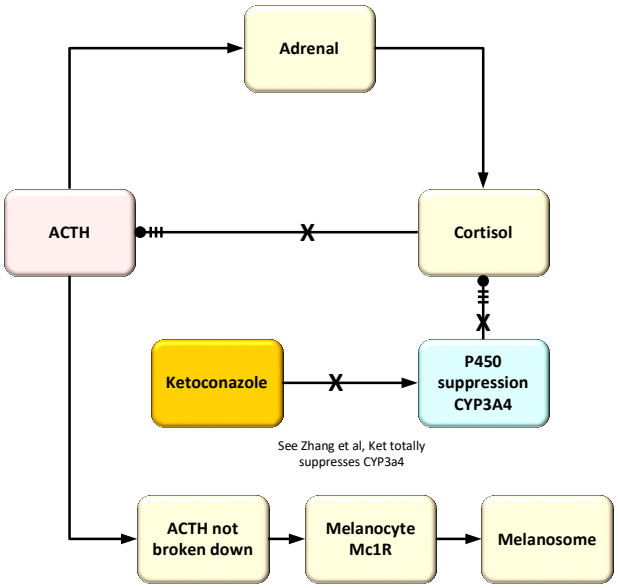

MELANIN AND KETOCONAZOLE: A PATHWAY PARADIGM

April 2024

ABSTRACT

We examine the impact of an antifungal, ketoconazole, on the generation of melanin. This is an interesting path (cause/effect) process that can be used in a variety of other approaches. Therapeutic options may be presented.

Terrence McGarty
TGL 207

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Communications relating to these documents and these should be sent to:
mcgarty@alum.mit.edu.

Access to prior Technical Notes can be made via Research Gate,
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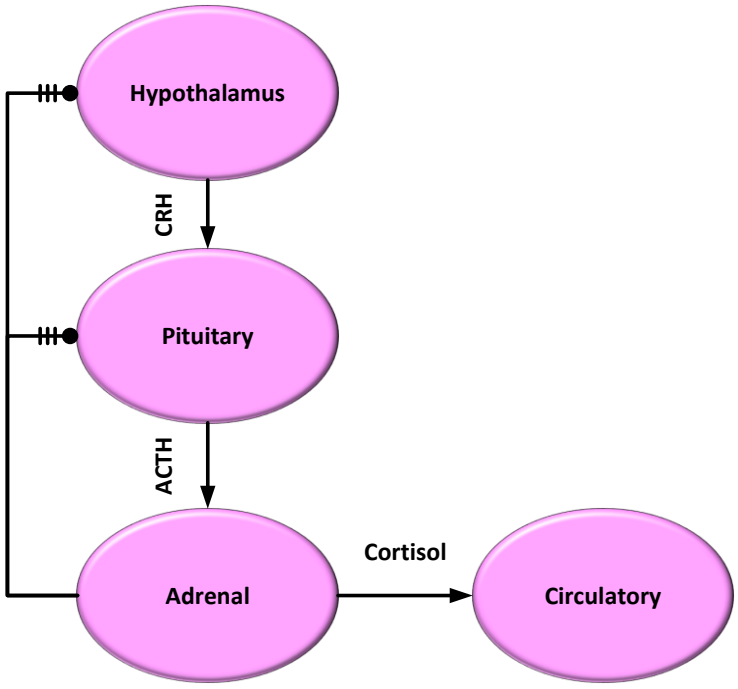
Contents

1 INTRODUCTION

Drugs often modify things we least likely expected. A key element in this process is the collection of P450 proteins that provide for this function. P450 proteins are a set of protein conjoined with a heme factor, with iron at its center. These proteins allow for the effective changes in therapeutics and facilitate their efficacy. One sees P450 almost everywhere.

Pigmentation of the skin is almost always the result of the production of melanosomes via activation of melanocytes. Melanocytes are activated to produce melanosomes by several factors, one being ACTH. For example in Addisons disease, an autoimmune disease of the adrenal gland, presupposes the skin often takes on a bronze like texture[1].

A driver in this process is ACTH (or corticotropin). In a normal metabolic process the figure below delineates a simplified version of ACTH, as a driver and as a feedback controlled element via cortisol. The hypothalamus releases CRH which induces the anterior pituitary to release ACTH and in turn drives the adrenal to facilitated release of cortisol.



There is a negative feedback process at play as well. As cortisol increases it inhibits both CRH and ACTH. There are models for this process. As Lightman et al have noted:

[1] See Felner and Umpoerrez p 215

The past decade has seen several critical advances in our understanding of hypothalamic–pituitary–adrenal (HPA) axis regulation. Homeostatic physiological circuits need to integrate multiple internal and external stimuli and provide a dynamic output appropriate for the response parameters of their target tissues.

The HPA axis is an example of such a homeostatic system. Recent studies have shown that circadian rhythmicity of the major output of this system—the adrenal glucocorticoid hormones corticosterone in rodent and predominately cortisol in man—comprises varying amplitude pulses that exist due to a subhypothalamic pulse generator.

Oscillating endogenous glucocorticoid signals interact with regulatory systems within individual parts of the axis including the adrenal gland itself, where a regulatory network can further modify the pulsatile release of hormone. The HPA axis output is in the form of a dynamic oscillating glucocorticoid signal that needs to be decoded at the cellular level. If the pulsatile signal is abolished by the administration of a long-acting synthetic glucocorticoid, the resulting disruption in physiological regulation has the potential to negatively impact many glucocorticoid-dependent bodily systems.

Even subtle alterations to the dynamics of the system, during chronic stress or certain disease states, can potentially result in changes in functional output of multiple cells and tissues throughout the body, altering metabolic processes, behavior, affective state, and cognitive function in susceptible individuals. The recent development of a novel chronotherapy, which can deliver both circadian and ultradian patterns, provides great promise for patients on glucocorticoid treatment.

Recently Ahmad et al have provided a detailed analytical model for the dynamics of this process. It is worth including this construct as an adjunct. They note:

A novel mathematical model for the hypothalamic–pituitary–adrenal (HPA) axis is proposed to comprehend the oscillations observed in hormone concentration and potential dysfunction within the HPA axis in stressful situation. This model integrates impact of hippocampal receptors on the secretion of corticotropin-releasing hormone (CRH), an additional signalling pathway involving Arginine Vasopressin (AVP) for the production and secretion of adrenocorticotropic hormone (ACTH), the inclusion of a daylight-related function for modelling circadian rhythms, and a short negative feedback loop from the pituitary to the hypothalamus in a minimal mechanistic model of the HPA axis.

This expansion allows us to estimate model parameters that led to a significant reduction in the mean absolute percent error, thereby enhancing the model's predictive accuracy with a demonstration of a strong fit to the validation dataset. Through sensitivity and correlation analyses, our study shows the parameters that exert the most significant influence on the dynamics of cortisol within the system. This study reveals intricate interdependencies within the model and among the various components of the HPA axis.

Despite current advancements in the model to comprehend the dynamics of the HPA Axis, these models are still lacking in addressing major factors such as the impact of genetic and

epigenetics, the role of the amygdala in processing and transmitting stress signals, and other minor impacting factors.

What we will examine here is the connection between this axis, the interference of an azole therapeutic, and melanin production. It is important to ultimately include the detailed temporal dynamics yet we have not done so herein.

Consider a clinical case[2]. A female patient has had a pruritic condition of head and neck. It appears as a seborrheic dermatitis, possibly fungal in origin. Thus a ketoconazole shampoo was prescribed. After an extensive period of use, 18 months, the itching was controlled but melanocytic lesions appeared. They were smooth, clear edges, blue-grey color, and not appearing to be a melanoma, but likely melanocytic in content. The question then is; is ketoconazole the driving factor and if so how does it react to do this.

Rosebush et al have noted:

Medications which have been most frequently implicated include antimalarials, hormones, oral contraceptives, phenothiazines, chemotherapeutics, amiodarone, minocycline, zidovudine, clofazimine and ketoconazole. Any patient taking these medications long term should be monitored for the development of oral pigmentation. Hard palate, gingiva and buccal mucosa are the most common locations affected. Clinically, the discoloration is flat and can be focal, multifocal or diffuse. The color may be black, gray, blue or brown. Pigmentation restricted to the hard palate is classically seen with antimalarials, which are commonly prescribed in the treatment of rheumatoid arthritis and systemic lupus erythematosus. A somewhat symmetric area of involvement centered on the midline may be seen.

Thus to understand this process we must understand:

1. Generation of melanin and its drivers.

2. The dynamics of ketoconazole and its impact

3. The dynamics and interactions of the P450 enzyme, CYP3a4 and ketoconazole

4. The flow of these interactions allowing excess ACTH to drive overexpression of melanin.

The overall objective is to obtain a "systems" understanding of this process. This may then allow for proper therapeutic management. However, the small example discussed herein, driven by a

[2] See Rosebush et al, Black and brown pigmentation of the oral mucosa can occur due to a multitude of non-neoplastic causes. Endogenous or exogenous pigments may be responsible for oral discoloration which can range from innocuous to life-threatening in nature. Physiologic, reactive, and idiopathic melanin production seen in smoker's melanosis, drug-related discolorations, melanotic macule, melanoacanthoma and systemic diseases are presented. Exogenous sources of pigmentation such as amalgam tattoo and black hairy tongue are also discussed. Determining the significance of mucosal pigmented lesions may represent a diagnostic challenge for clinicians. Biopsy is indicated whenever the source of pigmentation cannot be definitively identified based on the clinical presentation.

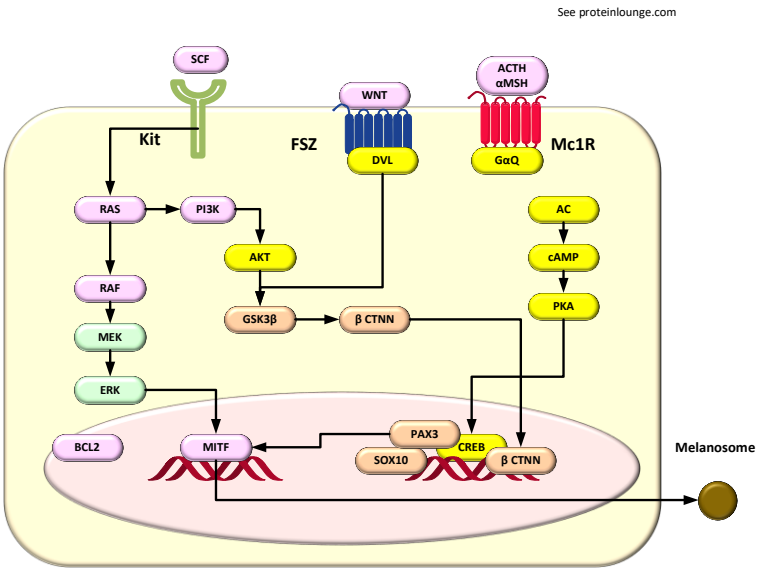
clinical issue, may be a possible explanation but may not be dispositive. The intent is to demonstrate a clinical example of a systems approach. Clearly a dispositive set of clinical investigates would be required.

