Salvestrols: A New Perspective in Nutritional Research

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Introduction

The cytochrome P450 enzyme is an enzyme complex which consists of the heme-thiolate P450 and its electron transport redox partner. NADPH-cytochrome P450 reductase is the common redox partner for mammalian microsomal P450s, whilst the bacteria and mitochondrial P450s receive electrons from a small soluble iron-sulfur protein, redoxin. The problem of redox partner recognition and mechanism of electron transfer has been one of the most important and intriguing area in P450 research. The involvement of both electrostatic and hydrophobic forces in protein-protein interactions between P450s and their redox partners has been demonstrated[1-6] but the questions of where and how P450s interact with electron donors and the precise nature of the electron-transfer remain unanswered. As a result of their abilities to metabolize xenobiotics, P450 researches are mainly focused on their metabolic capabilities to activate procarcinogens and to metabolize pharmaceutical drugs. One good example is the activation of the steroid hormone 17ß-estradiol by CYP1 isoforms (namely CYP1A1, CYP1A2 and CYP1B1) to its mutagenic metabolite 4-hydroxyestradiol.[7,8] It is thought that 4-hydroxyestradiol exerts its mutagenic effect by undergoing redox cycling that results in the generation of reactive semiquinone/quinine intermediates that can damage DNA by alkylation.[9-13] Earlier studies of CYP enzymes in ontogeny focused on inducible expression of P450s in terms of their role in deleterious responses in the conceptus, including teratogenesis and carcinogenesis. However, these studies did not examine the possibility that the presence of CYPs might be constitutive and may play a role in ontogeny by synthesizing or degrading signalling molecules required for normal patterning and differentiation. Yaffe and co-workers were the first to demonstrate CYP mono-oxygenase activity in human fetal tissues.[14] Later, CYP1B1 has been shown as an important modulator in normal human eye development and mutations in the *CYP1B1* gene linked to primary congenital glaucoma.[15] Given the constant exposure of fetal tissues to maternal 17ß-estradiol, as well as the rapid development and differentiation of fetal cells, one would expect the extinction of hominid species many years ago due to the carcinogenic effect of 4-hydroxyestradiol generated by fetal CYP1B1. This has shown the disparities in carcinogenesis models based on metabolism of endogenous substrates catalyzed by P450s. There is indisputable evidence to show that P450s, such as the CYP1 enzymes,[16-20] do activate procarcinogens to their ultimate mutagenic metabolites. Hence, these enzymes are considered to be carcinogenic. However, we have to comprehend most of these mutagenic compounds are man-made, synthesized perhaps in the past 200 years as a result of the industrial revolution and advancement

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in organic chemistry. Thus, CYP1 enzymes could not be evolved to metabolize these xenobiotics. The capabilities of CYP1 enzymes to metabolize these compounds are probably due to their structural similarity to 17ß-estradiol, the only known endogenous substrate for the CYP1 mono-oxygenases. It is accepted that after the animal-fungi-plant divergence, the main driving force of P450 evolution is by the great animal-plant "biochemical warfare."[21] This evolution event has seen the further divergence in plant P450s to enable plants to synthesize toxic products that acted as deterrents to herbivores. On the other hand, animals have evolved a battery of CYPs that could detoxify these harmful substances (the gene-for-gene theory). Many plant secondary metabolites do possess properties that can alter animals physiological and biochemical processes.[22-25] *Homo sapiens* did not emerge as a distinct species *circa* 1 million years ago[26] and it is widely accepted that our evolution was based on a largely vegetarian diet. Therefore, there is an intriguing possibility that plant substances, such as certain secondary metabolites, could be an integral part of the body's regulatory system that helps to maintain homeostasis. Animal P450s that evolved to metabolize these exobiotics may play a regulative role on the levels of these exo-regulatory compounds *in vivo*, as well as endogenous hormone metabolism. Based on the gene-for-gene theory, it is very reasonable to speculate that all P450s should have a very specific natural substrate, which is characteristic of any given P450 enzyme. Elucidation of the natural substrate for each P450 is of paramount importance given that this would probably lead to a better understanding of the biochemistry and molecular pharmacology of life.

