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Nephrotoxic Nephritis (NTN): Possible new therapeutic strategy using DHMEQ, a nuclear uptake inhibitor of NF-kappa B

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Dedicated to my late Parents

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Abbreviation index

fig.	Figure
BSA	Bovine serum album
BCIP	5 - Bromo - 4 - chloro - 3 – indolyl phosphate
bp	base pair
°C	degree of centigrade
cDNA	copy DNA
D, kD	Dalton, kilo-dalton
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
D-MEM	Dulbecco`s modified Eagle medium
DMF	N, N´ - Dimetylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreithol
ECL	*Enhanced Chemoluminescence*
EDTA	Ethylenediaminetetraacetic acid
ELISA	*Enzyme linked immunosorbent assay*
FCS	Fetal calf serum
FSGS	Focal segmental glomerulosclerosis
FITC	Fluorescein isothiocyanate
g	gram
g_o	Gravity / gravity, universal constant, represents the earth attractive force. The relative centrifugal force (rcf / relative centrifugal force / g force) is indicated in this work as multiple of g (x g).
GBM	glomerular basement membrane
GN	glomerulonephritis
Gen	genomic
HEPES	4 (2 - Hydroxyethyl) - 1 – piperazinethansulfic acid
IFN	Interferon
Ig	Immunoglobulin
IgG	Immunoglobulin of the class G
IgM	Immunoglobulin of the class M

IgE	Immunoglobulin of the class E
kg	kilogram (x 10^{3})
L	Liter
LDS	Lithium dodecyl sulfate
LPS	Lipopolysaccharide
m	milli (x 10^{-3})
M	Molarity
MES	Morpholinoethanol sulfonic acid
min	Minute(s)
MMLV	*moloney murine leukemia virus*;
MOPS	Morpholinopropanol sulfonic acid
mRNA	messenger RNA
n	nano (x 10^{-9})
NBT	4 – Nitro blue tetrazolium chloride
PAN	Puromycin aminonucleoside Nephritis
PAGE	Polyacrylamide Gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
s	second
Tab.	Table
TAE	Tris - acetate - EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline + Tween 20
TEMED	N,N,N,N-Tetramethylethylenediamine
TGF	Transforming growth factor
Tris	Tris - (hydroxymethyl) - aminomethan
Tween	20 Polyoxyethylenesorbitanmonolaurate
µ	micro (x 10^{-6})
U	Unit
rpm	revolutions per minute

Working Hypothesis and Problem:

Inflammatory glomerular kidney diseases (glomerulonephritis) are causing, in spite of some progress in the therapy, still in about 20-30% of the cases end stage renal disease. Understanding the pathogenesis of inflammatory diseases persistently improved further in the past years through following reasons among others:

By describing the role of transcription factors, that all ahead of NF kappa B, with that induction of inflammatory diseases occurs and

By showing, that NF kappa B has also a decisive importance in the resolution phase of inflammation processes.

The team was able to publish recently that it comes after induction of experimental kidney diseases into a two phase activation of NF kappa B. In the first peak, the p65/p50 heterodimer protein complex dominates, in the second peak the p50/p50 homodimers. Goal of the graduation work therefore is to examine the effect of the inhibition of the nuclear reception of NF kappa B complexes by means of in vivo application of DHMEQ at different times after induction of the NTN in wild type mice.

Preface

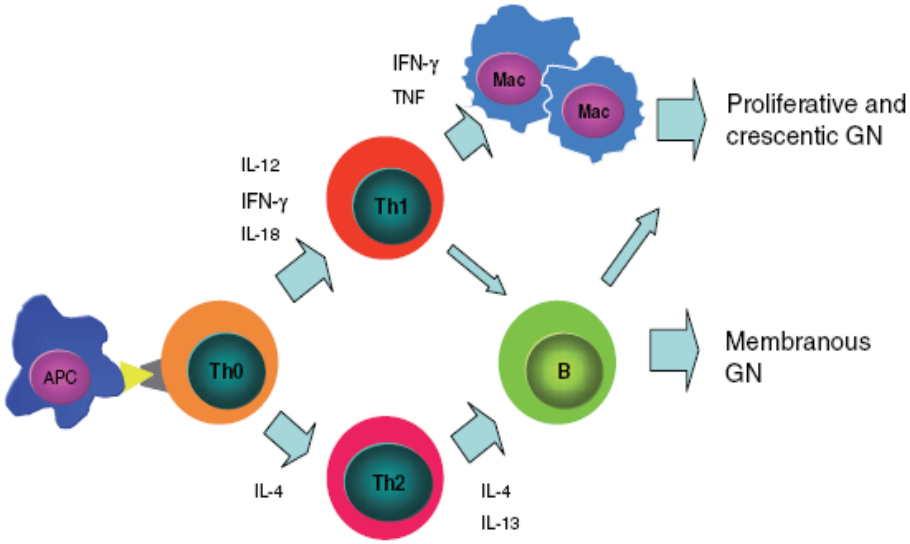
1.1. Glomerulonephritis (GN)

Glomerulonephritis is one of the major causes of chronic renal disease worldwide. Despite the increasing rate of diabetic renal disease, glomerulonephritis still remains a major problem and cause of chronic renal disease and end-stage renal failure requiring dialysis and renal transplantation. It shows a wide spectrum of histological patterns with different severity of injury and clinical outcome, which may be related to the nature of the nephritogenic immune response (P.G. Tipping and A. R. Kitching. 2005).

Inflammation is the body's primary response to infection or injury and is critical for both innate and adaptive immunity. Upon induction of inflammation resident tissue cells produces a variety of cytokines, chemokines, lipid mediators and bioactive amines which lead to an infiltration of inflammatory cells. Primarily these cells are macrophages, dendritic cells, mast cells and lymphocytes (T. Lawrence and C. Fonga. 2010). There is evidence that in the majority of cases both humoral and cellular effectors mechanisms play a central role in both human and animal models of glomerulonephritis (P.G. Tipping and A. R. Kitching. 2005).

In addition to providing help for production of immunoglobulins, that are involved in humoral effector mechanisms, T cells plays an important role in directing cellular immune mechanisms in GN. Th1 and Th2 cells are different in function, by their cytokine profile and their ability to generate different types of immune effector responses. They both are subsets of primed CD4+ T helper cells. Th1 cells produce tumor necrosis factor (TNF), interleukin (IL)-2, interferon (IFN)-γ and lymphotoxin-α. They activate macrophage and delayed-type hypersensitivity (DTH) effector responses and at the same time help B cells to produce complement fixing antibody isotypes that mediate opsonization and phagocytosis. On the other hand Th2 cells produce interleukin (IL)-4, IL-5, IL-10 (in mice) and IL-13 that stimulate the production of non-complement fixing IgG isotypes and IgE. They are important for elimination of infection and mediation of allergic response. While Th1/Th2 subsets do have a clear influence on injury in experimental GN, the dissection of their role in human GN has proven difficult due to the diverse range of underlying immune pathogenic mechanisms in human disease (e.g. organ specific or systemic autoimmunity, innocent bystander injury in the context of antigen immune responses to planted antigen, probably immune dysregulation as in IgA

nephropathy). In addition to this there are some other factors, like diversity in effectors pathways involved and the variable defined genetic influences on immune responses in human populations. Despite these obstacles, there is evidence which proved that the



differential activation of Th1 and Th2 mechanism is useful in explaining of human GN (P.G. Tipping and A. R. Kitching. 2005) (*FIGURE1*).

Figure1: A simplified scheme of Th1 and Th2 subset and cytokine involvement in glomerulonephritis. APC: antigen-presenting cell; Mac: macrophage; source: (P.G. Tipping and A.R. Kitching. 2005)

There is increasing evidence, in which glomerular crescent formation results from an underlying or superimposed Th1 response in severe and rapidly progressive forms of GN. In previous studies the presence of effector CD4+ cells and macrophages in crescentic lesions, has been observed. Biopsies of a variety of different forms of proliferative GN shows higher levels of IL-2 and IFN-γ compared with non proliferative forms and glomerular CCR5 expression associated with crescentic GN. Studies in human anti- GBM GN demonstrated IFN-γ -predominant antigen-specific effector cell responses in active disease and IL-10 predominance in remission further supporting a role for Th1 in injury. It has also been demonstrated in these studies a central role for CD25+ regulatory cells in suppressing pathogenic IFN-γ production human anti-GBM GN (P.G. Tipping and A. R. Kitching. 2005). T cells responses are able to cause the classical four types of hypersensitivity immune reactions, while these immune reactions can target the kidney and cause distinct forms of glomerulonephritis. CD4+ T cells can induce glomerular immunopathology by producing cytokines, by activating effector cells like macrophage and by mediating auto antibodies or immune-complexes, whereas CD8+ T cell response and failure of regulatory T cells may

represent two additional types of anti-renal hypersensitivity. The role of dendritic cells DC was proven as protective in renal diseases, while it is essential for T cell activation (C. Kurts et al. 2007). Cells with dendritic morphology and major histocompatibility complex (MHC) class II expression were described in normal rat and mouse kidney, and they were able to stimulate T-cell responses in vitro (S. Segerer et al. 2007).

1.2. Nephrotoxic nephritis (NTN)

Nephrotoxic nephritis is one of the most widely studied T cell-dependent models of kidney injury, which results in immunopathology similar to human crescentic GN. It is induced in mice, rats, or rabbits by injection of heterologous nephrotoxic serum (NTS), mostly generated in sheep or rabbits vaccinated with murine or rat cortex glomerular basement membrane antigens. It has been demonstrated in seminal animal studies by Tipping and Holdsworth that CD4+ Th1 cells play an important role in a type IV hypersensitivity reaction in murine NTN. Intravenously or intraperitoneally injected NTS predominantly binds to the glomerular basement membrane and is taken up and presented by antigen-presenting cells, presumably DCs to specific naive CD4+ T cells, causing their activation and differentiation into Th1 effectors cells. Depletion of CD4+ T cells attenuated NTN in mice and rats. Whereas CD8+ T cells depletion was protective only in rats but not in mice (C. Kurts et al. 2007).