2 MELANOSOMES AND MELANIN

Pigmentation is a product of the production of melanin in melanosomes by melanocytes. In this section we provide a focused overview of the key elements involved in melanin generation.

2.1 BASIC FLOWS

A simplistic process is shown below. We shall focus on more details as we progress. Note that the MC1R receptor is driven by ACTH. That will be a focal point in the entire process. Fundamentally melanocytes have receptors that are activated by sets of ligands and in turn flow throughs sets of pathways to the nucleus and act as activators and facilitators of transcription.



We now examine some of the most prominent elements in the above process. We shall deal with the key ones which will be involved in melanin generation.

2.1.1 MC1R

Let us begin with the most significant receptor, MC1R. Note that the MC1R receptor[3]:

The MC1 receptor (MC1R) is a 315 amino acid transmembrane protein which in humans is mapped to 16q24. It is the principal melanocortin receptor in the skin where it regulates its pigmentation. It exhibits high affinity for most MSH isoforms and a much lower affinity for ACTH. Its highest affinity is towards alpha–MSH (Ki = 0.033 nmol/l).

Stimulation of MC1R in the skin and the hair follicles by alpha-MSH results in induction of melanogenesis producing dark skin and hair in several species including the humans. The MC1R is also present in the adrenals, the leukocytes, lungs, lymph nodes, ovaries, testes, pituitary,

[3] https://www.ncbi.nlm.nih.gov/books/NBK279118/

placenta, spleen and the uterus. The agouti protein is an endogenous antagonist of alpha-MSH at the level of the MC1R in the skin. Over-expression of the agouti protein results in fair skin, reddish hair and disturbances of energy balance.

Variants of the MC1R in humans are associated with red hair, pale skin, and increased risk for skin cancer. The MC1R in leukocytes and macrophages has been associated with the immune effects of alpha-MSH

2.1.2 CREB

CREB is another transcription factor involved in melanogenesis. As Steven et al note:

Cyclic AMP (cAMP)-response element-binding protein 1 (CREB) is a 43 kDa stimulus-induced transcription factor (TF).

It can bind to the cAMP response element (CRE) sequence TGACGTCA or the conserved half CRE TGACG and was first identified in the somatostatin gene promoter. ***Genome-wide screening for CREB-binding sites suggested that more than 4000 genes might be controlled by CREB, postulating CREB as a general transcriptional activator.***

CREB is not a unique transcription factor but it is drawn to the mix driven by activated pathways as shown above.

Regarding its structure, CREB is made up of different domains with distinct functions. While the DNA binding and dimerization of CREB is mediated by a basic leucine zipper (bZIP) domain, CREB has nine serine residues in the kinase inducible domain (KID) that can be phosphorylated and activated by different kinases. Activated CREB can recruit coactivators, such as CREB-binding protein (CBP). The interaction between CREB and CBP is mediated via the interacting domain of CBP, named KIX. The CREB/ CBP complex recruits the transcription machinery at the gene promoter to initiate CREB-dependent gene transcription.

The CREB complex upregulates the methylation of histones H3 and H4, which is essential for the initiation of the transcriptional machinery. CREB activity is regulated by the phosphorylation of amino acid (aa) residues, which are mainly localized in the KID region, thereby influencing the dimerization of CREB and its binding to the CRE sequence. Phosphorylation of CREB at the Ser133 residue frequently occurs, whereas phosphorylation at other serine tyrosine and threonine residues of CREB is observed at a lower frequency.

Interestingly, the different phosphorylation patterns of CREB are correlated with distinct cellular functions and can exert opposite effects: CREBSer111 and CREBSer121 inhibit transcription, while CREBSer129 and CREBSer133 induce transcription.

2.1.3 MITF

MITF is another significant transcription factor and plays a key role in melanogenesis. As NCBI notes[4]:

The protein encoded by this gene is a transcription factor that contains both basic helix-loop-helix and leucine zipper structural features. The encoded protein regulates melanocyte development and is responsible for pigment cell-specific transcription of the melanogenesis enzyme genes.

Specifically unlike CREB it has a uniqueness to melanocytes. As Chauhan et al note:

Bidirectional interactions between plastic tumor cells and the microenvironment critically impact tumor evolution and metastatic dissemination by enabling cancer cells to adapt to microenvironmental stresses by switching phenotype. In melanoma, a key determinant of phenotypic identity is the microphthalmia-associated transcription factor MITF that promotes proliferation, suppresses senescence, and anticorrelates with immune infiltration and therapy resistance.

What determines whether MITF can activate or repress genes associated with specific phenotypes, or how signaling regulating MITF might impact immune infiltration is poorly understood.

Here, we find that MITF binding to genes associated with high MITF is via classical E/M-box motifs, but genes downregulated when MITF is high contain FOS/JUN/AP1/ATF3 sites.

Significantly, the repertoire of MITF-interacting factors identified here includes JUN and ATF3 as well as many previously unidentified interactors. As high AP1 activity is a hallmark of MITFLow, invasive, slow-cycling, therapy resistant cells, the ability of MITF to repress AP1-regulated genes provides an insight into how MITF establishes and maintains a pro-proliferative phenotype. Moreover, although β-catenin has been linked to immune exclusion, many Hallmark β-catenin signaling genes are associated with immune infiltration. Instead, low MITF together with Notch signaling is linked to immune infiltration in both mouse and human melanoma tumors.

MITF (microthalmia associated transcription factor) is a mediator of the pigmentation response in melanocytes.

It is also thought that the transcription of the MITF gene is facilitated by multiple transcription factors. MITF is both a transcription factor itself as well as a pathway mediator as we shall demonstrate.

It functions in both the nucleus and the cytoplasm.

MITF is required for the development, maintenance and survival of the melanocyte. It has been argued that MITF is one of the gene products that allows melanoma to survive the attack by

[4] https://www.ncbi.nlm.nih.gov/gene/4286

normal chemotherapy. … the disregulaton of transcription factors is putatively the prime reason for cancer. MITF disregulation is one of these transcription factors.

Continuing on MITD's importance, Yokoyama et al state:

So far, two genes associated with familial melanoma have been identified, accounting for a minority of genetic risk in families. Mutations in CDKN2A account for approximately 40% of familial cases[1], and predisposing mutations in CDK4 have been reported in a very small number of melanoma kindreds. Here we report the whole-genome sequencing of probands from several melanoma families, which we performed in order to identify other genes associated with familial melanoma. We identify one individual carrying a novel germline variant …

in the melanoma-lineage-specific oncogene microphthalmia-associated transcription factor (MITF). …

Consistent with this, the E318K variant was significantly associated with melanoma in a large Australian case–control sample. Likewise, it was similarly associated in an independent case–control sample from the United Kingdom. In the Australian sample, the variant allele was significantly overrepresented in cases with a family history of melanoma, multiple primary melanomas, or both. The variant allele was also associated with increased naevus count and non-blue eye colour. Functional analysis of E318K showed that MITF encoded by the variant allele had impaired sumoylation and differentially regulated several MITF targets.

These data indicate that MITF is a melanoma predisposition gene and highlight the utility of whole-genome sequencing to identify novel rare variants associated with disease susceptibility.

This identification of a mutated MITF and familial melanoma is a clear indication of the power that MITF has in establishing melanoma in general. As Wellbrock and Marais state:

Melanocytes are pigmented skin cells that protect us from ultraviolet radiation. The processes regulating melanocyte differentiation are intensely studied because melanocytes are thought to be the precursors of melanoma, a skin cancer whose incidence is increasing in Western societies. A master regulator of melanocyte differentiation is the microphthalmia-associated transcription factor (MITF). Strikingly, MITF levels are reduced in spontaneously transformed melanocytes, and low MITF expression correlates with poor prognosis in melanoma.

MITF regulation is complex. For example, the differentiation factor melanocyte stimulating hormone strongly increases its expression in a cAMP and cAMP response element binding protein (CREB) transcription factor–dependent manner. Another signaling module that regulates MITF is the RAS–RAF–MEK–ERK signaling cascade, which acts downstream of the receptor tyrosine kinase cKIT to stimulate MITF phosphorylation on serine 73 (S73) and enhances its transcriptional activity.

However, extracellular regulated protein kinase (ERK)–mediated S73 phosphorylation also targets MITF for ubiquitin-dependent degradation through the proteasome pathway. There are three RAS (H-RAS , K-RAS , and N-RAS) and three RAF (A-RAF , B-RAF , and C-RAF) genes

in humans. N-RAS is mutated in 5–20% of melanomas, and B-RAF is mutated in 50–70% of melanomas. The most common mutation in B-RAF (90%) is a glutamic acid for valine substitution at position 600, which produces a highly active kinase that stimulates constitutive ERK signaling and stimulates melanoma cell proliferation and survival.

In this study, we show that V600E B-RAF triggers MITF degradation in mouse and human melanocytes and that its re-expression inhibits proliferation. Furthermore, MITF up-regulation suppresses melanoma cell proliferation.

These data suggest that high MITF levels are anti-proliferative, and, therefore, its expression must be suppressed for transformation by oncogenic B-RAF.

The identification of V600E B-RAF triggering of MITF degradation is a powerful observation. The actual mechanism may not be fully understood but the causal basis is compelling. It is this type of cascade behavior that must be considered in such changes. The final conclusion is also compelling. MITF must be suppressed either by mutation or as seen here by suppression by another mutated gene. They conclude:

MITF re-expression in B-RAF–transformed melanocytes inhibits their proliferation. Furthermore, differentiation-inducing factors that elevate MITF expression in melanoma cells inhibit their proliferation, but when MITF up-regulation is prevented by RNA interference, proliferation is not inhibited. These data suggest that MITF is an anti-proliferation factor that is down-regulated by B-RAF signaling and that this is a crucial event for the progression of melanomas that harbor oncogenic B-RAF.

As Miller and Mihm state:

Mice lacking functional MITF are albino because they lack melanocytes, whereas those with partial MITF function have premature graying owing to the death of melanocytes. These experiments show that MITF is important in the differentiation and maintenance of melanocytes.

