
10	10	90	264	18
11	10	180	62	6
12	20	20	216	18
13	10	90	48	4
14	10	-	36	2
15	-	30	144	4
16	20	20	187	6
17	20	20	289	7

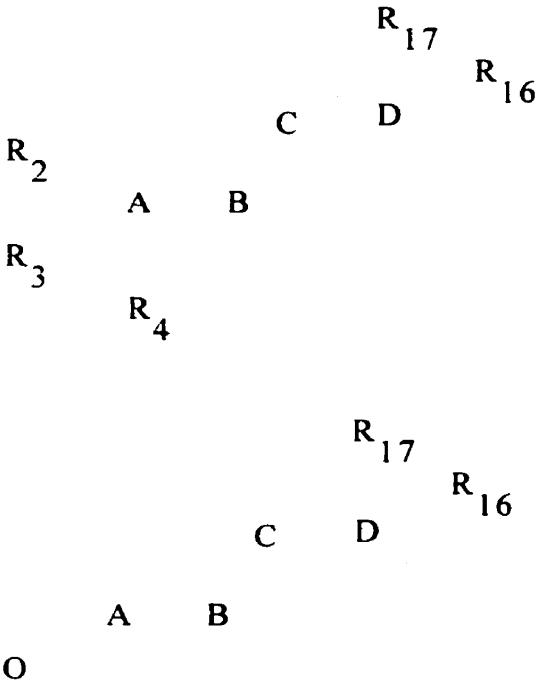
Table 2: The conformational analysis results. **IncD**: The increment angle in degree for the rotatable bonds on the D-ring. **IncA**: The increment angle in degree for the rotatable bonds on the A-ring. **Estimated**: The number of conformation estimated after steric evaluation. **Accepted**: The number of conformation estimated after optimization and removing duplicate conformations.

Compounds	%DNA	sd	n
1	72	17	4
2	115	9	3
3	108	17	2
4	144	19	2
5	118	26	2
6	103	19	3
7	200	6	2
8	99	25	2
9	52	16	2
10	53	6	2
11	150	26	3
12	157	41	2
13	67	17	2
14	111	3	2
15	92	19	4
16	53	1	2
17	63	1	2

Table 3: The percentage of DNA replication. (**%DNA**: the percentage of DNA replication. **sd**: standard deviation. **n**: number of assays).

Compounds	E min	Xdipmin	E act	Xdipact	ΔE	Biophπ	Hyd
1	-88.64	-2.25	-88.64	-2.25	0	0.056	-0.14
2	-72.45	0.11	-72.45	0.11	0	1.036	-0.22
3	-64.12	-1.32	-64.12	-1.32	0	0.196	-0.14
4	-128.86	-1.41	-126.99	1.05	1.87	0.056	0.16
5	-129.65	-1.25	-129.65	-1.25	0	0.056	0.16
6	-129.00	-1.00	-129.00	-1.00	0	0.056	0.16
7	-128.66	-1.23	-127.35	2.34	1.31	0.056	0.16
8	-106.21	1.80	-103.65	0.36	2.56	1.064	0.16
9	-128.02	0.55	-124.44	-1.08	3.58	0.196	-0.48
10	-126.88	-1.76	-126.88	-1.76	0	0.196	-0.48
11	-105.89	-0.49	-105.89	-0.49	0	0.056	0.16
12	-86.04	-0.22	-84.23	1.39	1.81	1.036	0.16
13	-68.54	-1.83	-68.54	-1.83	0	1.036	0.16
14	-54.91	-0.34	-54.91	-0.34	0	1.036	-0.14
15	-107.42	-0.64	-107.42	-0.64	0	0.196	-0.14
16	-97.20	-1.94	-97.20	-1.94	0	0.196	-0.14
17	-87.46	-2.49	-87.46	-2.49	0	0.168	-0.14

Table 4: Molecular descriptors found in Eq. 2 for minimum energy conformation and active conformation. (**E min**: The energy of the minimum conformation in Kcal/mol. **Xdipmin**: The dipole moment on The X axis for the minimum energy conformation. **E act**: The energy of the active conformation in Kcal/mol. **Xdipact**: The dipole moment on The X axis for the low-energy active conformation. **ΔE** : E act - E min. **Biophπ**: The π population on the heteroatom. **Hyd**: The hydrophobicity index).