Under the influence of TLR ligands IL-12 is secreted by DCs which is essential for murine NTN. After activation of T-cells, they are looking for their cognate antigen in non lymphoid tissues. Because of its specificity the planted antigen in the kidney is subsequently presented to CD4+ Th1 cells by local MHC II-expressing cells. In the kidney CD4+ T cells produced Th1 effector cytokines like IFNγ or tumor necrosis factor alpha (TNF-α), which at the same time stimulate DTH-like fashion resident macrophages and are essential effectors in NTN. Macrophages cause injury of Bowman's capsule and crescent formation by producing ROS and proinflammatory cytokines. In addition to this they also damage the basement membrane, which makes it easier for macrophage to escape from glomerluar capillaries (C. Kurts et al. 2007). The importance of Th1-associated cytokines has been shown in TNF-α or IL-12 deficient animals or animals after injecting Th2 cytokines such as IL-4. Besides this the hallmark Th1-chemokine receptor like CXCR3 was essential for immune pathology in NTN (C. Kurts et al. 2007).

However controversial results have been published on the role of Th1-associated cytokine IFNγ in NTN, where both aggravating and ameliorating effects were described showing that the role of IFN-γ is more complex than the simple Th1/Th2 dichotomy. One possible explanation from experiments on Th1 effector cells in various models is that IL-10 has been produced by Th1 effector cells as a co-product and it is one of the most potent immunosuppressive cytokines. Based on these results, it has been assumed that the cellular response and the co-production of further cytokines and chemokines are deciding to define whether INF-γ is protective or harmful (C. Kurts et al. 2007) *FIGURE2* (C. Kurts et al. 2007).

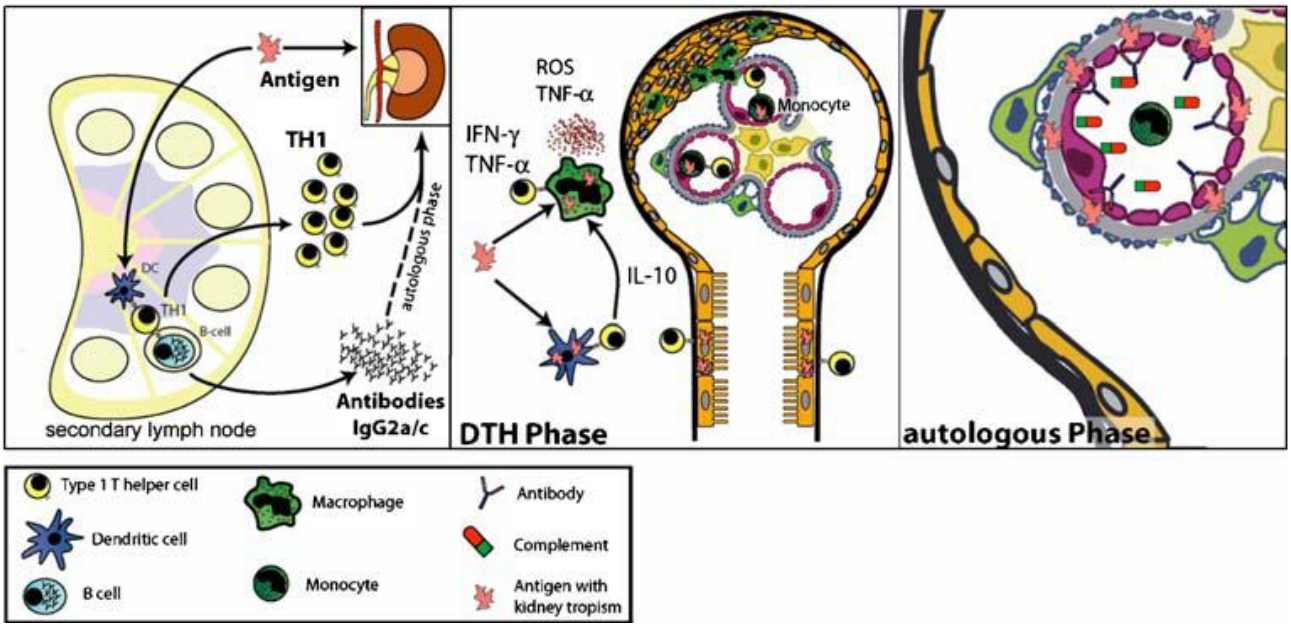


Figure2: Anti-renal macrophage-mediated delayed type hypersensitivity (type 4). Left panel DC in secondary lymphatics activate Th1 effector cells specific for an antigen with kidney tropism. Activated Th1 cells leave the node and travel via the bloodstream to the kidney. Th1 cells also induce antibody production, which will become relevant in the autologous phase illustrated in the right panel. Middle panel Tubulointerstitial and glomerular monocytes/macrophages, as well as intrinsic kidney cells, capture kidney-tropic antigen and present it to infiltrating CD4+ Th1 effector cells, which subsequently activate them, e.g., by IFNγ . Release of ROS and proinflammatory cytokine causes injury of Bowman's capsule and possibly in the glomerular basement membrane, resulting in compensatory crescentic cell growth. Membrane rupture may allow crescent infiltration by monocytes/macrophages, either from ruptured glomerular capillaries or from the interstitium. Dendritic cells represent a counter regulator and attenuate kidney disease, presumably by inducing IL-10 production. Left panel After the Th1 effector cell-mediated DTH phase, the autologous phase commences, in which specific antibodies bind to kidney-tropic antigen and induce additional type III hypersensitivity reactions (C. Kurts et al. 2007).

One of the master transcription factors in regulating T-cell response and cytokines and chemokines expression is NF-κB. Activation of NF-κB has been confirmed in a variety of inflammatory renal disease including NTN in several studies (T. Koska et al. 2008). Therefore

the focus of our studies was to elucidate the role of NF-κB in the NTN-model and to further define a possible therapeutic effect of DHMEQ on this model.

1.3. Nuclear factor-κB (NF-κB)

Nuclear factor-κB (NF-κB) was discovered as a nucleoprotein in 1986. It was bound to the enhancer region of the gene which encodes the immunoglobulin κ chain (Sen and Baltimore, 1986) (K. Umezawa and C. Chaicharoenpong 2002).

1.3.1. Structure of NF-κB

NF-κB is a dimer of members of the rel family of proteins. Each family member contains an N-terminal 300 amino acid, however the rel homology domain (RHD) known as conserved region. This is an important region and is responsible for DNA-binding, dimerization, and interaction with IκB family members. It also contains a nuclear localization sequence (S. Ghosh et al. 1998). It is one of the best characterized transcription factor. It has been shown that NF-κB typically exists as a heterodimer of p50 and p65 subunits (T. Suzuki et al. 2008). This heterodimer complex protein is what we commonly refer to as NF-κB, although the diversity of other members is also now known. The complex is been made up from the two rel proteins and each docking one half of the DNA binding site. A possibility of slightly variations in the 10 base pair consensus sequence, 5-GGGGYNNCCY-3, confer a preference for selected rel combinations of proteins and also has its own transactivating potential (S. Ghosh et al. 1998). The p65 and p50 proteins have a homologous sequence at their N-terminal regions and NF-κB can form various heterodimers or homodimers. There are more proteins such as p52, c-Rel, Rel B, v-Rel, Dorsal, Dif, and Relish that include to the NF-κB family (A. Ariga et al. 2002).

Although most of the NF-κB proteins are transcriptionally active there are some combinations considered as inactive or repressive complexes. Thus, p50/p65, p50/c-rel, p65/p65, and p65/c-rel are all transcriptionally active, whereas p50 homodimer and p52 homodimer are transcriptionally repressive. There is a lack of variable C-terminal domain in p50 and p52 found in the activating rel proteins, which is most likely responsible for transactivation of NF-κB-responsive genes (S. Ghosh et al. 1998).

1.3.2. Inducible activation of NF-κB

The transcription factor NF-κB has been the focus of intense investigation for more than two decades. Over this period of time, it has been made a considerable progress to understanding the function and regulation of NF-κB although there is still much to be learned in this important signaling pathway to understand (M. S. Hayden and S. Ghosh 2004). There is evidence that NF-κB plays an important role in both pro- and anti inflammation but the pathway depends on the cell lineage and pathophysiological context (T.Lawrence and C. Fonga 2010).

Nuclear factor-κB (NF-κB) is a well known transcriptional factor that induced expression of a variety of inflammatory genes and cell adhesion molecules such as TNF-α, interleukins, monocyte chemoattractant protein-1, ICAM-1 and VCAM-1 (T. Kosaka et al. 2008). It binds to the κB sequence and promotes transcription of cytokines such as IL-1, IL-2, IL-6, IL-8, TNF-α, and interferon-γ, cell adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM-1) and a vascular cell adhesion molecule 1 (VCAM-1), and a viral proteins (A. Ariga et al. 2002). However, there are significant differences in the precise sequence of different κB sites (F. Wan and M. J. Lenardo 2010). Activation of NF-κB is induced by extracellular signals such as TNF-α, IL-1, lipopolysaccharide, UV, and phorbol esters, in which the TNF-α receptor to NF-κB activation signal transduction pathways is well known and widely studied (A. Ariga et al. 2002). TNF-α is an apoptosis-inducing factor. However, in most cases usually TNF-α does not induce apoptosis, because at the same time it induces the activation of NF-κB, which inhibits the apoptosis (A. Ariga et al. 2002).