MITF appears to contribute to melanocyte survival by increasing the expression of the BCL-2 *gene, a key antiapoptotic factor.*[59] *In mice, deficiencies of both MITF and BCL-2 cause gray hair due to a loss of differentiated melanocytes. The loss of melanocytes is due to the apoptosis of melanocyte progenitor cells in the hair follicle.*

In melanoma cell lines, a reduction in BCL-2 protein also causes cell death, suggesting that the survival of malignant melanocytes depends on BCL-2. … MITF functions in a key pathway leading to melanocyte pigmentation. Intracellular signaling induced by α-MSH acting on MC1R increases MITF expression, which in turn increases the transcription of genes underlying melanin synthesis: tyrosinase, tyrosinase-related-protein 1, and dopachrome tautomerase.

*MITF also regulates the transcription of the melanocyte-specific genes silver homologue (*SILV*) and melan-A (*MLANA*), whose immunohistochemical detection points to the diagnosis of melanoma. In addition, MITF causes cell-cycle arrest by the induction of INK4A.*

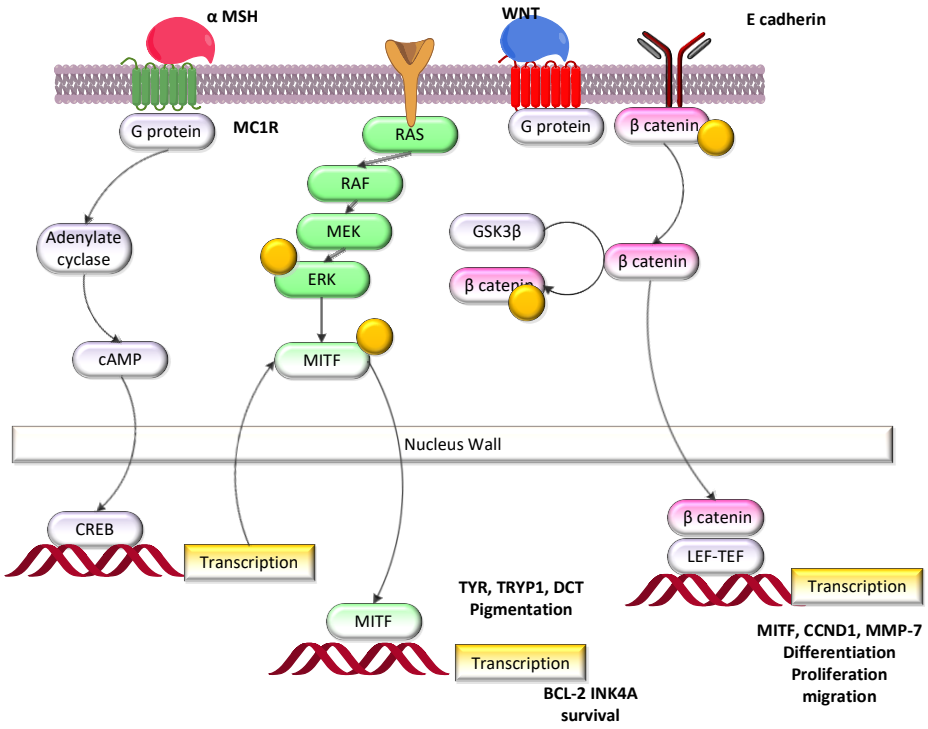
Decreased or absent pigmentation and decreased or absent expression of SILV and MLANA accompany the progression from nevus to melanoma.

*Tumors that are deficient in these proteins have a poor prognosis. Expression of the melastatin 1 (*TRPM1*) gene, whose function is unknown, is also controlled by MITF. Melanomas that are deficient in melastatin have a poor prognosis. The mechanism of decreased expression of these genes is a puzzle because MITF is present in nearly all melanomas.*[71-73] *Although MITF causes differentiation and cellcycle arrest in normal melanocytes, melanoma cells do not have these characteristics.*

Recently, a large-scale search for genomic changes in melanoma with the use of high-density single-nucleotide polymorphisms (SNPs) found an increased copy number (4 to 119 copies per cell) of a region of chromosome 3 that includes the MITF *locus. This increase was accompanied by the increased expression of MITF protein. The overexpression of both MITF and BRAF could transform primary cultures of human melanocytes, implicating MITF as an oncogene.*

Notably, MITF amplification occurs most frequently in tumors that have a poor prognosis and is associated with resistance to chemotherapy.[74] *Interference with MITF function increased the chemosensitivity of a melanoma cell line, making MITF a potential target for treatment.*

Miller and Mihm depict the MITF functions in the following Figure (as modified):



They state:

In the MITF pathway, MITF is regulated at both transcriptional and post-translational levels.

The post-translational activation can occur through the ERK component of the MAPK pathway.

The chief transcriptional pathways that are activated by extracellular signals are the melanocortin and WNT pathways.

Understanding the pathways is important to both proliferation as well a therapeutic targeting.

The melanocortin pathway regulates pigmentation through the MC1R. MC1R activates the cyclic AMP (cAMP) response-element binding protein (CREB).

Increased expression of MITF and its activation by phosphorylation (P) stimulate the transcription of tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and dopachrome tautomerase (DCT), which produce melanin; melan-A, silver homologue, and melastatin 1 (TRPM1) are melanoma markers; inhibitor of kinase 4A (INK4A) leads to cell-cycle arrest, and BCL-2 suppresses apoptosis.

In the β-catenin pathway, β-catenin plays a central role in cell adhesion and cell signaling. Signals from WNT ligands block the breakdown of β-catenin. When WNT proteins bind the G-protein–coupled receptor (called frizzled), they inactivate the kinase GSK3β, an enzyme that phosphorylates β-catenin and targets it for destruction in the proteosome.

Then β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it binds to LEF–TCF transcription factors and increases the expression of several genes, including MITF, the cell-cycle mediator cyclin D1 (CCND1), and matrix metalloproteinase 7 (MMP-7).

Further, regarding the relationship with other pathway elements, Liu et al state:

As a survival factor for melanocytes lineage cells, MiTF plays multiple roles in development and melanomagenesis. What role MiTF plays in the DNA damage response is currently unknown. In this report we observed that MiTF was phosphorylated at serine 73 after UVC radiation, which was followed by proteasome-mediated degradation.

Unlike after c-Kit stimulation, inhibiting p90RSK-1 did not abolish the band shift of MiTF protein, nor did it abolish the UVC-mediated MiTF degradation, suggesting that phosphorylation on serine 73 by Erk1/2 is a key event after UVC. Furthermore, the MiTF-S73A mutant ...was unable to degrade and was continuously expressed after UVC exposure.

Compared to A375 melanoma cells expressing wildtype MiTF (MiTF-WT), cells expressing MiTF-S73A mutant showed less p21 $^{WAF1/CIP1}$ *accumulation and a delayed p21*$_{WAF1/CIP1}$ *recovery after UVC. Consequently, cells expressing MiTF-WT showed a temporary G1 arrest after UVC, but cells expressing MiTF-S73A mutant or lack of MiTF expression did not. Finally, cell lines with high levels of MiTF expression showed higher resistance to UVC-induced cell death than those with low-level MiTF.*

These data suggest that MiTF mediates a survival signal linking Erk1/2 activation and p2 $^{WAF1/CIP1}$ *regulation via phosphorylation on serine 73, which facilitates cell cycle arrest. In addition, our data also showed that exposure to different wavelengths of UV light elicited different signal pathways involving MiTF.*

This demonstrates that UVC does have substantial mitogenic effects and may be a possible model for the mutation process.

Bourneuf et al state:

The incidence of cutaneous melanoma, the most aggressive form of skin cancer, is growing every year worldwide. Although most of the cases are sporadic and likely due to UV exposure, around 10% occur on a familial setting, and many studies have been performed to identify genetic variants conferring susceptibility to this type of cancer.

The familial setting is a powerful means to identify gene mutations that are germ line. The authors continue:

Two high-risk genes have been discovered in melanoma prone kindred, namely, CDKN2A and CDK4, both involved in cell cycle regulation through the p53/Rb pathway.

We will focus on both of these. Remember that CDK4 is a cyclin dependent kinase and plays a critical role in the cell cycle and mitotic change.

So far, the other genes, the variants of which are associated with melanoma, have been considered low-risk genes and are involved mostly in pigmentation, an important risk factor with a higher incidence of melanoma in fair-skinned patients.

For example, the melanocortin 1 receptor (MC1R) gene has been shown to enhance the penetrance of the CDKN2A mutations in patients. Its effect on melanoma, although it is also suspected to be related to UV sensitivity via unknown mechanisms, is due mainly to its major involvement in skin and hair pigmentation. Recent genome-wide association studies focusing on melanoma and number of nevi highlighted the potential role of several other genes such as MTAP (methylthioadenosine phosphorylase) and TYR (tyrosinase), which are also involved in pigmentation.

They then continue:

Numerous other genes have been shown to affect melanoma biology, but their involvement as predisposing loci for melanoma remains unelucidated.

One of these genes, MITF, is considered a master regulator of melanocyte function, including development, migration, survival, and differentiation, through complex mechanisms of regulation. Recently, MITF has been shown to be responsible for the melanocyte lineage specificity of DICER transcriptional regulation, thus contributing to melanocyte differentiation. In melanoma, Garraway et al. identified MITF as a lineage-specific oncogene, of which

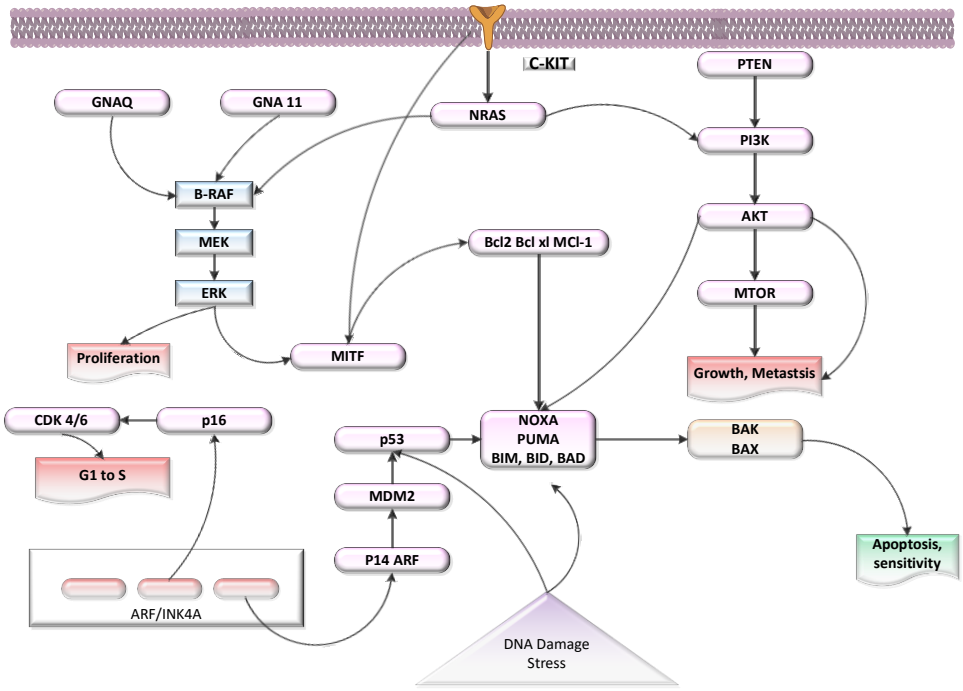
amplification in 10–20% of the melanoma samples was correlated with decreased patient survival.