Schemes 1: Structure of the steroids studied

Testosterone: R_{16} = H; R_{17} = OH (β)

Progesterone: R_{16} = H; R_{17} = CH3-CO (β)

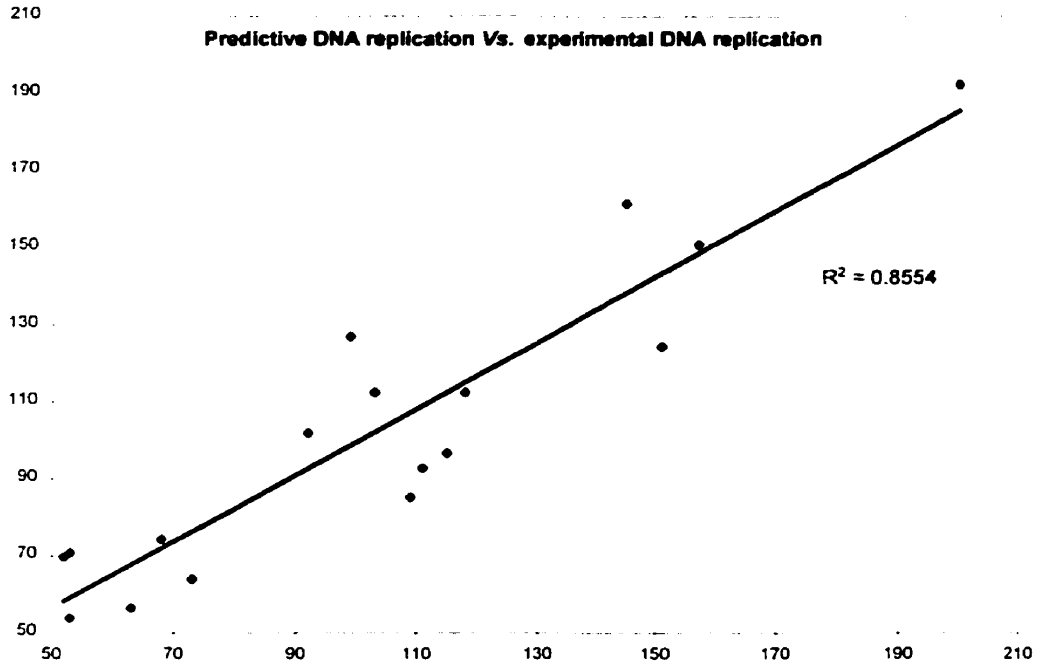


Fig 1: Predicted DNA replication *Vs.* experimetal DNA replication according to Eq. 2

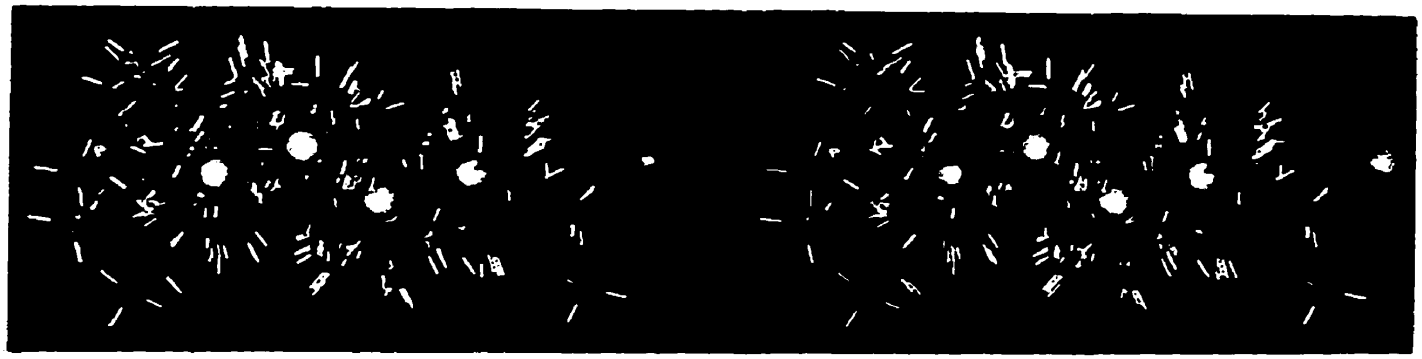


Fig 2: Stereo view for the best pharmacophore selected (Features color code: white: Center of ring; yellow: Hydrogen bonding site; purple: Hydrophobic site; blue: Heteroatom)