TNF-α receptors (TNFRs) include 55-kDa TNFR I and 75-kDa TNFR II. The ligand mainly uses TNFR-I to act, inducing its trimerization. TNFR-associated death domain protein recruits receptor-interacting protein and TNFR-associated factor-2 (TRAF-2) for activation of NF-κB. TRAF-2 activates MEKK-3 which is a mitogen-activated protein kinase. On the other hand MEKK-3 activates IκB kinase (IKK), which induces the phosphorylation of the inhibitor of NF-κB (IκB). Phosphorylation of IκB induces its ubiquitination and degradation by proteasomes. Liberated NF-κB molecules then enter the nucleus where they bind to the κB site of DNA (A. Ariga et al. 2002) *FIGURE*3 (K. Umezawa and C. Chaicharoenpong 2002).

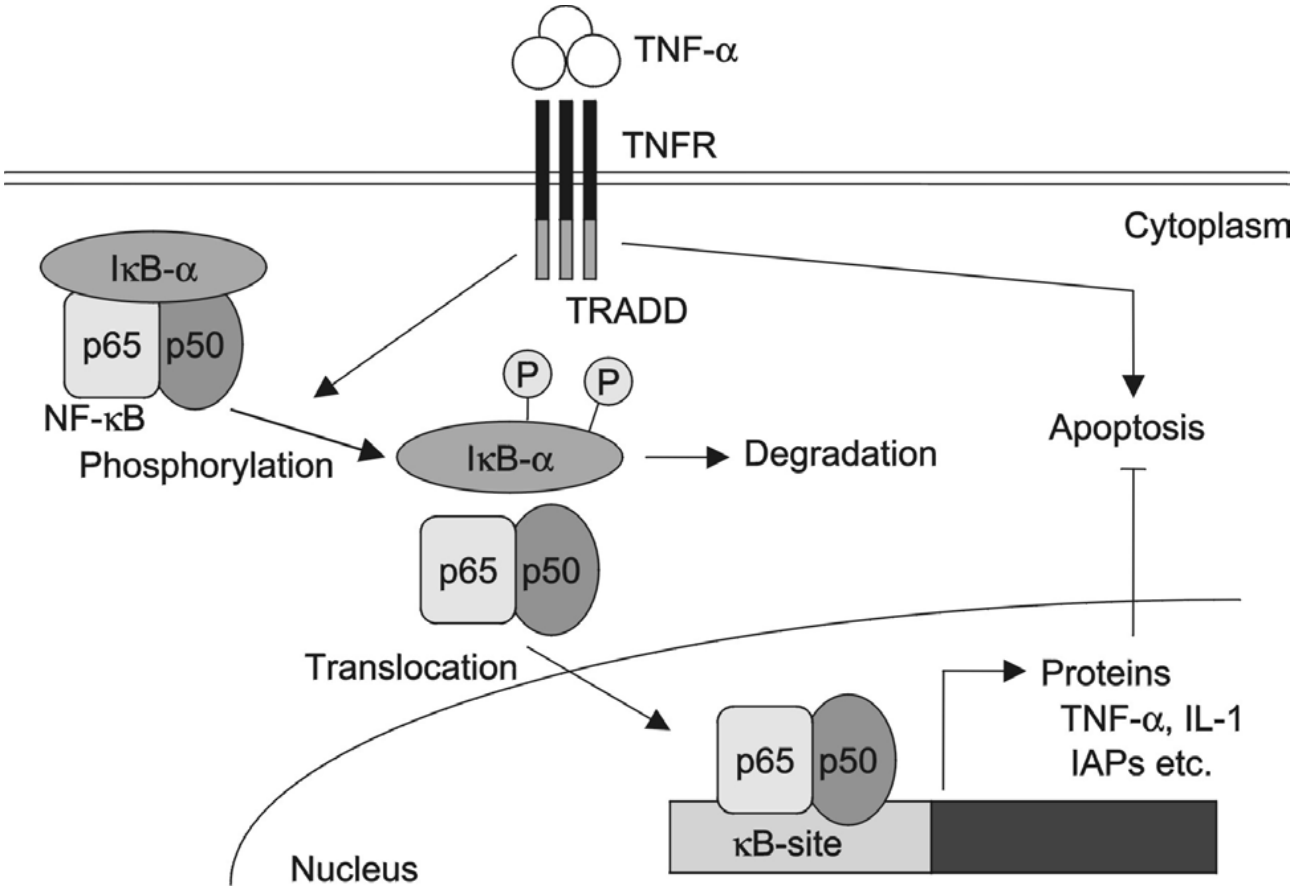


Figure3: Signal transduction pathway for TNF-α-induced activation of NF-κB (K. Umezawa and C. Chaicharoenpong 2002).

After gaining access to the nucleus, NF-κB must be actively regulated to execute its fundamental function as a transcription factor. Recent studies have highlighted the importance of nuclear signaling in the regulation of NF-κB transcriptional activity. There is increasing evidence that both the recruitment of NF-κB within the nucleus to target genes and the ensuing transcriptional events are actively and highly regulated. A growing list of novel molecules has been identified in recent studies, which customize NF-κB transcriptional activity in the nucleus. All of these observations giving the idea that the nuclear signaling of NF-κB is more complex than initially considered, adding another level of complexity to the complicated, but elegantly regulated, NF-κB signaling pathway (F. Wan and M. J. Lenardo 2010).

It is an area of intensive research to examine how NF-κB selectively recognizes certain κB sites to achieve specific gene transcription, but it remains still incompletely understood. The κB site sequence in target genes and the specific Rel dimer requirement are not consistently

correlate, although it has been assumed for a long time that the usage of certain Rel dimers could be dictated by the variability of κB sequences at specific promoters, based on the principle that each κB site variant could preferentially recruit one type of Rel dimer over others. Various κB sequences takes part in specific configurations for NF-κB binding, that showed if a single nucleotide change in κB sites made, it affected the formation of productive interactions between Rel subunits and coactivators (F. Wan and M. J. Lenardo 2010).

In several genome-wide studies, a large number of NF-κB-binding motifs have also been illustrated beyond recognizable κB sequences. These results strongly indicate that other integral non-Rel components in the NF-κB complex could not only regulate NF-κB activity but also contribute to the strength and specificity of DNA binding as an essential component. RPS3 has been identified as a one of a non-Rel subunit in certain NF-κB DNA-binding complexes, where it is essential for the recruitment of NF-κB p65 to the selected κB sites. RPS3 was demonstrated to interact with p65 in a proteomic screen and evidenced to be instrumental for NF-κB transactivation. The featured heterogeneous nuclear protein K (hnRNP K) homology (KH) domain of RPS3, a structural motif that binds single-stranded RNA and DNA with some sequence specificity, is essential for its association with p65 (F. Wan and M. J. Lenardo 2010).

RPS3 functions as an integral part of NF-κB revealed in several lines of evidence. It is also associated with the p65-p50-IκBα cytoplasmic sequestration complex in resting cells. In response of the stimulation, RPS3 is also able to translocate specifically to the nucleus and binds to κB sites in a large number of NF-κB-dependent genes. In addition to this RPS3 exists in endogenous NF-κB DNA-binding complexes, as demonstrated by the ability of RPS3-specific antibodies to supershift or diminish p65-containing DNA complexes in electrophoretic mobility shift assays (EMSAs). It has been shown a significant correlation between RPS3-dependence and p65-dependence in transcription of a subset of NF-κB target genes induced by T-cell receptor ligation. Therefore, RPS3 is an integral subunit of NF-κB rather than a co activator. It has also been demonstrated that RPS3 protein strikingly exerts a dramatic synergistic effect on the DNA-binding activity of both p65-p65 and p50-p65, but not of p50-p50 complexes. Furthermore, RPS3 stabilizes NF-κB association with certain cognate κB sites, as a DNA-binding component. These phenomena give us an important indication about unexplained extremely high affinity of native NF-κB complexes for κB DNA. No other

Rel-interacting protein has been clearly demonstrated to be integrated into endogenous NF-κB complexes or to substantially augment the DNA-binding activity of NF-κB, although over 100 proteins interaction with p65 were known. It has been emphasized that, in certain key physiological processes, including induction of immunoglobulin-κ light chain gene expression in B cells, and cell proliferation and cytokine secretion in T cells plays an important role of RPS3 in regulating NF-κB transcription. The inherent complexity of NF-κB itself is been highlighted by the identification of RPS3 (F. Wan and M. J. Lenardo 2010) *FIGURE4* (F. Wan and M. J. Lenardo 2010).