Also, Giuliano et al. demonstrated that MITF was preventing melanoma cells' senescence through a DDR/p53 signaling pathway.

In addition, somatic mutations of MITF were described in a fraction of primary tumors and metastasis. This gene therefore plays a major dual role between differentiation of melanocytes and proliferation of melanoma cells.

Thus the presence of a mutation in MITF as we have discussed is a significant factor. Clearly MITF as a transcription factor has a significant role in over production and as a pathway element can enhance such over-expression.

Finally the Figure below presents a summary of functions and results.



2.1.4 Melanin

Melanin is the product of the melanosome. As D'Mello et al note:

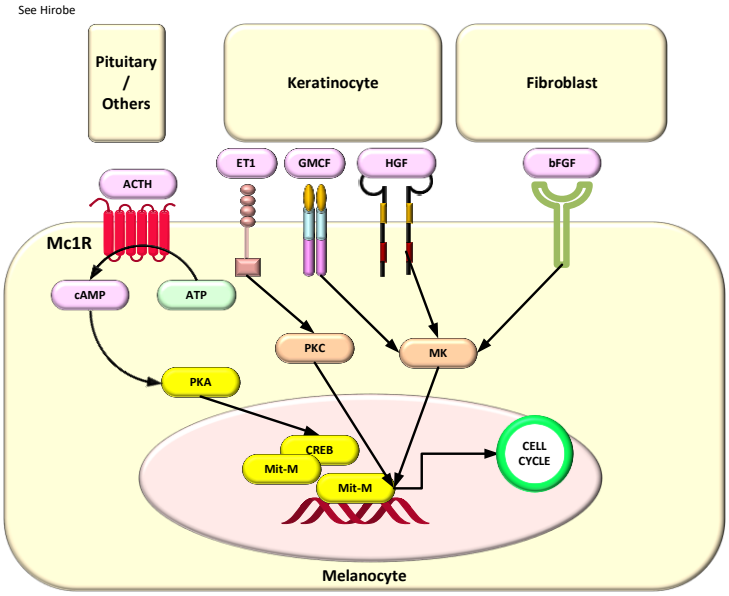
Melanocytes are melanin-producing cells found in skin, hair follicles, eyes, inner ear, bones, heart and brain of humans. They arise from pluripotent neural crest cells and differentiate in response to a complex network of interacting regulatory pathways. Melanins are pigment molecules that are endogenously synthesized by melanocytes. The light absorption of melanin in skin and hair leads to photoreceptor shielding, thermoregulation, photoprotection, camouflage and display coloring.

Melanins are also powerful cation chelators and may act as free radical sinks. Melanin formation is a product of complex biochemical events that starts from amino acid tyrosine and its metabolite, dopa. The types and amounts of melanin produced by melanocytes are determined genetically and are influenced by a variety of extrinsic and intrinsic factors such as hormonal changes, inflammation, age and exposure to UV light. These stimuli affect the different pathways in melanogenesis. In this review we will discuss the regulatory mechanisms involved in melanogenesis and explain how intrinsic and extrinsic factors regulate melanin production.

We shall develop this a bit in subsequent sections.

2.2 ACTH SOURCES

There are multiple sources of ACTH as well as other drivers, especially keratinocytes and fibroblasts. They drive both melanosomes and the cell cycle for proliferation[5]. The following is from Hirobe.



2.2.1 Keratinocytes

Keratinocytes play a significant role in melanin production. As Cirillo notes:

Neuropeptides have been known for over 50 years as chemical signals in the brain. However, it is now well established that the synthesis of this class of peptides is not restricted to neurons. For example, human skin not only expresses several functional receptors for neuropeptides but, also, can serve as a local source of neuroactive molecules such as corticotropin-releasing hormone,

[5] See https://www.researchgate.net/publication/264960157_Melanoma_Genomics for a detailed discussion of melanoma genomics. This was written in 2012 just as multiple immunotherapeutics were being deployed.

melanocortins, and β-endorphin. In contrast, an equivalent of the hypothalamic-pituitary axis in the oral mucosa has not been well characterized to date.

In view of the differences in the morphology and function of oral mucosal and skin cells, in this review I surveyed the existing evidence for a local synthesis of hypothalamic-pituitary, opiate, neurohypophyseal, and neuroendocrine neuropeptides in both epidermal and oral keratinocytes. The term neuropeptide was originally coined to indicate small protein molecules that are contained in neurons . They act either in an endocrine manner, where they reach their target cells via the bloodstream or a paracrine manner, as co-transmitters modulating the function of neurotransmitters .

Although by definition the biosynthesis of neuropeptides must occur in neurons, it is important to keep in mind that neuropeptides are not just in the brain—they act both in and out of the central nervous system .

Furthermore, it is now recognized that this class of molecules is expressed in many other tissues, including skinresident cells . The skin is considered an important peripheral neuro-endocrine-immune organ that is tightly networked to central regulatory systems. Specifically, epidermal and dermal cells produce and respond to classical stress neurotransmitters, neuropeptides, and hormones .

Keratinocytes cover both the skin and oral mucosa, but the morphology of these tissues and the behavior of the keratinocytes from these two sites are different .

One significant dissimilarity between the two sites is the response to injury, where oral mucosal wounds heal faster and with less inflammation than equivalent cutaneous wounds .

Unlike skin and its appendages, a mucosal equivalent of the neuroendocrine system has not been characterized. Here, I examine the evidence demonstrating the response to, and biosynthesis of, neuropeptides in the epithelial cells lining the skin and oral mucosa.

	Epidermal Keratinocytes		**Oral Keratinocytes**
Neuropeptide family Hypothalamic releasing factors Pituitary hormones	Local synthesis CRH, somatostatin, TRH ACTH, ct-MSH, prolactin, (TSH)	Response to exogenous stimulation CRH, (GHRH), TRH somatostatin ACTH, ct-MSH, (prolactin), (TSH)	No Local Synthesis Response to exogenous stimulation (GHRH), (somatostatin) ACTH, ct-MSH

Moreover they note:

It has recently become apparent that production of ACTH (corticotropin) is not restricted to the pituitary.

This is a powerful observation and may in a sense be the key to our analysis. ACTH is not pituitary only. In fact the keratinocytes are self-generating.

Among other cell types, it is synthesized and released by human keratinocytes in response to a number of stimuli including phorbol myristate acetate, ultraviolet light and interleukin-1 . For example, ultraviolet B (UVB) radiation stimulates increased expression of the proopiomelanocortin (POMC) gene which is accompanied by production and release of α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropin (ACTH) by both normal and malignant human melanocytes and keratinocytes .

Research suggests that corticotropin may have an immunoregulatory role in oral mucosa. Studies have investigated ACTH effects on a human oral keratinocyte cell lines and shown that corticotropin, acting via its specific receptor, stimulates a dose-dependent increase in DNA synthesis and induces cell proliferation, thus identifying corticotropin as a mitogenic regulatory peptide of keratinocytes . While the evidence for a local synthesis of ACTH in the oral cavity is debated, it is well known that the oral mucosa can respond to ACTH stimulation.

For example, high levels of circulating ACTH results in oral hyperpigmentation via stimulation of the α-MSH receptors in melanocytes . A point mutation in the ACTH receptor resulting in high plasma ACTH levels manifests with dark coloration of the oral mucosa and gums .

Corticotropin also exerts direct effects in oral mucosal cells . These observations are likely related to the fact that oral fibroblasts and keratinocytes express ACTH receptor (MC2R) and can activate its downstream signaling, e.g., de novo synthesis of cortisol . Activation of corticotropin-mediated biosynthetic pathways is functioning in oral tumours and may have clinical implications.

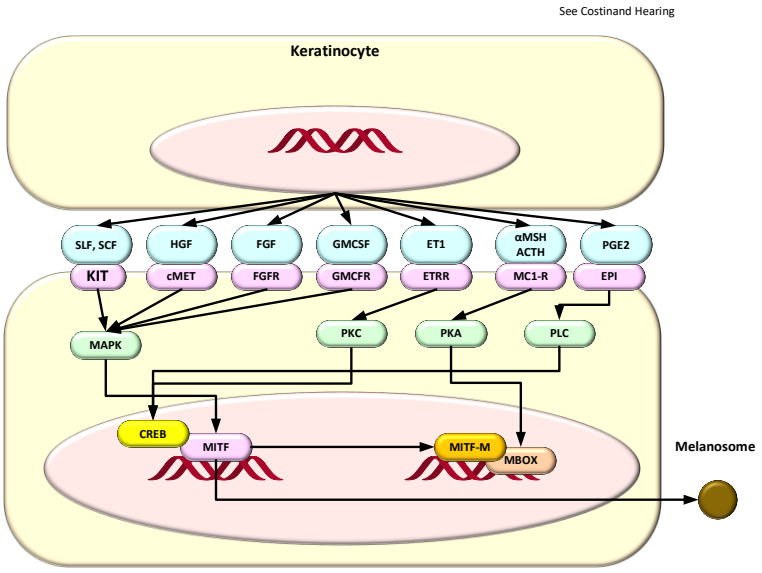
For example, ACTH can reduce the effectiveness of chemotherapeutic agents such as doxorubicin in oral squamous cell carcinoma (OSCC) cell lines, possibly via the autocrine effect of cortisol .

Cancer-derived cortisol induces a glucocorticoid receptor (GR)-dependent inhibition of tumour-specific CD8+ T cells and, consistent with this observation, higher levels of ACTH in the microenvironment of OSCCs are associated to a reduced density of the lymphoplasmacytic infiltrate .

Thus it is clear this polypeptide has remarkable effects in the physiological and pathological processes of the oral mucosa.

The last statement has a primary impact upon our analysis herein.

The keratinocyte in particular is a significant driver as shown below by Costain and Hearing.



2.2.2 Pigmentation

Pigmentation is an expression of melanin concentration and distribution. As Costin and Hearing have noted:

Cutaneous pigmentation is the outcome of two important events: the synthesis of melanin by melanocytes and the transfer of melanosomes to surrounding keratinocytes.