Evolution of Cytochrome P450s

Until the late 1980s, the nomenclature of P450s was usually based on how the enzymes were identified. A good example is the rat P450, formally known as P450PB1, where the designation PB was due to the enzyme ability to metabolize phenobarbital. Based on the current nomenclature devised under the leadership of Nebert,[27] the rat enzyme is now referred to as CYP2B1, while the corresponding gene became *CYP2B1*, to indicate family 2, subfamily B and individual enzyme number 1. As more and more genomes from different organisms were sequenced, the number of P450 genes/proteins detected also significantly increased. At the moment of writing, there are over 800 subfamilies and over 6,000 individual enzymes discovered in all life forms.[28] The large number of P450s identified has made the studies on how this superfamily of enzymes evolved over the last 3.5 billion years possible.[26] Using appropriate algorithms, it is feasible to compare two or more proteins sequences and their approximate evolutionary distance can be calculated.[29] The method is based on the specific rate of protein mutation[30] and how mutation rate is associated with the divergence of two related proteins.[31] This led to the unweighted pair group method of phylogenetic analysis (UPGMA),[32, 33] a method for the formulation of phylogenetic trees. The P450 nomenclature devised in 1987[27] is now choking with families as more and more P450 families are discovered and assigned. The explosion of family number has made the nomenclature cumbersome as well as creating inconvenience in evolutionary studies. Some families of P450 are clearly evolved from a common ancestor and therefore, a higher order of nomenclature is necessary to cluster these families collectively. The term used for these clusters is clan. The clan system devised by Nelson[34] has made the studies of P450 evolution manageable. P450s within a particular clan are diverged from a common P450. Comparisons of different clans have shown that P450s are probably descendants of an ancestral protein present in a prehistoric life form,

before the divergence of prokaryotes and eukaryotes. It is thought that the ancestral P450 was developed by ancient thermophilic archaebacteria which occupied the vicinity of deep sea volcano vents.[35]This is a sound hypothesis since there would be a plentiful supply of iron and sulphur, the key elements for P450 and the redoxin redox partner. It has been suggested that the early role of P450 enzymes may have involved detoxification of reactive oxygen species harmful to anaerobes[36] and it is plausible that early P450s could have utilized carbon substrates to detoxify oxygen in the pre-historic earth's atmosphere. According to recent dating, for the divergence between eukaryotes and prokaryotes took place around 2 billion years ago, which corresponds to the established time where the earth's atmosphere changed from being a reducing one to that of oxidizing nature.[37,38] This is thought to be brought about by the emergence of photosynthetic cyanobacteria.[39] The increase in atmospheric oxygen enabled life to develop to more complex forms, as a result of the ability of enzymes like P450s to unlock the chemical potential of oxygen, by converting this element from its ground state to active singlet excited state which allowed reactions with organic substrates. Once the oxygen level had risen significantly due to photosynthetic activity of blue-green algae, which started to proliferate on earth *circa* 1,800 million years ago,[40] the excess oxygen enabled metabolism of organic molecules that helped to drive the rapid development of eukaryotic organisms. The bifurcation of the eukaryotic P450 to microsomal and mitochondrial separated at around 950 million years ago.[41] This is close to the accepted divergence time between the plant and the animal kingdoms at about 1 billion years ago, which is also thought to represent the development of sexual reproduction. The evolution of life forms, which has progressed to complex multicellular organisms, presumably has necessitated signalling molecules that control inter- and intracellular communications. Ancestral P450s were expanded to accommodate these new roles, as evident that most of these signalling molecules, including steroid hormones and eicosanoids, are synthesized or partially synthesized by P450s.[42] By 450 million years ago, colonization of land began, first by plants followed by other animal species.[43] It is thought that via co-evolutionary process, animals elaborated the P450 system from an endogenous substrate metabolizing role to detoxify potentially lethal phytochemicals.[21] This scenario is commonly described as the animal-plant biochemical warfare, which has driven the further branching of the plants and animals P450 phylogenetic tree, as plants tried to synthesize phytochemicals as deterrents but herbivores also evolved P450 to detoxify these lethal substances.

The hominids probably started to roam the earth around 15 million years ago but our ancestors did not emerge as a species until 1 million years before present.[26] It is possible that during evolution, our body has adapted to utilize exobiotics, in the form of phytochemicals from the diet, as part of our body regulatory system that helps to maintain homeostasis. This is evident by the fact that many plant secondary metabolites do possess properties that can alter animals physiological and biochemical processes. Consequently, this may support the belief conceptualized by Hippocrates: "Let food be thy medicine and medicine be thy food."