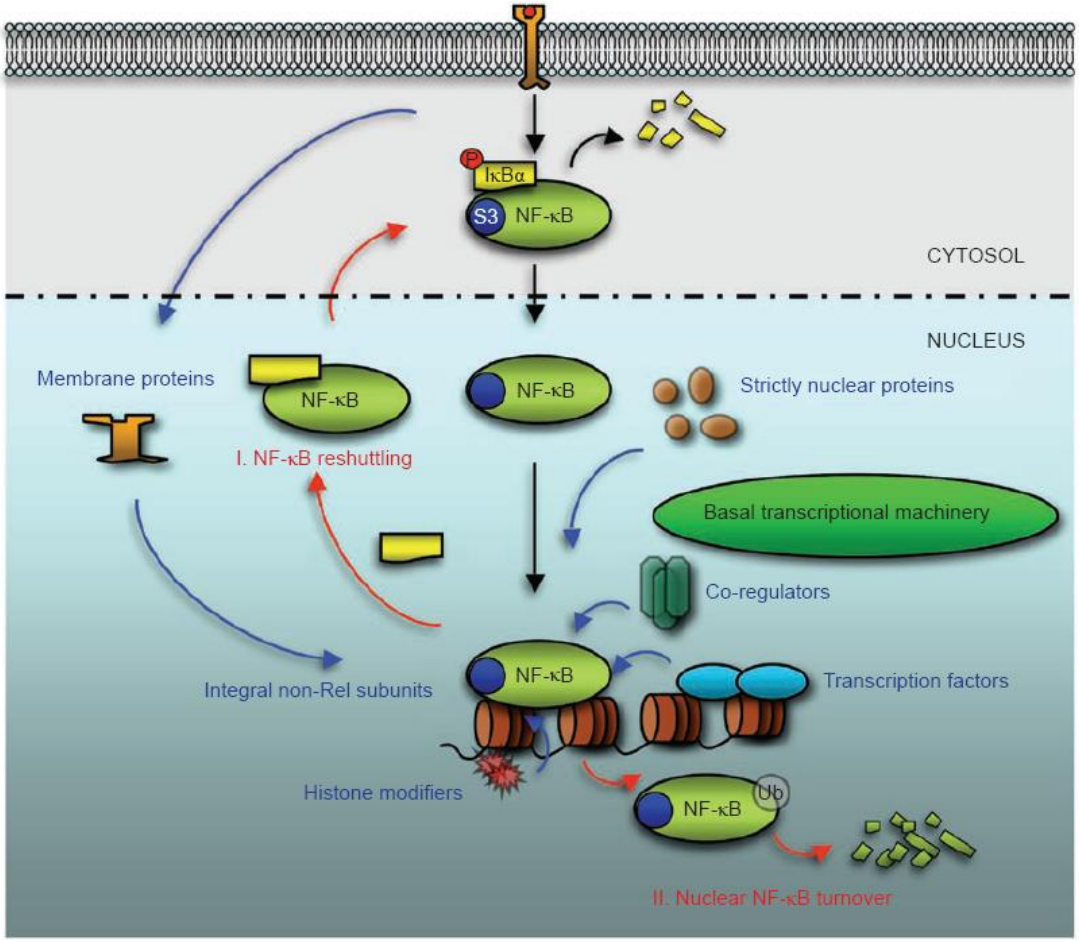


Figure 4. The nuclear regulation of NF-κB. After it gains access to the nucleus, NF-κB transcriptional activity is elegantly controlled by a number of nuclear regulators, as illustrated in blue, including non-Rel subunits such as RPS3 (S3) within the NF-κB complex, proteins restricted to the nucleus or translocated from the plasma membrane, other transcription factors, and chromatin modifiers. Two distinct pathways to terminate NF-κB transactivation, as illustrated in red, involve (I) IκB-mediated restitution of NF-κB to the cytoplasm and (II) ubiquitination-dependent nuclear degradation (F. Wan and M. J. Lenardo 2010)

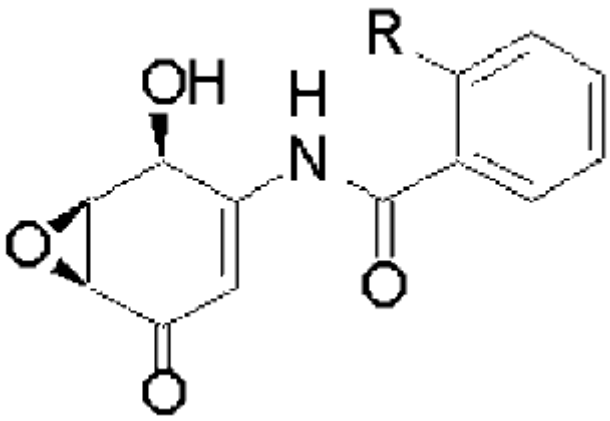
Some signaling molecules working close to the plasma membrane have also been shown novel function in regulating NF-κB transcriptional activity in the nucleus. Akt1, a serine/threonine protein kinase are the one of the examples and their functions are immediately downstream of plasma membrane tethered phosphoinositide 3-kinase. It was also observed that these molecules play an important role to translocate into the nucleus and complex with p65 and Ki-Ras to enhance NF-κB transcriptional activity (F. Wan and M. J. Lenardo 2010).

A non-Rel subunit of NF-κB, ribosomal protein S3 (RPS3), and numerous other nuclear regulators of NF-κB, including Akirin, Nurr1, SIRT6, and others, have recently been identified, unveiling novel and exciting layers of regulatory specificity for NF-κB in the nucleus. Further insights into the nuclear events that govern NF-κB function will deepen our understanding of the elegant control of its transcriptional activity and better inform the potential rational design of therapeutics for NF-κB associated diseases (F. Wan and M. J. Lenardo 2010).

Low molecular weight inhibitors of NF-κB therefore should be useful as immune suppressants and as anti-inflammatory, antiviral, and anticancer agents. Panepoxydone, cycloepoxydon and gliotoxin are among those inhibitors. Panepoxydone is isolated from the basidiomycete lentinus crinitus and inhibits TNF-α induction, and in this way inhibits the activation of NF-κB as shown in a promoter reporter assay by using COS-7 cells. It is been reported, that Gliotoxin producing by fungi, also inhibit the activation of NF-κB by preventing the degradation of IκB (A. Ariga et al. 2002).

1.4. Dehydroxymethylepoxyquinomicin (DHMEQ) NF-κB inhibitor

In the course of our research we therefore have used a new designed NF-κB inhibitor of low molecular weight inhibitors of NF-κB function. It was derived from the structure of the antibiotic epoxyquinomicin C (A. Ariga et al. 2002) *FIGURE 5* (A. Ariga et al. 2002).



R=OH; (-)-DHMEQ

R=H; 9-dehydroxy-(-)-DHMEQ

Figure5: Structure of DHMEQ and its inactive analogue. Racemic DHMEQ and 9-dehydroxy-DHMEQ were used throughout the experiments (A. Ariga et al. 2002).

This novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin, abbreviated as DHMEQ is synthesized from 2, 5-dihydroxyaniline in five steps as its racemic form. It has been shown that (-)-DHMEQ is ten times more potent than (+)-DHMEQ [12]. The designed molecule is called dehydroxymethylepoxyquinonmicin (DHMEQ, formerly DHM2EQ) and inhibits TNF-α-induced activation of NF-κB. It has been demonstrated in different studies, that DHMEQ was effective in suppressing rheumatoid arthritis (K. Wakamatsu et al. 2005), in Hodgkin/Reed-Sternberg cells (M. Watanabe et al. 2007), local and remote organ injury following intestinal ischemia/reperfusion in rats (T. Suzuki et al. 2008), anti-thy1.1-induced glomerulonephritis in rats (T. Kosaka et al. 2008), atherosclerosis in ApoE-deficient mice (T. Chiba et al. 2006) and osteoclastogenesis in mouse arthritis (T. Kubota et al. 2007).

DHMEQ inhibits the tumor necrosis factor (TNF)-α-induced NF-κB binding activity but not the phosphorylation or degradation of I-κB (T. Suzuki et al. 2008). DHMEQ inhibited the TNF-α-induced nuclear accumulation of p65. Thus, DHMEQ is a unique inhibitor of NF-κB that acts at the level of the nuclear translocation. DHMEQ has been reported to be effective on various hematologic and solid malignancies with constitutively active NF-κB (N. Dabaghmanesh et al. 2009). In these studies it has been noticed that the molecular target of

DHMEQ is directly bound covalently to the Rel family proteins through a specific Cys residue. This discovery may explain its highly selective inhibition of NF-κB and the low toxic effect of DHMEQ in cells and animals (M. Yamamoto et al. 2008). DHMEQ exhibits anti inflammatory, anti fibrotic, and anti tumor effects (K. Shinoda et al. 2010). DHMEQ completely inhibited NF-κB p65 DNA-binding activity. It has also been demonstrated that this inhibitory effect is dose dependent. The duration of the effect of 2 µg/ml DHMEQ pretreatment was limited to 6 hours, however pre-treating with 5 µg/ml DHMEQ indicated a dose-dependent inhibitory effect of DHMEQ of at least 24 hours. Moreover this study demonstrated that DHMEQ interfered with nuclear translocation of activated NF-κB without inhibiting phosphorylation or degradation of IκB in Mo-DC. In addition DHMEQ was shown to be cytotoxic to DC at concentrations of 10 µg/ml or more. The inhibition of cytokines production through DHMEQ is also dose-dependent. It has been noticed significantly inhibition of IL-6 and of TNF-α production at concentrations of 2 µg/ml or higher, whereas IL-12 p70 production was significantly inhibited at concentrations of 1 µg/ml or higher. Among these cytokines, IL-12 p70 production was most strongly inhibited by DHMEQ. On the other hand DHMEQ moderately affected IL-10 transcription and its production. There was an observation that CD40 and HLA-DR expression in DC was inhibited by DHMEQ, whereas CD80 and CD86 expression was moderately upregulated. DHMEQ also partially inhibited down-regulation of endocytosis during DC maturation (K. Shinoda et al. 2010).