Although the number of melanocytes in human skin of all types is essentially constant, the number, size, and manner in which melanosomes are distributed within keratinocytes vary.

The melanin content of human melanocytes is heterogeneous not only between different skin types but also between different sites of the skin from the same individual. This heterogeneity is highly regulated by gene expression, which controls the overall activity and expression of melanosomal proteins within individual melanocytes.

The above are important observation regarding melanin concentration and distribution.

It has been shown that melanocytes with a low melanin content synthesize TYR more slowly and degrade it more quickly than melanocytes with a higher melanin content and TYR activity (28). In general, highly pigmented skin contains numerous single large melanosomal particles (0.5–0.8 mm in diameter), which are ellipsoidal and intensely melanotic (stage IV). Lighter pigmentation is associated with smaller (0.3–0.5 mm in diameter) and less dense melanosomes (stages II and III), which are clustered in membrane-bound groups.

These distinct patterns of melanosome type and distribution are present at birth and are not determined by external factors (such as sun exposure). They are responsible for the wide variety of skin complexions. Epidermal melanin unit and the involvement of keratinocytes in melanin

production The epidermal melanin unit is a functional and structural complex within the epidermis consisting of two cell types: melanocytes and keratinocytes.

The variation in skin color among various races is determined mainly by the number, melanin content, and distribution of melanosomes produced and transferred by each melanocyte to a cluster of keratinocytes surrounding it. Once in keratinocytes, the melanin granules accumulate above the nuclei and absorb harmful UV-R before it can reach the nucleus and damage the DNA.

When melanin is produced and distributed properly in the skin, dividing cells are protected at least in part from mutations that might otherwise be caused by harmful UV.

The melanocyte-keratinocyte complex responds quickly to a wide range of environmental stimuli, often in paracrine and/or autocrine manners. Thus, melanocytes respond to UV-R, agouti signaling protein, melanocyte-stimulating hormone (MSH), endothelins, growth factors, cytokines, etc.

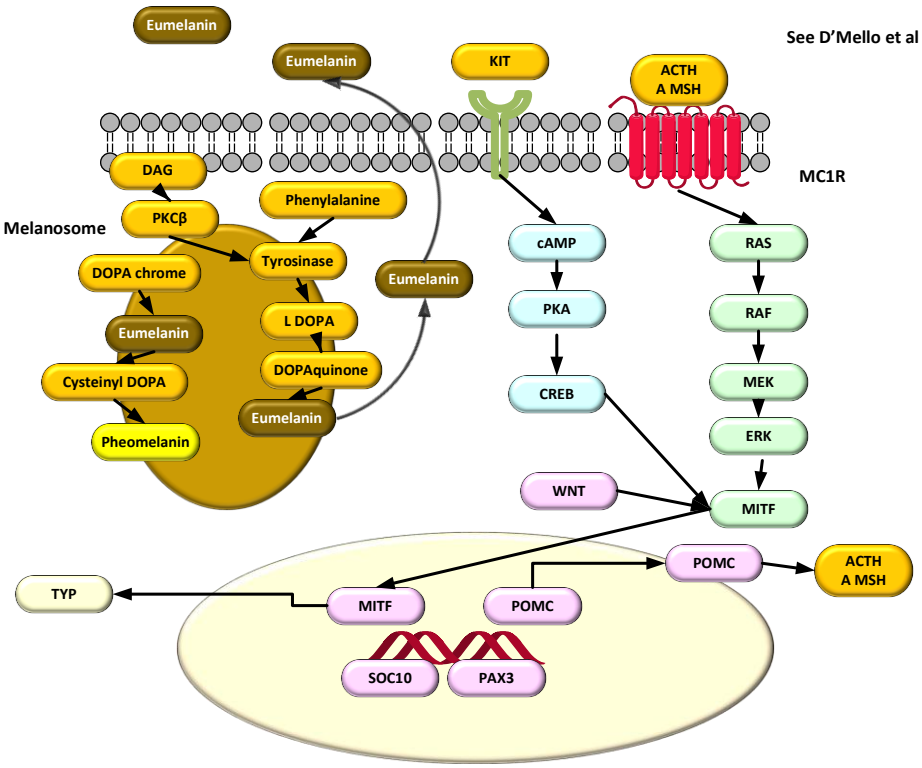
After UV-R exposure, melanocytes increase their expression of proopiomelanocortin (POMC, the precursor of MSH) and its receptor melanocortin 1 receptor (MC1-R), TYR and TYRP1, protein kinase C (PKC), and other signaling factors.

On the other hand, it is known that UV stimulates the production of endothelin-1 (ET-1) and POMC by keratinocytes and that those factors can then act in a paracrine manner to stimulate melanocyte function. In addition to keratinocytes, fibroblasts, and possibly other cells in the skin produce cytokines, growth factors, and inflammatory mediators that can increase melanin production and/or stimulate melanin transfer to keratinocytes by melanocytes.

Melanocyte growth factors affect not only the growth and pigmentation of melanocytes but also their shape, dendricity, adhesion to matrix proteins, and mobility.

2.3 MELANIN PRODUCTION

From D'Mello et al we have the following details showing the development of the melanin from the melanosomes.



The above is a more complete path for melanin production from an intracellular melanosome. As D'Mello et al note:

Eumelanin and pheomelanin are synthesized within melanosomes of melanocytes by a series of reactions that are catalyzed by specific melanogenic enzymes.

Production of these enzymes is driven by the MITF transcription factor whose activity is regulated by a number of signaling pathways including PKC, cAMP, MEK, and WNT.

These signaling pathways are activated upstream by receptors such as KIT (ligand: SCF) and MC1R (ligands: α-MSH, ACTH and ASP). The MITF transcription factor drives the expression of a number of genes including SOX10 and PAX3. Protein kinase C (PKC); cyclic AMP (cAMP); MAPK/ERK Kinase (MEK); Wingless-related integration site (WNT); Stem Cell Factor (SCF); Melanocyte-specific melanocortin-1 receptor (MC1R); α-melanocyte-stimulating hormone (α-MSH); adrenocorticotropic hormone (ACTH); agonist stimulating protein (ASP).

A higher overall melanin density results in darker skin, but the eumelanin to pheomelanin ratio also contributes to the differences seen in human skin pigmentation . Individuals with melanocytes that make more pheomelanin than eumelanin tend to have lighter skin that is more prone to blistering and burning.

Skin that has pheomelanin also produces more reactive oxygen species, which can accelerate carcinogenesis, compared with skin that produces eumelanin or has no melanin [31,32].

Following exposure to UV radiation, melanin can act as a photosensitizer to generate superoxide radicals that cause lethal cellular injury but melanin is important for skin homeostasis and tanning indicative of a distress signal . Hydroxylation of L-tyrosine to L-DOPA is the rate-limiting step in melanin synthesis and is catalyzed by tyrosinase, which is a copper-containing membrane-bound located in melanosomes. L-Phenylalanine in the cytosol may be converted to tyrosine by phenylalanine hydroxylase (PAH) in order to serve as the substrate for tyrosinase .

Aside from tyrosinase, TYRP1, and TYRP2 are present in melanosomes and also play a crucial role in catalyzing eumelanin-producing reactions. TYRP1 has been suggested to increase the eumelanin: pheomelanin ratio and protect against oxidative stress via its peroxidase effect . The biochemical pathway.

Eumelanin and pheomelanin are synthesized within melanosomes of melanocytes by a series of reactions that are catalyzed by specific melanogenic enzymes. ***Production of these enzymes is driven by the MITF transcription factor whose activity is regulated by a number of signaling pathways including PKC, cAMP, MEK and WNT.*** *These signaling pathways are activated upstream by receptors such as KIT (ligand: SCF)….*

The MC1R is a member of a subgroup of class A G-protein-coupled receptors that includes MC1R to MC5R.

Eumelanin synthesis is stimulated via the MC1R agonists α-MSH and ACTH while pheomelanin synthesis is simulated via ASP.

Note that eumelanin, the dark skin effect, is generated by ACTH. That is one of the keys in our analysis. There will be several questions we need to resolve. First, where does the ACTH come from, second, how is it spatially activated, and third, how long does it last.

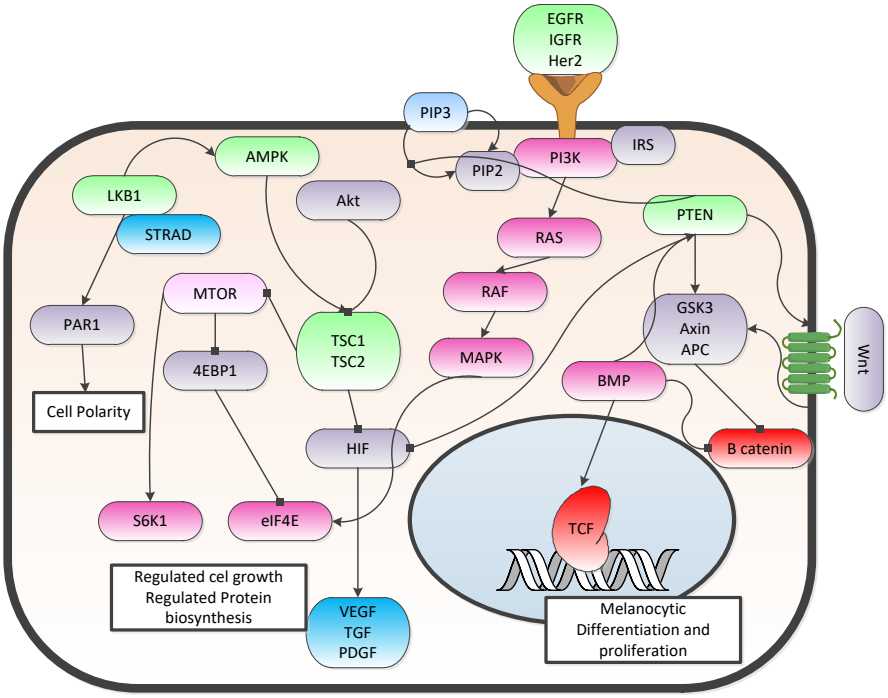
α-MSH also regulates pheomelanin and eumelanin proportions via the MC1R. α-MSH is cleaved from a precursor protein called pro-opiomelanocortin (POMC) produced by the pituitary gland and epidermal keratinocytes allowing for local paracrine regulation.

Differential expression of POMC has been shown during normal physiological hair growth, immune cytokine release, the presence of cutaneous pathology or UVR exposure. UVR acts as a stimulatory factor on POMC gene expression, and it is suggested that UVR-triggered oxidative stress leads to POMC peptide production.