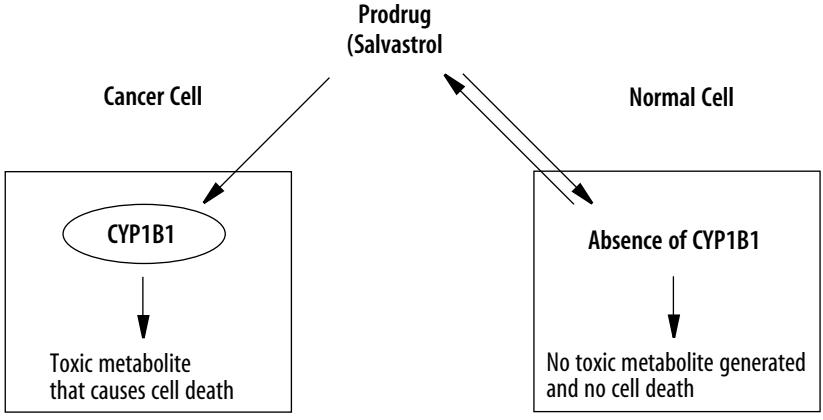
Salvestrols as Natural Anticancer Prodrugs

Prodrug is a pharmaceutical substance that is administered in an non-active form. Once absorbed, the prodrug is metabolized by an enzyme within the cell into the active compound. Providing the expression of the activating enzyme

is specific in diseased cells, the pharmacological effect of the activated drug will be limited to the target cells, since the normal ones do not contain the activating enzyme. (**Figure 1**, below)

Most of the current chemotherapeutics in clinical use are cytotoxic. As a general perception that natural anticancer agents have to be as cytotoxic as their synthetic counterparts, most medicinal phytochemists have so far been searching for increasingly cytotoxic natural compounds in the hope that these toxins can be developed into mainstream treatment. This may lead to novel therapeutic agents for cancer treatment but due to the non-specific toxicity of these compounds, patients may suffer from debilitating side effects or even death. The P450 enzyme CYP1B1 was first identified in mouse fibroblast[44] in the early 1990s and later the human CYP1B1 was identified in a keratinocyte cell line.[45] A few year after the discovery of CYP1B1, Murray and co-workers[46] have demonstrated that CYP1B1 enzyme was present in tumour biopsies but the enzyme was not detected in corresponding normal tissues. Apart from specific expression in different cancers,[46-53] CYP1B1 has been found in abnormal cells such as prostate cells from prostatic hypertrophy[54] and cervical cells from cervical intraepithelial neoplasia.[55] Many studies on the tissue expression of CYP1B1 have concentrated on the detection of *CYP1B1* mRNA using reverse transcriptase polymerase chain reaction (RT-PCR)[17, 56, 57] and it is clearly evident that *CYP1B1* mRNA is expressed extrahepatically. However, these authors did not address the existence of functional CYP1B1 protein in the tissues they had examined. It has been shown that there is a poor correlation between mRNA and the corresponding protein expression[58-60] and therefore, presence of *CYP1B1* mRNA cannot prove that CYP1B1 protein is also expressed. Using immunohistochemistry, Murray and co-workers[46] have demon-

Figure 1. ***Concept of Intracellular Activation of Anticancer Prodrug.*** Prodrug is the non-active form of a drug that requires metabolic activation by an enzyme. In this case, the specific expression of CYP1B1 in tumours only limits the activation of the prodrug in the diseased cell, leaving the normal one unharmed.



strated that CYP1B1 protein was present in tumours derived from the bladder, brain, breast, colon, connective tissues, kidney, liver, lung, lymph nodes, esophagus, ovary, skin, small intestine, stomach, testis and uterus. The presence of CYP1B1 was not detected in corresponding normal tissues though *CYP1B1* mRNA was detectable. This observation has led to the belief that regulation of CYP1B1 in tumours is predominantly post-transcriptional and a recent publication has shown that the expression of CYP1B1 protein is indeed post-transcriptionally controlled by microRNA.[61] In direct conflict with this finding is the reported expression of CYP1B1 protein, measured by western blotting and immunohistochemistry, in microsomal protein from non-tumour human liver and a variety of other tissues including bladder, breast, colon, kidney, lung, ovary, prostate and testis tissues.[62] Another study using the same antibody showed nuclear localization of CYP1B1 expression whereas negligible staining was observed in the cytoplasm.[63] This finding contrasts directly with the previous study where CYP1B1 was highly expressed in microsomal protein. Interestingly, all CYP1B1 positive liver samples used in the immunohistochemical study were obtained at autopsy, unlike the CYP1B1 negative liver samples, and the authors proposed that biogenesis of this enzyme may have been induced by terminal metabolic events.[63]

It is no exaggeration to say that the discovery of overexpression of CYP1B1 in tumours[46] is one of the most important revelations in cancer research for the past 25 years. The presence of a specific yet functionally competent enzyme within tumours has provided medicinal chemists a gateway to design prodrugs that would be selectively activated by this enzyme.[64, 65] Potter and co-workers have successfully synthesized a range of novel tumour selective anticancer prodrugs that were designed to be activated by CYP1B1. One such novel compound is DMU212, which is currently undergoing extensive preclinical studies. DMU212 has shown significant improvement in terms of tumour selectivity[66] and has been shown to cause no side effects in toxicology studies.[67] The same research group later has found that the anticancer prodrugs synthesized are very similar to natural compounds in our diet. Further screening processes have identified the first Salvestrol, namely resveratrol, which is activated *in vitro* by CYP1B1 to cytotoxic species.[68] This finding has led Potter et al. to believe that CYP1B1 may function as tumour specific rescue enzyme, utilising natural anticancer prodrugs in the diet to selectively destroy the malignant tumours.[69] Since this discovery, more of these beneficial natural molecules have been discovered and these newly identified Salvestrols have turned out to be far more powerful and life-protecting than resveratrol (Nature's Defence Investments; unpublished observations). (**Figure 2,** page 44)