In another study there is evidence that DHMEQ inhibits LPS-induced histamine secretion and induction of HDC. DHMEQ inhibits C/EBP transcription factor activity, whereas C/EBP is known to be involved in the transcription of the HDC gene (E. Suzuki et al. 2009). It has been observed that DHMEQ significantly effects nuclear factor-κB nuclear translocation in a murine model of arthritis and cultured human synovial cells (K. Wakamatsu et al. 2005). Through inhibition of constitutive NF-κB activity DHMEQ reduces cell growth and induces apoptosis of H-RS cells. In the same study it has been observed that DHMEQ has a potent inhibitory effect on the growth of H-RS cells in NOG mice model. DHMEQ increase anti-tumor effect of topoisomerase inhibitors by blocking inducible NF-κB in H-RS cells. DHMEQ induce apoptosis in cells in which the activation of caspases 3, 8 and 9 is involved (M. Watanabe et al. 2007). In such a study it has been shown that the activity of NF-κB is higher in tumor initiating TIC-like cells when compare to control cells. Moreover, DHMEQ, a highly specific inhibitor for NF-κB, suppressed tumorgenesis in the TIC-like cells in mouse

model. After treatment with DHMEQ it has been shown a decrease in tumorgenesis in CD24-/low/CD44+ populations (M. Murohashi et al. 2010).

DHMEQ reduces NF-κB p65 DNA-Binding capacity, cell growth, and induces apoptosis in human hepatoma cells. ERK1/2 was involved evidenced based in cell proliferation and apoptosis. DHMEQ enhances the phosphorylation of ERK1/2 and, in combination with the MEK-specific inhibitor U0126 in order to reduce cell growth. This study showed that induction of ERK1/2 phosphorylation in both cell lines through DHMEQ is dose-dependent. MEK/ERK pathway is activated as a pro-survival signaling during DHMEQ treatment. This antitumor effect of DHMEQ in human liver cancer cells are mediated through a Reactive Oxygen Species (ROS)-dependent mechanism (N. Lampiasi et al. 2009).

DHMEQ inhibits TNF-mediated localization of c-Rel and RelB, which retain RelA RSAGSI sequences, but not that of p52 having p50 PSHGGL in the nucleus (M. Watanabe et al. 2008). In another study it has been demonstrated that DHMEQ suppresses the development of atherosclerosis in apoE-deficient mice (T. Chiba et al. 2006). DHMEQ does not inhibit TNF-α induced phosphorylation and degradation of IκB in Jurkat and COS-1 cells (A. Ariga et al. 2002). It does not inhibit the nuclear transport of Smad2 and the large T antigen, which means that it does not inhibit the TNF-α-induced activation of JNK, but synergistically induced apoptosis with TNF-α in human T cell leukemia Jurkat cells. In addition to all these, DHMEQ inhibits 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced NF-κB activation in Jurkat cells. TPA is considered to activate IKK through the activation of protein kinase C. Therefore, it was assumed that DHMEQ inhibits the NF-κB activation at a level that is downstream of IKK. DHMEQ also inhibits the TNF-α-induced DNA binding of nuclear NF-κB in the electrophoresis mobility shift assay (EMSA). However, it does not inhibit the direct binding of NF-κB after subcellular fractionation. On the other hand TNF-α induces phosphorylation and proteasome-mediated degradation of IκB to release NF-κB from the inactive complex. Interestingly, DHMEQ does not inhibit the TNF-α-induced phosphorylation and degradation of IκB (K. Umezawa and C. Chaicharoenpong 2002).

It has been illustrated that DHMEQ does not suppress spontaneous expression of RANKL nor macrophage colony-stimulating factor in culture of fibroblast like synovial cells obtained

from patients with rheumatoid arthritis. These observations suggest that DHMEQ suppresses osteoclastogenesis in vivo through down regulation of NFATc1 expression without significantly affecting expression of upstream molecules of the RANKL/receptor activator of NF-κB/osteoprotegerin cascade (T. Kubota et al. 2007).

2. Material and Procedure

2.1. NTN-induction and treatment of the experimental animals

For the experiment male C57Black6 mice were used. The mice were injected with 0.6 ml NTN antiserum i.p (animal experiments were approved by a positive vote of the local ethical committee FII 18/04). DHMEQ was injected subcutaneously in a dose of 3g/KG body weight starting 12 hours before induction of NTN and once every 48 hours. For the vehicle control animals were injected with 10% Cremaphor solution dissolved in PBS or DMSO with methylcellulose.

2.2. Urine collection in metabolic cages

Urine was collected at different time points after the induction of NTN while mice were placed in metabolic cages for 6 hours. Urine collected was transported into prepared 1.5 ml sample containers and frozen at -20°C until further analysis**.**

2.3. Blood collection and kidney harvest

Animals were sacrificed at 2, 4, 10, 14 days after NTN-induction. At the end of the observation period 0.5 ml blood was collected by direct puncture of the Aorta. Blood was transferred into a 1.5 ml serum sample container (Sarstedt) and stored at -20°C. Kidneys were harvested and further processed for total protein and RNA isolation and morphological examination.

2.4. Freeze and thawing of tissue

Kidney tissue for RNA and protein extraction was shock frozen in fluid nitrogen and stored at -80°C until further processing. Nuclear protein was done immediately after kidney harvest from fresh isolated tissue.

2.5. Protein biochemistry

Protein electrophoresis is based on ions migration within an electric field. The proteins have different entire electrical charge and different pK values that are dependent on the amount of sour and basic amino acids. If an electrical charge molecule is brought into an electric field, the electrophoretic mobility depends on the size of molecule, shape, the total net electrical charge, the pH-value, the pore size of the carrier, the temperature, the ion strength of the buffer and on the electric field strength. The protein separation is carried out usually in form of a gel (Agarose, Polyacrylamide) in a so-called carrier matrix. Through that variable cross linked networking the gels serve as a filter effect, that separates the molecules after its electrical charge and after its size, shapes and design.

2.6. One-dimensional SDS Polyacrylamide gel electrophoresis

If proteins are charged through the anionic detergent SDS (Sodium-Dodecyl-Sulfate), separation in the electric field is possible almost solely through molecular weight. SDS bonds to the hydrophobic regions of proteins, whereby most of the proteins dissociate into its lower units. Through this bonding, a strongly negative electrical charge establishes in that polypeptide chains. It emerges a complex with negative sulphate group directed outward, i.e. the amount of negative charge is directly proportionally to the protein size. The proteins move differently from the cathode to the anode depends to its electrical charge. Due to the narrow pore of separation gel, there is an additional molecular filter effect, whereby the proteins are separated corresponding to its size. To the successful execution of a gel electrophoresis, the protein samples must be brought into a medium, which reduces or prevents an intermixture of the samples. The medium may allow neither to react with sample nor inhibiting the movement of the protein sample. These conditions are fulfilled with polyacrylamide gel. The network of the gel is constructed through radical polymerization of the acrylamide monomer and the cross linked networking of bifunctional N,N'-Methylen-Bisacrylamide. As a radical starter, ammonium peroxide disulphide and the tertiary amine N, N, N N' –Tetramethylethylen diamine (TEMED) were used. The pore size can be varied by the quantity ratio of both components. Due to different molecular weights, an optimal separation conditions can be created.

2.6.1. Standard proteins for the electrophoresis

A marker is a mixture out of high or low molecular standard proteins whose molecular weights are well known. It has been applied in the electrophoresis in addition on the gel in order to detect proteins according to its corresponding molecular weight. For that the marker SDS-7B has been used from (Sigma). The standard proteins are mixed here with a blue dye which changes the molecular weight of the proteins. For this reason a renewed calculation of the molecular weight of the respective standard protein after every staining is necessary. The sublime has been dissolved in the urea solution and NuPage 4x LDS sample buffer (4x LDS + 0.4 MS DTT), given from the manufacturer (Sigma), heated for 10 min at 80°C and frozen in form of Aliquots at -20°C.

Molecular weight:

-Macroglobulin, human plasma 190 kD

-Galactosidase, E. coli 108 kD

-Fructose- 6-phosphate-kinase, muscle, rabbit, 84 kD

-Pyruvate-Kinase, muscle, hen, 67 kD

-Fumarase, heart, pig, 55 kD

-Lactate-dehydrokinase, muscle, rabbit, 39 kD

-Triose-phosphate-isomerase, muscle, rabbit, 35 kD

2.6.2. Extraction of cellular and nuclear proteins

2.6.2.1. Preparation of sample for cellular protein

Tissue lysate of mouse kidney were used to separate cellular protein. Tissues have been lysed with T-PER Tissue protein Extraction Reagent from (Pierce) and with complete Protease inhibitor from (Roche). In addition to this the tissue was transferred with 10 µl Lysis buffer per 1 mg tissue and minces mechanically with a pestle. After fifteen minute intubation time on ice, the suspension should bring to Centrifugation for 15-20 min in (full speed) 16100x g and at 4°C. That supernatant was transported into a new sample container and frozen on -80°C.

2.6.2.2 Determination of the cellular protein concentration

The determination of the protein concentration resulted after Lowry Assay method. Firstly we prepare a BSA standard with a concentration of 10mg/ml. BSA serum albumin solves in T-PER Tissue protein Extraction Reagent from (Pierce) and it is dilute 1:2 (10; 5; 2.5; 1.25; and 0.6 mg/ml). After thawing of proteins, they have been mixed gently and well. 5µl proteins were given into each wells of a 96 holes disk and then 5µl BSA standard, comparably solved in lysis buffer, was given into each wells of 96 holes disk. Each protein sample were pipetting twice. 25µl a mixture out of solution A/S (1ml solution A+20µl solution S from manufacturer) of Bio Rad and 200µl of solution B from Bio Rad were add into in it and subsequently leave to incubate for 15 min at room temperature (RT). The measurement resulted in 550nm in the Mithras.