Fig 3: Stereo view of the Inactive Volume

Fig 4: Stereo view for the Inhibitor Volume (blue) and Promotion Volume (yellow)

CHAPTER SIX
GENERAL DISCUSSION

Steroids, especially estrogens, have been extensively studied, because of their physiologic importance and a potential role in carcinogenesis, i.e. hormone-dependent cancers, such as breast cancer. Despite a great deal of experimental and clinical data, the exact mechanism underlying their actions in carcinogenesis is not clearly understood. Data from *in vitro* and *in vivo* studies indicate that some of their actions may involve non-receptor mediated mechanisms. Better understanding of the targets and mechanisms of action of estrogens in carcinogenicity is of great importance, and needs to be understood in order to design more effective treatments for breast cancer, and to limit causative factors such as may arise from environmental or dietary exposure. Therefore, since cell proliferation is a constant feature in the development of carcinogenesis, and the fact that steroids have proliferative effects on normal and neoplastic cells, we have chosen to study the effect(s) of steroids on DNA replication.

DNA replication was studied using a mammalian *in vitro* DNA replication assay (Pearson et al., 1991). Several investigators have used such *in vitro* systems to demonstrate autonomous replication of cloned mammalian DNA sequences (e.g. the human 5' c-myc promoter region [Berberich et al., 1995]; hamster DHFR [Zannis-Hadjopoulos et al., 1994]; *ors* clones [Pearson et al., 1991]). We have used this system to study the effects of drugs and compounds on DNA replication. The validation of this mammalian *in vitro* DNA replication assay showed that the method was reproducible (Chapter Three). The system contains among other components, mammalian cell extracts from HeLa cells, and a specific mammalian origin of replication, the hamster DHFR origin, oriβ. Among all mammalian origins, DHFR is one of the best characterized. A chromosomal initiation site, *oriβ*, mapping 3' to the hamster DHFR gene was demonstrated by several methods, including nascent strand PCR, Okazaki strand switching (Burhans et al., 1990), and 2DGE (Vaughn et al., 1990). Additionally, fragments from the hamster 3' DHFR region replicated autonomously in both *in vivo* and *in vitro* assays (Zannis-Hadjopoulos et al., 1994). A DNA fragment derived from the known mammalian chromosomal initiation site of DHFR was used in this study. Specifically, we have confirmed the autonomous replication of plasmid, pX24 containing the 4.8 kb fragment from the *oriβ* region 3' to the hamster DHFR gene (Zannis-

Hadjopoulos et al., 1994). Additionally, we demonstrated by the earliest labeled DNA fragment method that *in vitro* replication of pX24 initiates within the DHFR fragment (Chapter three). The preferred initiation site for replication in pX24, is localized close to the origin of bidirectional replication (OBR) (Burhans et al., 1990). Moreover, the inhibitory effect of the anthracycline, doxorubicin, did not change the apparent initiation site. We tested drugs (doxorubicin, araC) which are known to interact with the DNA replication machinery, and we observed, as expected from other studies, an inhibitory effect on *in vitro* DNA synthesis. We had to use the active form of araC, the phosphorylated nucleoside analog araCTP, in order to see the inhibitory effect on DNA synthesis. The suboptimal metabolism afforded by these HeLa cell extracts apparently failed to adequately phosphorylate araC to the levels attainable in intact cells. The mammalian autonomous replication assay we have used provides a tool for the study of the effect of drugs and compounds on DNA synthesis (Chapter Three). This system which allows us to study the effect of drugs and compounds on DNA replication has several advantages; it lacks the inconvenience of requiring transport and getting metabolism of the drugs as occurs in whole cells, and the extreme simplicity of *in vitro* reconstituted systems that ignore indirect or unforeseen interactions. Additionally, this *in vitro* system is closer to *in vivo* mammalian DNA replication conditions than other similar existing systems, since it does not need a viral protein. We also tested the steroid progesterone, which is thought to exert an indirect effect on DNA synthesis by binding to the progesterone receptor. However, measurement of progesterone receptors in the HeLa nuclear and cytoplasmic extracts used in the *in vitro* assay did not reach the necessary threshold for positivity (20 fmol/mg total protein). Thus, this hormone showed an effect on DNA replication whose mechanism seems to be unrelated to receptor-ligand nuclear interactions.