It is believed this signaling pathway is critically involved in physiological adaptations of the skin to environmental factors such as UV exposure. Activation of MC1R by α-MSH or ACTH increases cAMP synthesis which indirectly induces a switch from the production of pheomelanin to eumelanin synthesis

2.4 MELANOCYTE FUNCTIONS

Finally from Bauer and Stratakis we have a more detailed picture of multiple processes. The authors use this model to explain multiple melanin generating syndromes. Now in the paper the authors provide an excellent overview of the controlling pathways. We provide a revised version of their pathway controls in a normal melanocyte below. This provides a description of the normal homeostatic pathways within a melanocyte.



From: Bauer and Stratakis

The LKB1 gene, also called STK11, which encodes a member of the serine/threonine kinase, regulates cell polarity and functions as a tumour suppressor. This is clearly demonstrated in the above. Now recall that mTOR is a protein kinase and is a key regulator of cell growth[6].

mTOR stimulates mRNA translation thus facilitating the conversion into proteins. mTOR also facilitates the formation of ribosomes which as an important condition of cell growth under specific physiological conditions. Through the effects of mTOR on the ribosome machinery it becomes a significant factor in increasing translational activity in a cell.

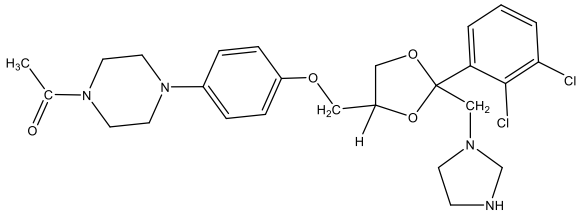
[6] See Marks et al pp 335-345.

3 KETOCONAZOLE

Ketoconazole is a therapeutic used for fungal infections. It can be used systemically or topically. Systemically it does have negative effects on the liver and should be closely monitored. Topically it still can be absorbed systemically and thus should be equally considered and monitored. Also ketoconazole is used in Cushing's disease systemically. In this section we present some basic facts about this therapeutic.

3.1 BASIC STRUCTURE

The basic structure of ketoconazole is shown below.



The specific mechanisms of interactions are not discussed herein.

3.2 MECHANISM

From NCBI[7]:

Ketoconazole works as an antifungal agent by inhibiting the cytochrome P450 14α-demethylase enzyme.

This enzyme is responsible for inhibiting the biosynthesis of triglycerides and phospholipids by fungi. More specifically, ketoconazole inhibits the synthesis of lanosterol, a necessary precursor for ergosterol biosynthesis. Ergosterol is needed to maintain the integrity of the membrane of fungi. Without ergosterol, the fluidity of the membrane increase, which in turn prevents fungal growth.

Ketoconazole, in high doses, can competitively bind to androgen receptors, such as that of testosterone and dihydrotestosterone, which can decrease the activity of testosterone and dihydrotestosterone in prostate cancer.

Ketoconazole can also inhibit the enzymes 17-alpha-hydroxylase and 17,20-lyase, which are necessary for the synthesis of steroids in the adrenal cortex, including testosterone. Ketoconazole inhibits the activity of the enzyme 21-hydroxylase. This enzyme is essential for synthesizing mineralocorticoids and glucocorticoids, such as cortisol, in the adrenal cortex. By

[7] https://www.ncbi.nlm.nih.gov/books/NBK559221/ also see https://pubchem.ncbi.nlm.nih.gov/compound/Nizoral

inhibiting enzymes involved in cortisol synthesis, ketoconazole can be a treatment option for Cushing syndrome.

3.3 ACTH ACTIONS

From Shirley:

Ketoconazole, an imidazole derivative, was originally developed and approved for use as an antifungal agent . In the early 1980s, following observations that ketoconazole was a potent inhibitor of steroidogenesis through its broad inhibition of cytochrome P450 (CYP) enzymes in the adrenal glands, it was hypothesised that ketoconazole may have a role in clinical situations where steroidogenesis inhibition is a therapeutic goal. One such situation is in the treatment of endogenous Cushing's syndrome, a rare but life-threatening condition resulting from cortisol hypersecretion.

In Cushing's syndrome, chronic hypercortisolism can result in a range of complications or co-morbidities, including hypertension, hypokalaemia, growth retardation in children and diabetes mellitus.

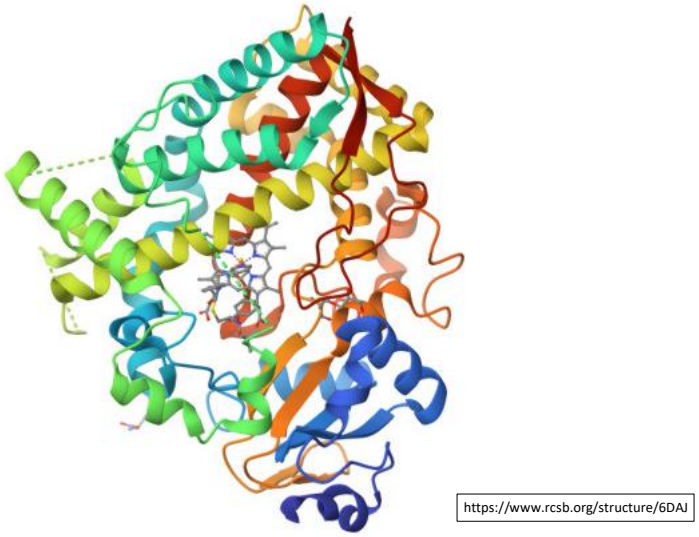
Endogenous Cushing's syndrome can have a range of aetiologies, broadly divided into adrenocorticotropic hormone (ACTH)-dependent disease [including pituitary corticotroph adenoma (Cushing's disease; ~ 60–70%) and extrapituitary or ectopic ACTH syndrome (EAS; ~ 5–10%)] and ACTH-independent disease (~ 20–30%). Although surgical resection of the underlying tumour is the recommended first-line treatment for Cushing's syndrome, medical therapies (including steroidogenesis inhibitors) also play a role in treatment, including when surgery is not possible, unsuccessful or contraindicated, or while awaiting the effect of radiation therapy of the pituitary gland.

4 P450

P450 is a class of enzymes that are key to metabolizing various factors such as drugs[8]. A small number of the P450 enzymes are involved in human drug metabolism. If the P450 enzyme for a specific target is encumbered in some manner then the results could be significant for the individual. A classic concern is the use of grapefruit for example.

4.1 CYP3A4 Overview

A significant one is CYP3a4. The CYP3a4 enzyme is shown graphically below. Note that at its center is a heme molecule, containing iron.



As NCBI notes[9]:

This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases that catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.

This protein localizes to the endoplasmic reticulum and its expression is induced by glucocorticoids and some pharmacological agents.

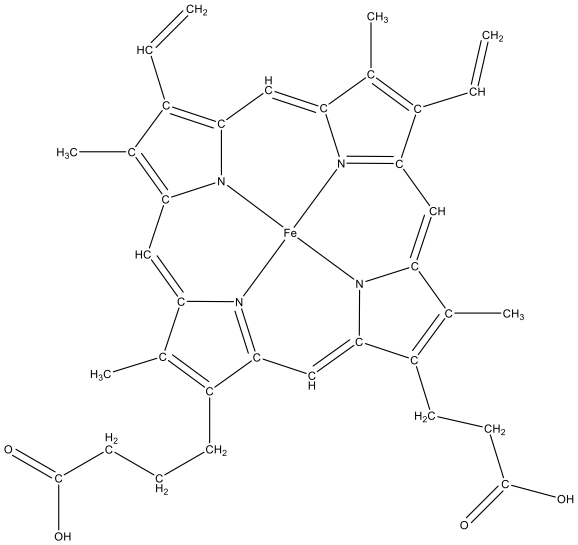
This enzyme is involved in the metabolism of approximately half the drugs in use today, *including acetaminophen, codeine, cyclosporin A, diazepam, erythromycin, and chloroquine.*

[8] See Brunton Chapter 6

[9] https://www.ncbi.nlm.nih.gov/gene/1576

The enzyme also metabolizes some steroids and carcinogens. *This gene is part of a cluster of cytochrome P450 genes on chromosome 7q21.1. Previously another CYP3A gene, CYP3A3, was thought to exist; however, it is now thought that this sequence represents a transcript variant of CYP3A4. Alternatively spliced transcript variants encoding different isoforms have been identified*

Now the heme element at the center of P450 enzymes is a critical factor in the enzymes effectiveness. Recall that heme is shown below. This binds itself to the CYP3a4 protein and become a key elements in the action as an enzyme.



4.2 P450 ACTIONS

An excellent summary of P450 enzymes in therapeutics has been prepared by Guengerich. The author notes:

The history of drug metabolism began in the 19th Century and developed slowly. In the mid-20th Century the relationship between drug metabolism and toxicity became appreciated, and the roles of cytochrome P450 enzymes began to be defined in the 1960s. Today we understand much about the metabolism of drugs and many aspects of safety assessment in the context of a relatively small number of human P450s.

P450s affect drug toxicity mainly by either reducing exposure to the parent molecule or, in some cases, by converting the drug into a toxic entity.

Some of the factors involved are enzyme induction, enzyme inhibition (both reversible and irreversible), and pharmacogenetics. Issues related to drug toxicity include drug–drug interactions, drug-food interactions, and the roles of chemical moieties of drug candidates in

drug discovery and development. The maturation of the field of P450 and drug toxicity has been facilitated by advances in analytical chemistry, computational capability, biochemistry and enzymology, and molecular and cell biology.

Problems still arise with P450s and drug toxicity in drug discovery and development, and in the pharmaceutical industry the interaction of scientists in medicinal chemistry, drug metabolism, and safety assessment is critical for success.

As Zhao et al note:

The CYPs are hemoproteins; embracing about 400–500 amino acids in their sequences and a single heme prosthetic group in the active site .

There now are 104 unique structures of CYPs that have been deposited in the Protein Data Bank (PDB), and this accumulating evidence suggests that the overall CYP folds are quite conservative. Members of the CYP family share about 40% sequence homology; with 55% sequence identity shared between subfamilies .

To date, nonheme proteins with CYPs folds have not been discovered, and a small handful of enzymes, including the CYP450nor , prostacyclin synthase , and allene oxide synthase , with CYPs folds do not catalyze traditional CYP chemistry. All CYPs involve a heme–iron center in the active site, tethered by a cysteine thiolate ligand localized in a characteristic FXXGXXXCXG element in their amino acid sequence. The shared tertiary structures usually include 12 common helices (A-L) and four common β-sheets. …

The closer to the heme, the more conserved is structure; especially helices I and L, which connect to the heme directly.

The most conserved elements of CYPs center on heme–thiolate oxygen activation chemistry, such as the β-bulge segment housing the Cys ligand.