2.6.2.3 Preparation of sample for nuclear protein

Tissue lysate of mouse kidney were used to separate nuclear protein. We prepare firstly a Low salt buffer (Buffer A) and high salt buffer (Buffer B) solution. Buffer A contained 10mM HEPES pH 7.9, 10mM KCl, 0,1mM EDTA pH 8.0, 0,1mM EGTA pH 8.0 and 1mM DTT and subsequently added a freshly Protease Inhibitor Mix M (Serva,1:100) into in it. Buffer B contained 20mM HEPES pH 7.9, 40mM NaCl, 1mM EDTA pH 8.0, 1mM EGTA pH 8.0 and 1mM DTT with an addition of freshly Protease Inhibitor Mix M (Serva1:100). Firstly the kidney has been mashed on Petri dish with a scalpel and subsequently homogenized in 1ml Buffer A in the douncer. The mixture brings into marked Eppi-tubes and subsequently let for 15 min on ice for incubation. An addition of 100µl of 10%NP-40 is to follow. The Eppendorf tubes were shaken gently and placed immediately at 16000xg for 5 min to centrifuge. The supernatant was thrown away and tissue pellet has been resuspended in 200-400µl Buffer B and subsequently let for 15-20 min to incubate at 4°C in the cool room with rotation. Then centrifugation is to follow with 16000xg for 5 min and subsequently the supernatant has been aliquot and frozen after fluid nitrogen shock at -80°C.

2.6.2.4. Determination of the nuclear protein concentration

The determination of the protein concentration resulted after Lowry Assay method. First one prepares a BSA standard with a concentration of 10mg/ml. BSA is solved in Buffer B (even produced) and is diluted 1:2 (10; 5; 2.5; 1.25; and 0.6 mg/ml). After thawing of proteins, they have been mixed gently and well. 5 µl proteins were given into each wells of a 96 holes disk

and then 5µl BSA standard, comparably solved in lysis buffer, was given into each wells of a 96 holes disk. Each Protein sample were pipetting twice. 25 µl a mixture out of solution A/S (1 ml solution A+20µl solution S from manufacturer) of Bio Rad and 200 µl of solution B from Bio Rad were add into in it and subsequently leave to incubate for 15 min in room temperature (RT). The measurement resulted in 550nm in the Mithras.

2.7. Preparation of samples for Western Blot

It has been loaded a 50 µg of cellular protein as well as nuclear protein per lane of a NuPAGE. The concentration of gels depends on the molecular weight of the protein to be detected. Volume for 50 µg proteins has been calculated on the basis of protein determination. Volume difference has been filled with 16µl of corresponding Protein lysis buffer. All samples were poured in cups with the 8 µl corresponding quantity of 3x sample protein buffer (NuPage loading buffer + 0.4 MS DTT) and were heated to the complete denaturing for 10 min at 95°C.

2.7.1. Separation of samples

For the separation of proteins, a prefabricated, reducing 4-12% NuPage Bis-Tris gradient gel was used (Invitrogen). The gel was taken out of its packet and wash shortly with water in order to remove non pure particles of the Butyl alcohol, which used for the preservation. After that the pockets of the gel has been washed with MOPS or MES (50 mM of MOPS and/or MES, 50 mM of Tris base, 0.1% SDS, 1 mM of EDTA) course buffer. The gel is been fixed into an electrophoresic chamber from (PeqLab) and the chamber is been filled up completely with course buffer. After loading of the pockets with 4 µl protein marker and 22 µl samples, the chamber is been attached to a designed power supply (Organic wheel) with a Voltage of 200 V and is been let to run for at least 45-50 minutes. This voltage has retained until the first proteins reaches the lower end of the gel.

2.7.2. Western Blot

The proteins separated by the SDS PAGE is been transfer out of the gel through electrophoretic transfer on a positively charged carrier membrane from PVDF (Amersham). This procedure is known as a wet procedure. Because the proteins are further by SDS negatively charged, they move from cathode to the anode. A novex mini cell was used to transfer the proteins. The blotting resulted like in the sandwich procedure between three filter

papers. PVDF membrane is been activated for 1 min with methanol before use. The membrane brought air bubble less to the gel and put between three pieces of watman paper and between three pieces of sponges in the blotting device. The fixation has been followed, in order that the gel to the cathode side and membrane to the anode side directed. The novex minis cell was filled up then with blotting buffer (25 mM of Tris base, 0,192 MS Glycin, 20% methanol in aqua dest). The transfer takes place at a current strength of 65 mA (1mA/cm²) and at a temperature of 4°C in about 1.5 hours. After successful transfer, the Blot was stored at 4°C in TBST (10 mM of Tris pH 7.4, 107 mM of 5 MS NaCl, 0.05% Tween 20) for the further use.

2.7.3. Immunoblot

In the Immunoblot, separated proteins through gel electrophoresis and transferred proteins on a membrane are detected with the help of specific antibodies. The antibodies do not bind covalently with its antigen. The PVDF membrane incubates and has been blocked with dried milk solved 5% in 30ml TBST for approx. 3h on seesaw at RT, after the removal out of the transfer unit. After short washing with TBST, the membrane is been incubate with the primary antibody in TBST solved 5% lean dried milk. The length of the incubation time in which the antibody can bind depended on the assumed affinity of the antibody and the quantity of the product to be detected. In our experiments, the gels were incubates overnight at 4°C with primary antibodies IκBα (C-21 Santa Cruz) or NF kB p65 (F-6 Cell Signaling) in blocking buffer on a seesaw. After removing the membrane from the primary antibody, the membrane was washed twice in TBST for over 10 min each time. After that the secondary antibody coupled with horseradish peroxide has been poured. The antibody was solved in 20 ml 5% lean dried milk in TBST. After removing of the secondary antibody, further wash steps are followed with TBST for over 30 min and as well as with assay buffer twice for 5 min. All steps occur on a shake table/seesaw. Immune complexes were visible with the help of enzyme conjugation. In order to reinforce the effect, the detection takes place indirectly via marked secondary antibodies, which are directed against the primary antibody bound to the antigen. There are different possibilities of detection. In the frame of this work, the detection with ECL super signal resulted. It is based on the principle of a highly sensitive method of chemiluminescence. This is based on that, in which the secondary antibodies coupled with peroxide aces oxidize in presence of H_2O_2 luminol. At the same time light is released, that after corresponding exposure time on x-ray film exposes. The detection takes place in the

darkroom with a help of automatic film processor. The sensitivity of the Western Blots lies between 10 to 100 fmol. This corresponds in a molecular weight of 50 kD approx. 0.5 to 5 ng proteins.

2.8. Molecular biology

2.8.1. Isolation of RNA

For the extractions of RNA out of mouse kidneys, a Trizol method was used. The one third of kidneys placed on Petri dish, after detaching the hilus mechanically and minced gently with scalpel and tweezers. The kidney pieces are homogenized then with pestle in a 1.5 ml sample container cups containing 200 Trizol. It is given in addition to this 800µl Trizol and mixed well. Then let the samples to incubate for 5-10 min in RT. It is been mixed gently and thoroughly in between. After a shortly centrifugation 200µl chloroform has been added. The sample were mixed gently for 15 second and then let the samples for 3 min at RT to incubate and subsequently centrifugation does take place with 10000 rpm for 15 min at 4°C. The clear supernatant of approx. 500 µl led into new RNA Cup with 200 µl pipette. It has been added a 500 µl isopropyl alcohol in it and mixed well. Subsequently let the sample for 15-30 minutes at RT to incubate. These have been placed for centrifugation at 13000 rpm for 10 minutes in 4°C. Supernatant were through away and RNA pellets were put with 75% 1 ml of Ethanol/DEPC-H_2O in centrifugation at 13000 rpm for 10 minutes and at 4°C. The next both step of washing is followed with 500µl Ethanol/DEPC-H2O on seesaw. Supernatant is through away and pellets were dry in speed vac. for 5 minutes without temperature. In between time a mixture of RNase out and DEPC/H_2O for solving the peeling has been made. 30 µl mixture of RNase poured to the peeling and well mixed. For the further processing the RNA is been stored at -80°C.

2.8.2. Concentration of RNA determination

For inspection of the quality and evaluation of the RNA quantity, a photometric measurement was carried out. To this the RNA is firstly diluted with RNase and dist. water 1:100 and the extinction measured in λ=260 nm and λ=280 nm. The quotient of the measurement serves as a measure for the purity of the RNA. The quotient should lie between 1.5 and 1.8, smaller values speaks for a contamination of RNA with proteins. The concentrations calculated themselves out of the extinction after the formula 40 x extinction in 260 nm x diluting factor.

2.8.3. RT-PCR

The total RNA extracted out of the cells was reverse transcribed in cDNA, which served as a raw material for the following two steps of RT-PCR reaction. The reverse transcription takes place with the help of reverse transcriptase out of the Moloney-Murine-Leukemia-virus (MMLV-RT), which shows the activity of RNA dependent DNA polymerase. Possibly available RNases is been deactivated through the addition of RNase inhibiting material. It has been pipetted a control (without MMLV RT) for every extension. To the RT-PCR, first of all two master mixtures were prepared in a new Eppendorf cup. Master mixture 1 (MI) contained 1µl HEX Primer/sample, 1µl dNTPs/sample, 8µl DEPC-water/sample. Mastermix 2 (MII) contained 4µl first-strand-buffer 5x/probe, 2µl DTT/sample, 1µl DEPC-water/sample and 1µl MMLV-RT/sample. The RNAs are diluted to 100ng/µl in a new Eppendorf cup. From them 2 µl were put in a new cDNA-cup and to every cup 10 µl of sample has been added. Oligo dt served in this extension as a Primer for the reaction. These were heated for 5 min at 65°C, on which secondary structure of the RNA melt up. Subsequently put all samples for 5 min on ice to cool down. This prevented the new formation of the RNA secondary structure and enabled the hybridization of the Primer. 8 µl Mixture II MII was added in it after this. Subsequently put the Eppendorfer-cups in PCR machine for the synthesis of cDNA. To the RT-PCR, 1.5µl of prepared cDNA pipetted in a 96 hole disk after pipetting plane of real Time PCR. It has been added 11 µl of Mastermix of Primer (18S, CCL-2, CCL-5, CXCL-10) per wells, made with 1.25 µl Primer Fw/sample, 1.25µl Primer Rev/sample, PCR water 2µl/Probe and syber Green 6.25µl/Probe. It's been covered with a plastic foil and subsequently brings it to for a short centrifugation and then put it into a RT-PCR machine. The analysis is followed thereafter.