Since there is strong evidence from several systems supporting the hypothesis that steroids, particularly estrogens, are carcinogens acting via stimulation of cell proliferation, we have investigated the effect of steroid hormones on DNA replication, using this *in vitro* DNA replication system. Most of the carcinogenic studies have been

focused on estrogens, and therefore, the majority of the steroid hormones tested belong to the estrogen family.

Initially, we tested several estrogens (Chapter Four) which are presumably involved in the development of carcinogenesis. These estrogens, 17ß-estradiol, estrone, estriol and 16α-hydroxy estrone produced an enhancement of *in vitro* DNA synthesis, which is supported by other *in vitro* and *in vivo* studies. Schneider and coworkers (1984) found that 17ß-estradiol, estriol and 16α-hydroxy estrone were effective stimulators of MCF-7 cell proliferation. Moreover, these hormones produced an enhancement on DNA synthesis in mouse mammary epithelial cells (Suto et al., 1993; Telang et al., 1992). In addition to the mammary tissue, these estrogens have been tested in other tissues, such as primary renal epithelial cells (Li et al., 1995) which resulted in an increase on *in vitro* cell proliferation. We have used as a negative control, the inactive epimer of 17ß-estradiol, 17α-estradiol, which did not produce any effect on this system, as has been shown in other *in vivo* studies (Li et al., 1995). Interestingly, the enhancement observed with the estrogens is probably due to a mechanism of action different than the receptor-mediated mechanism, because estrogen receptors (ER) in the HeLa cell extracts could not be detected. Evidence exists that certain estrogens can be involved in carcinogenesis by mechanisms that do not involve the classical estrogen receptor. Evidence supporting the hypothesis of other modes of action include the lack of correlation of biological action with affinity for the steroid receptor, and carcinogenesis by estrogens in cells that do not contain the estrogen receptor. For instance, 16α-hydroxy estrone exhibited greater potency in stimulation of MCF-7 cell proliferation than was expected from its estrogen receptor affinity (Schneider et al., 1984). Newbold et al., (1990) have shown that DES treatment of neonatal mice, which contain few uterine epithelial cells that are ER positive, results in uterine adenocarcinoma. There are several possible mechanisms for estrogen carcinogenicity, in addition to the receptor-mediated mechanism. It has been suggested that there is a direct interaction with DNA and/ or proteins by some steroids (Chapter One). Several estrogens have been found to lead to covalent adduct formation with DNA in different tissues (for example, 17ß-estradiol and its metabolites, 2-hydroxy estradiol and 4-hydroxy estradiol in hamster embryo cells [Barrett and Tsutsui, 1996];

DES in hamster kidney [Liehr et al., 1991]; and 16α–hydroxy estrone in mammary epithelial cells [Telang et al., 1992]). Estrogens also have been found to form covalent adducts with several proteins or peptides, such as 2-hydroxy estrone with glutathione (Liehr and Roy, 1990) and 16α–hydroxy estrone with albumin (Bucala et al., 1982). Additionally, it has also been suggested that the intercalation of steroids into partially unwound double stranded DNA is another non-receptor mediated mechanism. The fit into the DNA of the estrogen compounds has correlated well with hormonal activity (Hendry and Mahesh, 1995).

This *in vitro* DNA replication assay has identified an additional potential mode of action for these estrogens, i.e. the direct interaction of estrogens with the replication components, independent of classical receptors. Thus, it is possible that estrogens and other steroid hormones act through different modes of action to exert such biological actions, as carcinogenesis. Depending on the cellular conditions and on the hormone, there may be a predominant mechanism of action for a particular hormone. However, its final biological effect may result from a combination of several modes. Understanding these modes of actions can lead to the design of new related compounds, useful in cancer therapy.

