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Summary and general discussion

This thesis is composed of two parts. In the first part the focus lies on glucocorticoids. Glucocorticoids have been used worldwide for more than half a century. This would suggest that it is a safe drug to use but this is not the case. Soon after it was introduced in the clinic it became apparent that its use is accompanied by adverse effects. Sixty years later the drug is still first line treatment for many inflammatory conditions. In this thesis a new mechanism of glucocorticoid action is described which includes effects on T cell activation that does not include the regulation of genes. We found that this mechanism is important to the overall effect of glucocorticoid treatment and used it to develop a screening assay with the intention of finding a glucocorticoid ligand that induces exclusively non-transcriptional effects, possibly providing a safe alternative to conventional glucocorticoids. Because only a limited number of these non-transcriptional effects of GC treatment have been described, it is certainly possible that some of the adverse effects are also the result of non-transcriptional effects. The two compounds S3.1 and S3.4 described in chapter 7 are certainly not intended as a final product. These compounds were used to develop a pharmacophore, a chemical model describing what properties a molecule needs to exhibit to behave similarly. From this model more compounds were successfully developed. Work on these compounds is in progress.

The second part of this thesis describes the use of an existing cytostatic drug for immunosuppression. Interestingly this drug was developed for the treatment of cancer but came from a screening for anti-inflammatory drugs. As a cytostatic drug it wasn't very successful but it got a lot of attention when it was found that it could be used to cure visceral leishmaniasis. Further research on the mechanism of action resulted in studies that showed a potential for immune suppression. We too describe successful use of miltefosine for the treatment of chronic inflammation of the colon using a transfer mouse model. Miltefosine knows very few adverse effects and is a cheap drug that can be taken orally. However, it can cause nausea resulting in reduced appetite and emesis. Although we feel that the transfer model compares best to actual inflammatory conditions, it remains to be a mouse model and may not reflect IBD in humans well enough.

Chapter 1

The first chapter is a simple introduction to glucocorticoids (GCs) in general. Mentioned are the advantages and disadvantages of glucocorticoid treatment, outlining the general problem of developing save GCs.

Chapter 2

Glucocorticoid analogues have been around for more than half a century. Just like so many other drugs, GCs were not developed with the intention to suppress inflammation. Isolation, characterization and further development were fueled by war time rumors. Afraid the enemy would gain an advantage over them, allied forces initiated large scale research which eventually led to post-war development of GCs. As the rumors turned out to be false, a real purpose for the drug was lacking. Justification for the major investment led to the idea of dividing the few milligram GC available amongst lead scientists. Eventually it was Philip S. Hench who first tested the drug on rheumatoid arthritis patients and discovered its anti-inflammatory effect.

Chapter 3

The major downside of glucocorticoids as a drug is the induction of a great number of adverse effects. If not for these, the drug would be almost ideal. Most adverse effects have few consequences for the long term and can in general be ignored. But with higher concentrations and longer periods of treatment, the severity and the number of complications increase. If development of a GC analogue lacking adverse effects turns out to be impossible, it may be of great importance to understand how GCs cause these adverse effects on a very basic level. This may eventually provide a way to suppress adverse effects by concomitant treatment targeting the underlying cause.

In the last decades biomedical science has benefitted greatly from the development of new methods in the various fields such as genetics and proteomics. With it our understanding of GC signaling and how adverse effects are caused, has increased. This chapter is a broad overview of our current understanding of the underlying molecular mechanism responsible for GC side effects. The different side effects are addressed separately, focusing on the common major adverse effects associated with GC treatment. These include obesity, thinning of the skin and appearance of striae, muscle atrophy, osteoporosis, diabetes, hypertension, cataract and

glaucoma and finally effects on the central nervous system. In general, tissues in which GCs are part of the normal homeostasis are effected most by addition of exogenous GCs.

Chapter 4

From the first moment they entered the clinic, GCs have been remodeled to improve their properties, resulting in a whole range of GC analogues. Initially steps aimed for more potent anti-inflammatory properties by increasing half-life, followed by decrease of off-target effects by selecting for a higher affinity for the GR and lower affinity for other nuclear hormone receptors such as the mineralocorticoid receptor. Although this approach did provide GC analogues with more potent anti-inflammatory properties and a reduction in off-target effects originating from activation of other nuclear hormone receptors, it also increased adverse effects that arise from GR related transcriptional regulation of genes that are not involved in inflammation. In the last decades we have started to unravel how GCs work. And while we learn our notion of what to take into account while developing new GCs changes. Not so long ago it was believed that GCs repress pro-inflammatory genes while activating genes responsible for adverse effects and that this could be exploited to develop safe GCs. This chapter is a brief overview of what is currently known about GC action and what should be considered when developing GCs with more favorable properties.

Chapter 5

Classically GCs act through the regulation of genes. This mechanism requires a certain amount of time before actual changes can be noticed. However, it's not uncommon for GC effects to appear after a much briefer stretch of time. Patients suffering from osteoarthritis can be treated palliatively by injecting GCs directly into the affected joint. The pain relief that follows is almost instantaneously. This is just one example of GC effects that are too fast to involve regulation of genes. And because these effects do not include transcriptional regulation, they are termed non-transcriptional or non-genomic GC effects.

This chapter addresses the rapid effects of GC treatment on T helper cells and more specifically on a complex of proteins that is associated with the T cell receptor (TCR). In a previous study[1] the kinome profile of T helper cells, activated and treated with GCs, was assessed using a kinome array

(PepChip). The results indicated that two kinases, LCK and FYN, are inhibited by a short GC treatment of 10 minutes. These kinases, LCK in particular, are the first messengers to transfer a signal from the activated TCR. LCK is vital to T cell activation. T cells lacking this kinase cannot be activated through the TCR[2].