2.9. Enzyme linked immunosorbent assay (ELISA)

The ELISA serves for the quantitative determination of different ingredients out of samples. A specific monoclonal antibody is added to a 96-hole immunosorbent disk. By adding the sample, these are bound to the detecting components through the antibody. Through a second antibody coupled horseradish peroxide aces, the sample bound at the first antibody is detected by supplement of peroxide ace substrate by color development. This color reaction can be grasped photometric. In this work, an ELISA Kit was used. All related materials and reagent ions were taken from the Kit. The related 96-hole flat grounds disks had to be poured first of all with the binding first antibody. An 8-channal-pipette was used in order to pipette possibly

the same amount of the antibody on the disks. 100µl/well of the antibodies is been added (anti mouse album out of goat, diluting 1:100) with the binding buffer (0.05 M carbonate bicarbonate pH 9.6 was transferred) on the micro disk and let the overnight at 4°C to incubate. Now started three times washing of the not bounded remainders with wash solution 200µl/well (50 mM of Tris, 0.14 MS NaCl, 0.05% Tween 20, pH 8.0), All was treated, which not occupied binding sites, with 150µl/well post coat Buffer block solution (50 mM of Tris, 0.14 MS NaCl, 1% BSA, pH 8.0). The contained BSA occupied yet free binding sites let for the thirty minute to incubate. Again a wash step followed. Then 100 µl/well of diluted urine, samples added to examine and let for 60 min at room temperature for incubation. It followed five-time thorough wash steps with 200 µl/well wash buffer in order to remove not bounded albumin. To the bounded albumin, the second antibody (anti mouse album out of goat, horseradish peroxide aces coupled 1:20,000) has been given and let for 60 min at room temperature to incubate. It followed a five-time washing with 200 µl/well wash buffer. In order to make the coupled antibodies visibly, 100 µl/well of the provided enzyme substrate mixture is been added and let for 20 min to incubate. The horseradish peroxide aces transformed the substrate. The color reaction is directly proportional to the amount of the bound Albumins. Through supplement of 2 MS sulfuric acid this reaction was stopped. For the analysis an ELISA harvest device was used. The measured extinction could be converted then on the basis of the formula absorption = (A-D)/1+(x/C)B+D) into the concentrations of the Albumins contained in the samples. The values A to D resulted from the related standard curve. For the accuracy of the fair results, it was calculated the regression values of the standard values of the ELISAs. Valued nearly 1 indicate a high accuracy of the fair curve. No results with a regression were used smaller than 0.9950.

2.10. Histology

2.10.1. General

In this work, routine PAS-stained histology and immune histology were prepared of the treated animals and the control animals. In general sliced with a thickness from 3 µm to 5 µm were prepared.

2.10.2. Principle of the immune histology

The basic goal of the immune histology is to identify the epitope on or in the cells or tissues, with the help of antigen antibody reaction. The epitope is bound through a specific first antibody. After corresponding bonding time, the Fc-fragment of the first antibody is detected by means of a second antibody. The selection of the secondary antibody depended on the origin species of the related primary antibody. The second antibody is bound either with an enzyme, with biotin vitamin, or with a fluorescence dye. The so formed complex of antibody and antibody becomes visible either through enzymatic color reaction or through fluorescence. The method permits a signal strengthening through the second antibody.

2.10.3. Complex of streptavidin and Biotin

The representation of the antigens takes place partially by the indirect method of avidin and biotin- complex (ABC). It is based upon the affinity of Streptavidin, a protein out of Streptomyces avidinii, to the vitamin biotin. The first antibody bound specific at the antigen is bounded with a secondary antibody coupled with biotin, against the Fc-fragment of the primary antibody directed to the epitope. The detection of the secondary antibody does occur through alkaline phosphatase coupled with the Biotin. The related streptavidin complex has four binding sites for biotin. To the preparation, substance A (Avidin complex) with substance B (biotin coupled with alkaline phosphatase) was mixed for 30 min at room temperature and incubates in 5x TBS. In this time three of the four free binding site of the Avidin complex were occupied biotin in the ideal case. Before the incubation of AB-Complex with sample takes place, it has to be washed twice with 5x TBS, because the phosphate of PBS could the further reaction disturb. All following wash steps were followed in absence of phosphate with 5x TBS.

2.10.4. New fuchsine development solution

The sliced, colored with AB-complex is been developed with a bath of new fuchsine solution in the dark. It has been added 300 mg sodium nitrite solved in 7.5 ml Aqua dist. and mixed with new fuchsine stem solution (2.5 g new fox and 50 ml 2 MS hydrochloric acid). Then 150 ml TNT buffers (6.35 g Tris, 9 g NaCl, 25 ml became 1 M HCL of all 1 g Tween 20, in 1025 ml Aqua dest) is been added. In this mixture 800 µl of a naphthol solution (20 mg naphthol-ACE-buses-phosphates and 750 mg NN-dimethylformamid) is been added and mixed. The alkaline phosphatase coupled with the biotin splits phosphate from the naphthol-ACE-

biphosphate, which formed then an insoluble color complex with the new fuchsine. Cells that showed specific colour, represent themselves red after this development.

2.10.5. Nuclear coloring with hematoxylin by Boehmer

The slices have been colored in hematoxylin, in order to make the specifically developed new fuchsine prominent. 10 g of 12 x aluminum potassium sulphate were solved in H_2O and 0.1 g sodium iodate for overnight in 200 ml Aqua dist were solved. Moreover 1 g Hematoxylin and 10 g NN-dimethylformamid were solved also for overnight. After mixing of these solutions, it colored the cell nuclei blue. If cells were already colored red through new specific fuchsine, its cell nucleus becomes represented itself now violet.

2.10.6. Analysis

The analysis of the colorings resulted at a microscope (Axioskop Zeiss) and the documentation took place with the help of connected camera (Axiocam Zeiss).

2.10.7. PAS-Staining

This standard coloring served to the morphological judgment of the kidney tissue. The tissue slice is given for 1 min in periodic acid (Merck). After washing off of the remainders for 3 min under flow of tap water and rinsing with Aqua dist. the slice is given for 40 min in shiff reagent (Sigma). Again it was washed under flow of tap water and in Aqua dist. the slice and then it were colored for 90 sec in hematoxylin after Boehmer. After washing off of the residues, the slides is been covered with glass (Menzel).

2.10.8. F4/80-Staining

To detect issue macrophages kidneys were stained with F4/80. The related antibody (MCAP497) detect the mouse F4/80-antigen, a 160 kD recognizes large glycoprotein which is expressed on murine macrophages. F4/80 is not expressed on lymphocytes or other polynuclear cells. After three time washing for respectively 5 min in PBS, the tissues let to incubate with 0.05% Trypsin EDTA at 37°C for 15 min. To stop the trypsin reaction the slice were immersed shortly in 100% ethanol. A three time wash step with PBS followed it for 5 min. Subsequently the slices were blocked in blocking buffer (2% serum of horse, 1% BSA,

0.1% gelatin from fish, 0.1% Triton X-100, and 0.05% Tween 20 in PBS) for 30 min to the saturation of unspecific binding site. The one-hour incubation with the primary antibody resulted to a concentration of 1:50 in antibody buffer (1% BSA, 0.1% gelatin in PBS). After three time of washing with PBS for 5 min, the incubation takes place with the secondary antibody (biotinylated antibody) in PBS in a concentration of 1:500 for 30 min. After washing off the not bounded remainders of secondary antibody with PBS twice for 5 min at room temperature, the tissue slice buffered with 5x TBS and subsequently let to incubate for 30 min with the prepared streptavidin-biotin-complex. After three times washing of the slice with 5x TBS, the slide is been developed in new fuchsine solution in the dark. After sufficient staining, the reaction was stopped in Aqua dist. In the end, the nuclear coloring resulted after Boehmer for 90 s before the slices were covered with Fluoromount-G (Southern Biotechnology). The analysis resulted with the help of microscope.

2.10.9. CD3-Staining

The CD3-staining serves to the identification of T cells in the tissue. The staining is a double staining procedure. The slices were cooked for 35 min at 680 watts in the microwave with citric buffer to the Permeability of the tissue. For cooling off the slices were given in citric buffer for 10 min on ice and subsequently washed with Aqua dist. twice for 5 min. It has been transferred in blocking solution for 30 min (1:20 goat serum in PBS, Vector). Then the slices were incubated with the secondary antibody (biotinylated anti-rabbit-antibody) in blocking buffer with a concentration of 1:100. After washing twice with PBS and with 5x TBS for 5 min, the slice were transferred in the prepared streptavidin-biotin-complex with the concentration of 1:100. After 30 min of incubation and washing with 5x TBS, slices were developed with NBT/BCIP (Dako) till the glomeruli a grayish to black color showed. The slices were washed subsequently three times with PBS and were transferred again in blocking buffer. After 30 min actual primary antibody (anti-CD3, Vector) were incubate with the slices in blocking buffer with a concentration of 1:20 for overnight at 4°C. At the next morning, not bounded elements were removed through triple washing with PBS before the secondary antibody (biotinylated anti-rabbit-antibody, Vector) in block buffer in a concentration with 1:100 added. After 30 min incubation, a new washing process started with PBS. We changed the buffer to 5x TBS and the incubation with the prepared 1:100 concentrated streptavidin-biotin-complex is to follow. After triple washing for respectively 5 min with 5x TBS, the slice has been developed in the dark with a new fuchsine solution, till the T cells became visible as single red cells. The reaction was stopped in aqua dist. and was colored with hematoxylin

after Boehmer for 90 s. Subsequently the slices were washed in aqua dist. cleanly and covered.