Another highly conserved region involved in O2 activation is the portion of helix I near the heme. An outstanding structural characteristic of CYPs is their ability to adapt to substrates of various sizes and shapes. Most of our detailed understanding of CYP–substrate interaction derives from highly specific CYPs that bind to their respective substrates tightly. The size and shape of the various substrates for CYPs are fairly diverse. Substrates usually enter the active site near the connection between the F and G helices, which is the main entry point for substrates in many CYPs.

The structural changes of regions including F and G helices in CYPs may be responsible for the requirement for substrate specificity . CYP101 and cytochrome P450epoK represent the two extremes of substrate size and shape. Some of the most different regions when comparing these two enzymes are the F, G, and B' helices. The B' helix is rotated 90◦ in cytochrome P450epoK compared to CYP101. This reorientation opens the substrate-binding pockets, making room for specific regions of the substrates

As Zhao et al note:

Drug metabolism is the process of altering their molecules chemically after entering the body . In general, the metabolism of drugs decreases their therapeutic effects . The majority of drugs lipophilic centers are converted to hydrophilic centers during drug biotransformation, which can increase their water solubility, to allow elimination in urine or bile . This is an important progress for drug metabolism, because the lipophilic nature of drugs can keep them staying for longer in the body, which may in turn lead to toxicity.

Drug metabolism can be divided into phase I and phase II reactions . Figure 1 shows the known generalized pathways associated with drug metabolism catalyzed by cytochrome P450 (CYP) enzymes. Phase I reactions introduce reactive or polar groups (-OH, -COOH, -NH2, -SH, etc.) into drugs, including oxidation, reduction, and hydrolysis, where drugs cannot be excreted from bodies. The modified drugs are then conjugated to polar compounds in phase II reactions, which are catalyzed by a variety of transferase enzymes, such as uridine diphosphate (UDP)-glucuronosyltransferases, sulfotransferases, and glutathione S-transferases .

The conjugated drugs may be further processed, before being recognized by efflux transporters and pumped out of cells. However, the same metabolic process can also lead to the generation of reactive metabolites, which are toxic to the human body. This is termed the bioactivation of drugs, which depends specifically on important structural feature present in these compounds. Drug metabolism is the metabolic breakdown of drugs through specialized enzymatic systems . CYPs are involved in more than 90% of the reported enzymatic reactions .

Regarding drug metabolism, CYPs are the most well-known drug-metabolizing enzymes and are mainly expressed in the liver , but other organs are also involved: kidney, placenta, adrenal gland, gastrointestinal tract, and skin .

Among the 57 putatively functional human CYPs, the isoforms belonging to the CYP1, 2, and 3 families are mainly responsible for the metabolism of about 80% of clinical drugs .

CYP-mediated drug metabolism not only converts lipophilic products into hydrophilic products to facilitate elimination, but also plays a critical role in determining treatment outcomes, by influencing drug action, safety, bioavailability, and drug resistance, through the metabolism in both metabolic organs and local sites of action . CYPs, as the most diverse catalysts known in biochemistry, contribute to interindividual variations in drug responses, resulting from genetic and epigenetic variants, as well as environmental factors, such as gender, age, nutriture, disease states, and pathophysiological factors .

In particular, CYPs can be inhibited or induced by concomitant drugs and circulating metabolites, which can influence treatment outcomes through drug–drug interaction (DDI), drug–gene interaction (DGI), and drug–drug–gene interaction (DDGI)

4.3 P450 AND DRUG INTERACTIONS

P450 enzymes have a significant influence on drugs. As Zhang et al noted:

Antifungal imidazole derivatives are frequently used both systemically and topically (depending on the particular agent) in the treatment of systemic candidal infections and mycoses. These derivatives, ***including ketoconazole (KET),*** *miconazole (MIC), tioconazole (TIO), clotrimazole (CLO), and sulconazole (SUL), are* ***recognized as potent ligands of the heme iron atom of P450s***

The heme ligand plays a key role. It is suspected that ketoconazole may break down P450, a CYP, and release the iron element of the heme and thus part of the pigmentation may be iron enrichment.

The interactions of antifungal imidazole derivatives with P450 enzymes have been studied to an extent, with information on all of the major P450s and some newer antifungals lacking. KET is frequently used as a CYP3A-selective inhibitor in in vitro P450 identification studies. KET has shown to be selective up to 10 times its Ki value in human liver microsomes. …

In keeping with the lack of CYP3A selectivity of CLO, MIC has been shown to strongly inhibit both CYP3A and CYP2A6 in vitro.

Moreover, in vivo both fluconazole and MIC have shown to potently inhibit CYP2C9, as demonstrated by clinically significant drug interactions observed in the presence of concomitant CYP2C9 substrates, including warfarin and phenytoin.

Our observation that KET is capable of inhibiting CYP3A4 and to a lesser extent 2C9 is in agreement with previous studies. CYP1A1 was not investigated in the present study because of its general low involvement in therapeutic drug metabolism. In addition to previous studies demonstrating inhibition of CYP3A4 and 2A6, CLO was shown to potently inhibit CYP2C19. The coadministration of CLO with low therapeutic index compounds in which clearance primarily depends on CYP2C19 may thus be potentially hazardous. Previous studies showed inhibitory interactions between MIC and CYP3A4, 2A6, and 2C9 in vitro and in vivo. Our data show that in addition to these, MIC potently inhibits CYP2C19, 2D6, 2B6, and less so CYP1A2 in vitro in cDNA-expressing microsomes.

Therefore, one may expect significant in vivo inhibition of several of the major P450s, posing potential drug interactions with a number of drugs metabolized by these enzymes.

4.4 CYP3A SUBFAMILY

The CYP3a family is the dominant one in humans. As Klyushova et al note:

CYP3A is an enzyme subfamily in the cytochrome P450 (CYP) superfamily and includes isoforms CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A enzymes are indiscriminate toward substrates and are unique in that these enzymes metabolize both endogenous compounds and diverse xenobiotics (including drugs); almost the only common characteristic of these compounds is lipophilicity and a relatively large molecular weight.

CYP3A enzymes are widely expressed in human organs and tissues, and consequences of these enzymes' activities play a major role both in normal regulation of physiological levels of endogenous compounds and in various pathological conditions. ...

The CYP3A subfamily is affiliated with the cytochrome P450 (CYP) superfamily, which represents monooxygenases that catalyze the breakdown of various substances via hydroxylation and epoxidation with the participation of an electron donor (NADPH) and molecular oxygen . CYP enzymes function as the first line of defense against exogenous chemical agents . CYP enzymes are responsible for approximately three-quarters of all drug metabolism reactions in the human body.

CYP enzymes are involved in many critical metabolic reactions, including the metabolism of steroid hormones, bile acids, polyunsaturated fatty acids, leukotrienes, and eicosanoids . Genes of CYP enzymes have been found in the genetic material of representatives of all kingdoms of living organisms, including plants. There are 57 known functional CYP genes in the human genome, aside from 58 pseudogenes whose protein products are enzymes metabolizing a wide range of endogenous and exogenous chemical compounds. The genes of CYP enzymes are categorized into 18 families and 43 subfamilies based on the percentage of amino acid sequence homology.

Just 3 families—CYP2, CYP3, and CYP4—contain more genes than the other 15 families combined.

The human CYP3 family consists of a single subfamily, CYP3A, which contains four genes (CYP3A4, CYP3A5, CYP3A7, and CYP3A43) encoding four functional enzymes. CYP3A is a major subfamily in the cytochrome P450 superfamily. CYP3A enzymes are involved in the metabolism of more than 30% and according to other reports 45–60% of all pharmaceutical drugs currently on the market.

CYP3A enzymes also metabolize some endogenous substrates, including hormones and bile acids, as well as nonpharmaceutical xenobiotics. Expression of CYP3A enzymes is regulated and varies under the influence of various exogenous (drugs, chemicals, and diets) and endogenous factors (fatty acids, hormones, cytokines, and microRNAs [miRs or miRNAs]) ...

CYP3A enzymes play an important part in hormonal homeostasis. With regard to steroid hormones, the CYP3A subfamily plays an important role in the metabolism of androgens (testosterone, androstenedione, dehydroepiandrosterone, and dihydrotestosterone), progesterone, cortisol, and their metabolites.

CYP3A metabolizes testosterone, progesterone, and cortisol via the 6β-hydroxylation reaction unique to this P450 subfamily.

The highest 6β-hydroxylation activity is observed in CYP3A4, followed by CYP3A5 and CYP3A7. In this context, testosterone stimulates progesterone 6β-hydroxylation, whereas progesterone inhibits CYP3A-mediated 6β-hydroxylation of testosterone …

Extrahepatic CYP3A4 is more often responsible for the metabolism of hormones in situ, for example, it takes part in irreversible oxidation of testosterone in the prostate, thereby terminating its androgenic effect . …

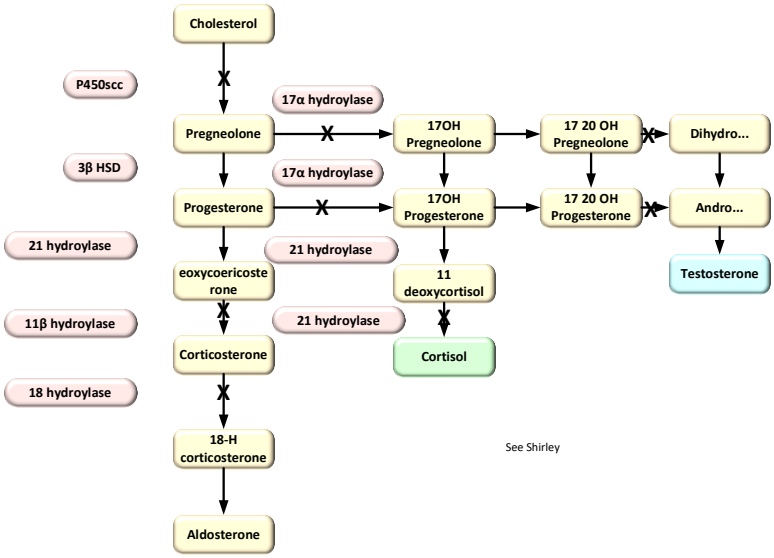
The last observation is of great significance. CYP3a4 is extrahepatic, namely in cells beyond the liver, and in our analysis this most likely includes the cells in the oral mucosa. Again, there does not appear to be any direct evidence of this currently.

5 INTERACTIONS

We now consider the multiple iterations involved in this process. Let us first look at how cortisol is produced and the impact of P450, namely CYP3a4, is involved.

5.1 FLOW OF ENZYME CONTROL

From Shirley, we have the following linked elements. Cholesterol progresses catalyzed by CYP3a4, a P450. It then controls the process whereby cortisol is produced in the adrenal gland. Ketoconazole blockage is shown by the X marks the blockage in the flows.