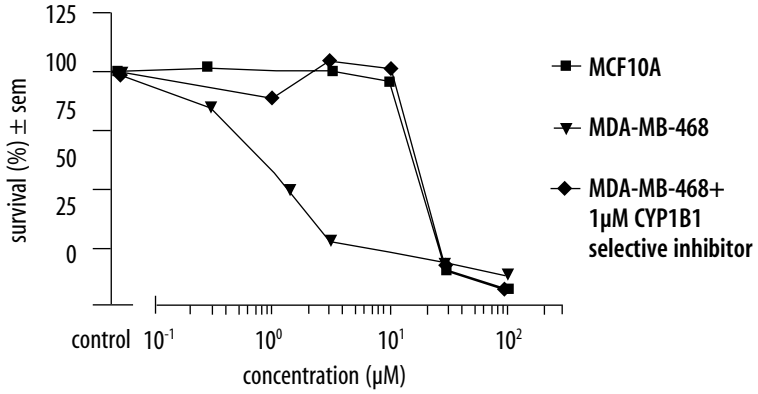
Depletion of Salvestrols in Modern Diet

Salvestrol is a new pharmacological definition and it is derived from the Latin word *salve*, meaning to save. A new definition is necessitated because although these natural molecules come from diverse classes of chemicals, they do share a common pharmacological effect which is they are non-toxic dietary phytochemicals that will be activated to toxic metabolites by tumour specific enzymes, such as CYP1B1. These compounds are present in our diet and have been safely eaten for many millions of years. Initial screening of fruits and vegetables at Nature's Defence yielded no result until organic produce was used in the studies (unpublished observations). This interesting observation has led to the discovery that modern agricultural techniques have depleted the level of these compounds in the modern diet and

consequently, may help to explain the rise of cancer incidence in human populations even though the consumption of fruits and vegetables is increasing year by year. Salvestrols belong to a group of phytochemicals called phytoalexins, which are produced by plants as a direct challenge by pathogenic organisms. The start of the agricultural revolution in the 18th century has necessitated that crops be grown to a standard height to permit mechanization of farming processes. This monoculture farming practice requires the use of agrochemicals for crop protection, since the absence of genetic diversity in the field may render the whole crop susceptible to diseases. Since the plants are no longer challenged by diseases due to protection from chemical sprays, the production of Salvestrols, such as resveratrol,[70,71] is not induced, thus, only very low levels of Salvestrols are being synthesized in non organic produce. Most of the Salvestrols have a bitter or sharp taste. Modern palate demands ever sweeter and better tasting fruits and vegetables. This has driven botanists to breed and produce modern varieties of food crops that are sweeter. Moreover, government initiatives of "no added sugar" have forced the food manufacturers to utilize technologies, such as ion-exchange chromatography, once only found in the chemical and pharmaceutical sectors, to non-selectively remove bitter agents in fruit juices to satisfy the "no added sugar" demand. Unfortunately, these practices are removing the life-protecting Salvestrols from our diet.

The discovery of natural anticancer prodrugs in our diet has revolutionized our view on the relationships between diet and cancer. There is an intriguing

Figure 2. ***Bioactivation of Salvestrol Q40 in Human Cell Lines.*** Human cancer cell containing CYP1B1 (MDA-MB-468) and human cell line lacking CYP1B1 (MCF10A) were each treated with a range of concentrations of Salvestrol Q40 and the percentage survival of both cell types were measured spectrophotometrically (MTT cytotoxic assay). The results are average values of four determinations +/- standard error of the mean (sem). The activation of Salvestrol in MDA-MB-468 can be abolished by a specific CYP1B1 inhibitor developed at the Cancer Drug Discovery Group, De Montfort University.



probability that other chronic diseases may be regulated by similar mechanisms utilizing dietary substances. Currently at Nature's Defence, we are trying to develop these natural compounds into clinical anticancer agents. As we are also believe that prevention is better than treatment, we have started to work with local farmers and other organizations in an attempt to try to revive older varieties of fruits and vegetables, as well as exploring alternative farming techniques, with the aim of increasing our intake of Salvestrols from food sources.

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