This chapter describes how, after stimulation of T cells, a complex is formed at the TCR. In this complex LCK associates with CD4, and FYN associates CD3. Both are associated with the chaperone protein Hsp90 and the GR. When stimulated T cells are treated with GC, all of these associations are lost. From this a model is proposed in which the GR forms an essential part of the TCR related complex. When the GR binds ligand, it breaks it bonds with the other proteins an leaves the complex, thereby destabilizing it causing all associations to be lost and the complex to break up. As a result TCR signaling is abrogated.

Chapter 6

Another aspect of GCs, besides the adverse effects, less desirable in a drug, is loss of sensitivity or even total loss of response. This group of patients can be subdivided into separate groups based on the underlying mechanism responsible for said loss. Mechanisms include increased clearance of GCs, increased pro-inflammatory transcription factors, altered posttranslational modifications to the GR, defective histone acetylation and finally overexpression of the dominant negative beta form of the GR. At least *in vitro* it is possible to induce steroid insensitivity in T cells by activation with super antigens. This chapter describes how the superantigen staphylococcal enterotoxin B (SEB) activates T lymphocytes through stimulation of a signaling cascade that deviates from the normal TCR-LCK mediated transduction pathway. This pathway leads to activation of T cells without the need for LCK activation. It includes a guanine nucleotide-binding protein Gαq and the phospholipase PLCβ2 and converges with the canonical TCR signaling cascade at the level of PKC. Stimulation with SEB triggers T cell proliferation that is less sensitive to GC treatment than with a normal mitogen. Direct activation of PKC with the phorbol ester PMA in combination with the ionophore ionomycin induces proliferation that is completely resistant to GC treatment. Taken together this suggests that the inhibitory effect of GC treatment on T cell proliferation is primarily a non-genomic effect on LCK and associated proteins. This places the relevance of non-genomic GC effects for the

overall anti-inflammatory effect of GC treatment in a whole new perspective.

Chapter 7

Inhibition of T cell proliferation is one of the primary features of the anti-inflammatory effect of GC treatment. The findings in the previous chapter suggest that this is based on a non-transcriptional effect. This chapter describes how through a combination of *in silico* and *in vitro* screening two GR ligands were discovered that do not induce transcriptional effects but do cause dissociation of the TCR associated molecules leading to inhibition of T cell proliferation.

For the screening, potential GR ligands were selected from a public database of commercially available compounds. Selection was based on similarity to the basic cortisol structure or gonane. This resulted in a quarter of a million potential candidates which in a second round of screening were fitted to a virtual GR binding pocket in four different known conformations. A selection was made of the compounds that fitted best and 80 of these were purchased. Each compound was then tested for its capacity to inhibit T cell proliferation. Using a cell line stably transfected with a glucocorticoid responsive element (GRE) reporter construct, compounds that induced transcriptional activation were excluded. Applying this strategy two compounds, S3.1 and S3.4, were identified that repress T cell proliferation with a potential comparable to prednisolone. Further examination of these compounds revealed an inhibition of TCR related signaling similar to the non-genomic effect of dexamethasone. But unlike dexamethasone these compounds did not induce adipogenesis or muscle atrophy, as was assessed through *in vitro* assays.

Chapter 8

In search for a PLCβ inhibitor (Chapter 5) we came across a drug called miltefosine. Indeed as had been previously reported[3], miltefosine inhibits PLCβ but not very specifically. We did however notice a very strong inhibitory effect on T cell proliferation and wondered if miltefosine could be used as an anti-inflammatory drug. In inflammatory bowel diseases (IBD) T lymphocytes play an important role in the underlying cause[4]. Using a mouse $CD4^+$ transfer model we tested miltefosine as a means to suppress chronic inflammation. The SCID mice in this model lack T and B cells and replacement with CD4 effector cells only, results in the induction

of chronic inflammation of the colon. The level of inflammation was assessed by colon weight and length, stool consistency, spleen weight, pathology score, influx of macrophages, neutrophils and T cells and the presence of cytokines. Oral treatment with miltefosine reduced inflammation significantly.

Chapter 9

Miltefosine (or hexadecylphosphocholine) was originally intended for the treatment of cancer but failed in most of the clinical trials due to lack of significant improvement of the patient's condition. In the end it was only effective as a topical treatment for skin metastasis of patients with breast cancer. But just as the drug was threatened to be forgotten completely, it was discovered that miltefosine was an excellent treatment for patients suffering from visceral Leishmaniasis. This parasitic disease is relatively unknown to people in the western part of the world because it only affects the economically less developed areas near the equator. This is because Leishmaniasis is transmitted through a type of sandfly that is only indigenous to those particular areas. Nevertheless, Leishmaniasis is, next to malaria, the second biggest parasitic killer worldwide with an estimated 25.000 fatalities each year. Although an effective treatment existed, it required a trained physician to administer the drug. Miltefosine can be taken orally, has few adverse effects and is relatively cheap.

This chapter mainly focusses on what has been published on miltefosine in relation to suppression of the immune systems and cells belonging to the immune system. These publications can be broadly divided into three separate groups. Effects on macrophages, effects on T cells and effects on mast cells. This data is used to build a case in favor of testing miltefosine as an immunosuppressive drug. And finally the underlying molecular mechanism is discussed based on what is currently known about how miltefosine affects cells.

References

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