First, note that P450 is blocking the cholesterol conversion.

Second, pregneolone is then also blocked inhibiting the cortisol path

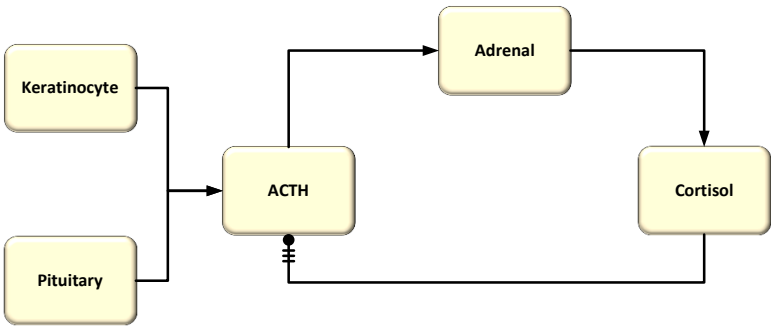
Third, progesterone is also blocked on the cortisol path.

Finally, the conversion of 11 deoxycortisol is blocked again in the cortisol path.

Thus cortisol is inhibited and the negative feedback controlling ACTH is blocked. This will be a critical factor. ACTH can rise thus driving melanin production. We now provide control pathways demonstrating this effect.

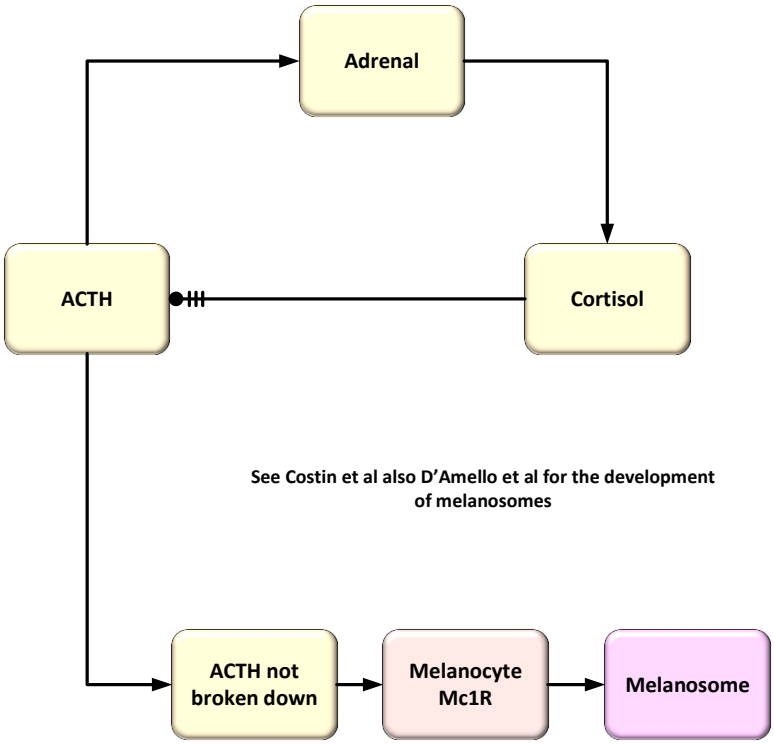
5.2 STEP 1: ORGAN FLOW

We can now begin to lay out the process. Step 1 is below. This is the classic control loop. Cortisol blocks ACTH. A simple loop.



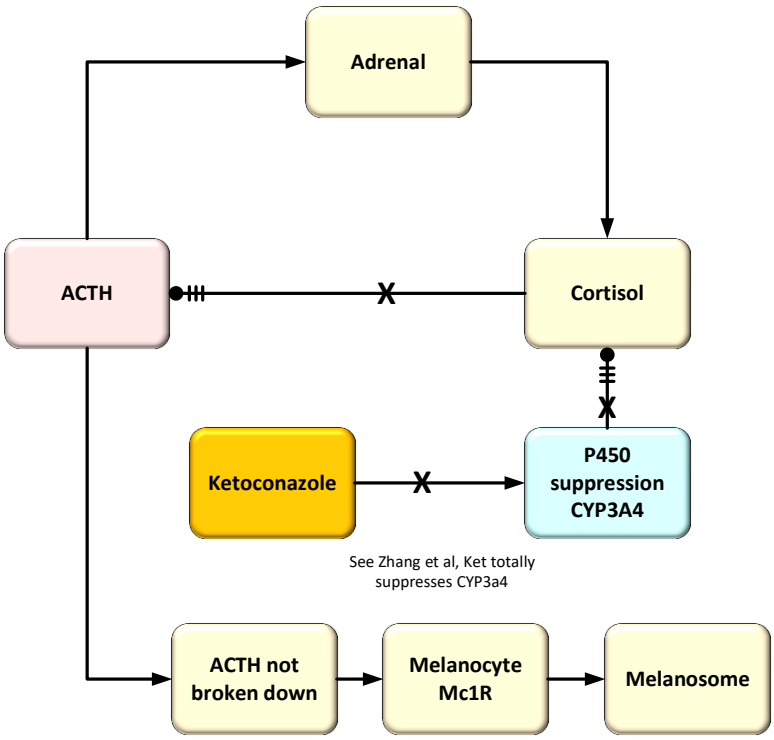
5.3 Step 2 Melanocyte and Melanin

If ACTH is not broken down but active it the drives the receptor MC1R and in turn drives the melanosome to produce melanin. We have shown that process above. The network describing this is below.



5.4 Step 3 Ketoconazole Control

Now with ketoconazole we have a blockage. It blocks several paths, one being the cortisol to ACTH control path allowing the persistence of ACTH. Thus excess ACTH generates the impact on melanocytes and likely other cells and melanocytic paths via the MC1R receptor.



It is not known to the author if this pathway has been examined previously. However it is a useful and simple example of a systems approach to genomics of diseases. The above process with ketoconazole is simply:

1. Ketoconazole blocks P450

2. Blocked P450 disables the cortisol flow

3. Disabled cortisol disable the negative feedback on ACTH

4. ACTH then is produced in an excess amount.

It should be remembered that keratinocytes also produce ACTH. It is not clear how that control mechanism can be fully incorporated.

6 OBSERVATIONS

We can make several observations resulting from this simple analysis.

6.1 HOW CORRECT IS THIS SYSTEM MODEL?

We have tried to create a model to account for the production of melanin resulting from the use of ketoconazole. Each step has experimental validation yet we have connected the steps in a logical yet unproven manner.

6.2 WHY DO PIGMENTS LOCALIZE IN A DELIMITED MANNER?

Lesions seem to be spatially delimited. Other than Addisons systemic result, the ones we examine herein are localized. What then is the process of delimitation and further localization?

6.3 WHAT ARE POSSIBLE THERAPEUTIC TARGETS?

If this type of lesion is to be delimited and reduced we have some possible targets such as MC1R. Also perhaps ACTH reduction.

6.4 ARE THERE SEQUELLAE THAT MAY RESULT?

ACTH modulation has extremes such as Addisons and Cushing. Yet what other are there?

6.5 IS ACTH THE ONLY TARGET INITIATOR?

Is ACTH the sole driver? There are clearly many other possibilities.

6.6 CAN REDUCTION IN ACTH BE PRODUCTIVE?

This is worth a try[10]. Newfield notes:

It is hypothesized that blocking the adrenocorticotrophic hormone (ACTH) receptor, using either a blocking antibody or a drug will result in a medical cortical-adrenalectomy, with relative sparing of mineralocorticoid hormone production. This would be similar to the clinical findings in familial glucocorticoid deficiency type 1, an autosomal recessive condition due to inactivating mutations of the adrenal receptor for ACTH, also known as the melanocortin 2 receptor (MC2R). It is further hypothesized that MC2R blockade should allow using lower glucocorticoid doses to treat congenital adrenal hyperplasia (CAH) due to enzyme deficiency of either 21-hydroxylase (CYP21B) or 11-hydroxylase (CYP11B1), thus reaching a better final adult height than with current therapeutic strategies. Blocking the ACTH receptor can also be employed to medically treat Cushing's due to excess pituitary or ectopic ACTH production.

Actual clinical experience in oral mucosa is lacking.

[10] See Newfield, ACTH receptor blockade: a novel approach to treat congenital adrenal hyperplasia, or Cushing's disease, Med Hypotheses, . 2010 Apr;74(4):705-6

6.7 WHAT SITE (CELLS) IS THE MAJOR SOURCE OF ACTH?

Clearly adrenal glands dominate and we see keratinocytes. But ACTH impacts melanocytes. How strong that impact is relative to the adrenal gland is not clear.

6.8 IS CYP3A4 THE PRIMARY P450 ENZYME AND IF NOT WHAT OTHERS?

As we have noted there are many P45 enzymes and many CYP3 particularly.

6.9 WHAT HAPPENS WITH BLOCKING MC1R?

This is not clear. One could develop a monoclonal antibody (MAb) approach and examine its impact.

6.10 WHAT ARE THE SYSTEMATIC EFFECTS?

Our discussions have focused on localized delimited lesions. Ketoconazole has its own system effects which are primarily hepatic.

6.11 THERE ARE TEMPORAL EFFECTS POSSIBLE IN THIS SPECIFIC LESION.

We know that under UV activation skin melanosomes are produced and result in a tan[11]. Remove the UV stimulation and the tan disappears, namely the melanosomes are removed, via phagocytosis. Now in the care of ketoconazole induced melanin induction, if the ketoconazole is removed, will we see a similar effect. There have been studies regarding melanin generation and some results on its degradation.

The work of Diaz et al is useful for generation by UV sources. As noted in Science News[12]:

A protein called MITF coordinates skin-darkening melanin production with other skin protection mechanisms in response to UV light, molecular geneticist Carmit Levy of Tel Aviv University and her colleagues discovered.

The team shone UV-B light on mice every 24, 48 or 72 hours for 60 days. Mice exposed to UV-B radiation on the 48-hour schedule developed darker skin and had less DNA damage than mice in the other groups. Mouse and human skin cells grown in lab dishes that were exposed to UV light every other day also made more melanin than cells that were irradiated daily.

Other experiments with skin cells in dishes suggest that within minutes of UV exposure, MITF turns on genes involved in skin cell survival. Those genes make proteins engaged in inflammation, DNA repair and recruitment of immune cells to the skin. Only later does MITF

[11] See Guo et al.

[12] https://www.sciencenews.org/article/get-deeper-tan-dont-sunbathe-every-day

give the OK for melanin production to begin. Hitting cells with daily UV interrupts melanin production, leaving skin without its protective shield, the researchers found.

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