Drug molecules or compounds work through a structural interaction with targets, that play key roles in biological processes; these interactions may be neutral or inhibit or enhance the normal function of the targets. A series of related steroids were tested in the *in vitro* DNA replication assay (Chapter Five), showing a diversity of effects. When information about the target is lacking, quantitative structure-activity relationship (QSAR) studies can be used to relate the structure of the compound to the biological effects. Since we do not have information about the target, among the components of the DNA replication machinery, the observed effects on *in vitro* DNA replication of the steroids were related to their structures through the construction of a QSAR. The results from the initial QSAR studies have identified structural features, which produce an increase or decrease in DNA replication. An equation was derived from the QSAR studies that directly relates the activity in DNA replication primarily with the dipole moment (dmx). To design a better inhibitor of DNA replication in the steroid family, we

should design a molecule that has a low energy conformation with low dmx. Since each molecule has many conformations, finding the biological active conformation would be time-consuming; thus, the development of a pharmacophore model in combination with QSAR studies can help in the design of new inhibitors or promoters of DNA synthesis.

For each steroid, the conformation that fits the QSAR model was used to build pharmacophores. The pharmacophores that presented a match of superimposition over 0.75 were selected, and a predictive equation of the percentage of DNA replication was developed.

Although we have looked at fifteen estrogen related compounds and observed an entire range of activities from enhancement to inhibition of DNA replication, we have studied only one androgen, testosterone. Progesterone was also studied as a single member of its subfamily of steroid compounds. The limited assessment of these other steroidal compounds may contribute to the evaluation of QSAR analyses of estrogen compounds; however, we also realize that testing of a wider number of variants of these other steroid compounds may also exhibit wider ranging effects on DNA replication allowing independent QSAR analyses to be performed.

Therefore, this *in vitro* DNA replication system can be used to design new drugs, including drugs that can be used to inhibit or enhance cellular proliferation. The pharmacophore will help us to understand structure-activity relationships and to design active analogs. Additionally, the building of a pharmacophore for the site of enhancement (or inhibition) will help us to better understand the mechanisms of biological activities, which in our case is the mechanism of steroids in carcinogenesis. Further refinement of the QSAR analysis and construction of more accurate pharmacophores will greatly facilitate our understanding of the ligand (steroid) structure and target interactions that produce receptor-independent effects upon DNA replication. Thus, future work to improve the predictability of the target-steroid interaction model would be done using a larger number of steroids. Part of these compounds would be used to refine the system and part to test the system.

We now need to consider the nature of the targets for these steroids. With the pretreatment of a mixture of replication proteins present in HeLa cell extracts, the

potential for interaction of different estrogen compounds with different targets that could collectively impact on DNA replication is significant. Therefore, in order to assemble homogenous groups of compounds interacting with a common target in order to perform QSAR analyses, further extension of this work will require *in vitro* binding studies. These *in vitro* binding studies may best be performed as competitive binding assays to obtain association constants and indications of cooperative interactions between compounds binding at different sites of the same protein.

In order to make the best predictions regarding a compound's direct effect on DNA replication, the multi-regression approaches generally give a low predictive power. The multi-regression assumes a linear relationship between activity data and the molecular descriptors. The non-linear relationships must be incorporated explicitly into the regression model. In contrast, Artificial Neural Networks (ANN) make no assumptions about the linearity of the data (Hertz et al., 1991). ANN is a layered system of processing units that are interconnected to facilitate the ordered transfer and processing of data. It is intended to model the functioning of the human brain. Training of ANN consists of presenting pairs of input/output data to the system in an iterative fashion. If the final output of the network is in discrepancy with the expected output, the weights and biases are adjusted to minimize the error and another iteration is performed. ANN, using back-propagation algorithm (Hertz et al., 1991), can be trained using molecular descriptors in the Input layer and biological activity in the Output layer to build a non-linear model and improve the predictive power of the QSAR/pharmacophore approach. Members of our laboratory have already successfully used ANN to predict the binding of small molecules to biopolymers, albeit, in a chromatographic system with the binding of aromatic carboxylic acid to amylose (Booth et al., 1997).

CHAPTER SEVEN

CLAIMS TO ORIGINALITY

CLAIMS TO ORIGINALITY

The main observations of this work were as follows:

1. Initiation of replication originates within the DHFR origin in an *in vitro* DNA replication system, using human cell extracts.

2. Demonstration of the reproducibility and usefulness of this mammalian *in vitro* DNA replication assay for the study of the effect of drugs and compounds in the mechanism of DNA synthesis.

3. Several estrogens (17β-estradiol, estrone, estriol, and 16α-hydroxyestrone) enhance DNA replication by a receptor-independent mechanism.

4. A series of related steroids show a diversity of effects (no effect, enhancement or inhibition) on mammalian *in vitro* DNA replication.

5. Development of a QSAR study that leads to a equation, relating the biological activity and the dmx (dipole moment), as the major factor.

6. Development of a pharmacophore, which indicates the minimal key structural moieties in the steroid structure to produce specific effects of enhancement or inhibition of DNA replication.

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ADDENDUM

The results showing the effects of steroids on *in vitro* DNA replication obtained after preincubation of the essential proteins that are required for replication (contained in the HeLa cell extracts) with these compounds are provided here as a supplementary information. The steroids were tested under the *in vitro* assay conditions described in the **Materials and Methods** section presented in Chapters Three, Four and Five. The results of those experiments are summarized in Table 1. The effects of 17β-estradiol, 17α-estradiol, estrone, estriol, and 16α-OH-estrone at 10 nM concentrations on *in vitro* DNA replication are reported in Chapter Four.

Table 1. Effects of steroids on *in vitro* DNA synthesis. The HeLa cell extracts were pretreated with the compounds (10 nM). % DNA: the percentage of DNA synthesis compared to no treatment with compounds; s.d.: standard deviation; n: number of assays.

	Extracts		
Compound	**% DNA**	**s.d.**	**n**
Testosterone	99.43	12.2	3
Progesterone	207.9	30.4	3
17β-estradiol	173.4	56	4
17α-estradiol	111.5	28.5	2
Estrone	171.9	29	3
Estriol	363.4	117	3
16-epiestriol	104.8	29.8	2
17-epiestriol	109.6	16.2	2
16,17-epiestriol	114.1	9.6	2
16keto-β-estradiol	150.0	19.4	4
2-OH-estradiol	267.4	66.6	3
4-OH-estradiol	228.6	52.7	3
16α-OH-estrone	165.9	23.3	2
16β-OH-estrone diacetate	99.7	19.7	3
β-estradiol-17 acetate	85.9	19.1	2
Estrone acetate	89.9	34.5	2
2-OH-estrone	155.8	47.2	3
4-methoxy-estrone	153.3	27.2	2
2,3-methoxy-estrone	70.0	5.8	2

The data reveal that these steroids behave differently if pretreated with replication proteins present in HeLa cell extracts; the entire range of effects from inhibition to no effect to enhancement of *in vitro* DNA replication was seen. The variability of effects suggests several possibilities including the existence of different targets, different affinities for the same target, or differential effectiveness of compounds. Following a determination of whether all these compounds have a common target or can be associated into groups with a common target, separate QSAR studies will be performed for each group of compounds to a common target.

In summary, the hormones studied in this thesis, which include estrogens, testosterone, and progesterone, have different effects on *in vitro* DNA replication. The effects are varied and may differ with different targets, e.g. preincubations with DNA (Chapters Four and Five) or HeLa cell extracts (Chapter Four, and results summarized in Table 1 of this Addendum). The steroids can be separated into two classes through the effect on *in vitro* DNA replication. One class would include those hormones which differ only quantitatively in their effects on DNA replication when they are either pretreated with DNA or with HeLa cell extracts, e.g. 17β-estradiol, estriol, which enhance, or 2,3-methoxy estrone, which inhibits DNA replication. The second class of compounds have qualitatively different effects when used for pretreatment of either DNA or HeLa cell extracts, e.g. 2- and 4-hydroxy estradiol, which enhance DNA replication when used in pretreatment of HeLa cell extracts, but inhibit DNA replication when used in pretreatment of DNA.