2.10.10. Cell counting of CD3- and F4/80-positive cells

The number of the CD3- and F4/80-positive cells was determined in a blinded fashion. At the same time the number of the specifically colored cells in the glomeruli and in the stroma was determined in 400 x enlargement in every 20 fields of vision. At least two glomeruli in one visual field had to be contained per definition. The significance of the results was determined per T test for independent random samples.

3. RESULTS

The aim of this M.D. thesis was to demonstrate a potential new therapeutic opportunity using DHMEQ, a recently described NF-κB inhibitor, in the model of rapid progressive glomerulonephritis in mice. The NTN-model has been established and described in detail previously, first pharmacokinetics and the solvent for DHMEQ have been determined.

3.1. Effect of DHMEQ *in vivo* treatment in mice with nephrotoxic serum nephritis

3.1.1. Effect of DHMEQ with vehicle DMSO in NTN treated animals

First we used DMSO as vehicle for DHMEQ *in vivo* NTN experiments. Four groups of animals were examined: *1)* control animals, *2)* control animals treated with DHMEQ, *3),* animals injected with NTN only, and *4)* animals with NTN and treated with DHMEQ dissolved in DMSO. Observation period was 2 and 4 days after NTN induction.

3.1.2.2. Analysis of Serum and Result of Urine-ELISA

Mice treated with DHMEQ dissolved in DMSO showed reduced serum creatinine levels at days 2 and 4 after induction of disease (p values 0.525 and 0.368 respectively). Serum creatinine in control mice injected with DMSO as a vehicle, however, was slightly, non-significantly increased when compared to non-treated control mice (*FIGURE 6*).

Serum-urea levels were also decreased at 2 days (*p value 0.048) and 4 days (p value 0.163) after NTN-induction in animals treated with DHMEQ when compared with non-treated nephritic mice (*FIGURE 7*).

Even more, the alb/crea quotient was significantly reduced both at 2 days and 4 days after NTN-induction in treated animals with DHMEQ (*p values 0.030 and 0.002) (*FIGURE 8*).

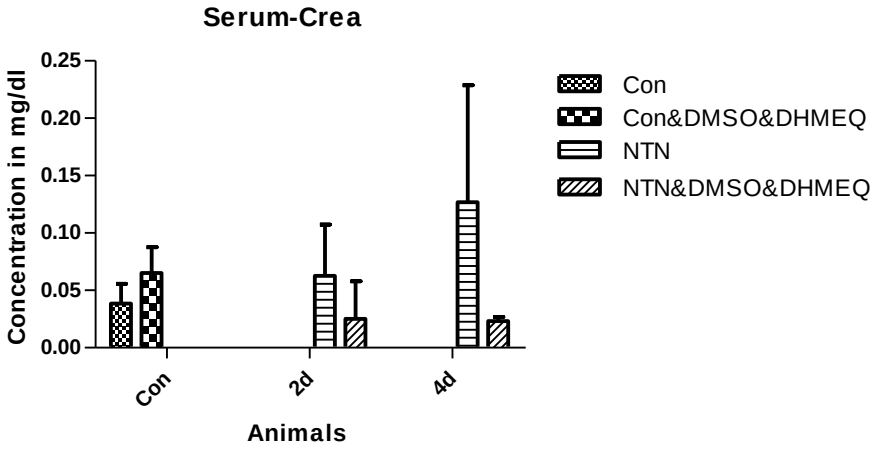


FIGURE 6: Serum level of creatinine after 2 days and 4 days treated animals and data are expressed as mean ± SD with P values > 0.05

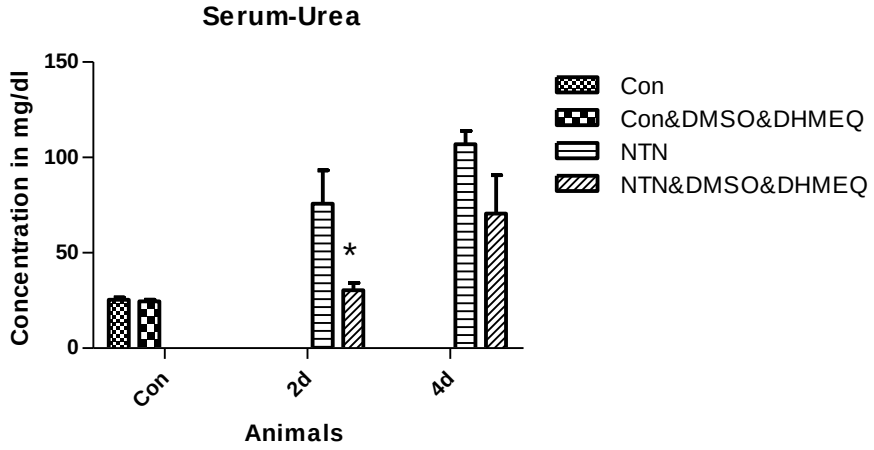


FIGURE 7: Serum level of Urea after 2 days treated animals and data are expressed as mean ± SD with *P values < 0.05 and 4 days treated animals P value > 0.05

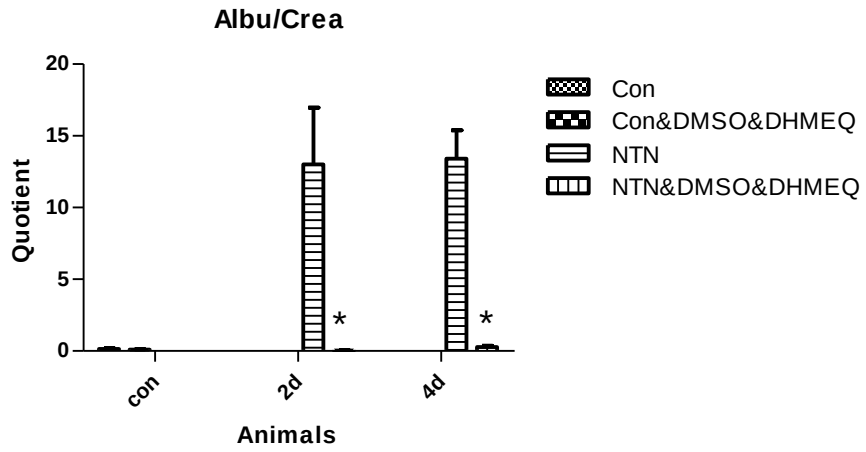


FIGURE 8: Serum level of albumine/creatinine quotient after 2 days and 4 days treated animals and data are expressed as mean ± SD with *P values < 0.05

3.1.2.3. Results of Histology and Immunhistology

Next, morphological examinations were performed to demonstrate whether improved functional parameters described above correlated with improved renal morphology.

In PAS-stained kidney sections glomerular score index of glomerular lesions was significantly reduced at 2 days and 4 days after NTN induction in mice treated with DHMEQ when compare to untreated nephritic animals (*p values 0.010 and 0.005) (*FIGURE 9*).

By immune-histochemistry the number of CD3-positive lymphocytes ((*p value 0.0006) and the number of F4/80-positive monocytes/ macrophages (p value < 0.05) infiltrating the kidneys was significantly reduced at 2 days after NTN induction in animals treated with DEMEQ, not however at day 4 after disease induction (*FIGURES 10 and 11*).

However, in control mice treated with DHMEQ dissolved in DMSO the number of CD3-positive and F4/80-positive cells was significantly increased ($p<0.05$) per kidney cross sectional area when compared with non-treated controls demonstrating that the solvent DMSO might be toxic to some extend for kidneys of control mice (*FIGURES 10 and 11*).

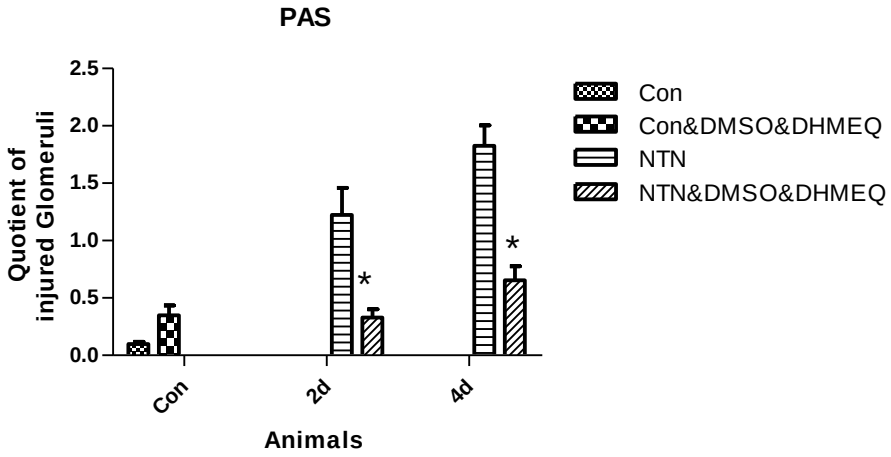


FIGURE 9: Histological scoring of injured glomeruli after 2 days and 4 days treated animals and data are expressed as mean ± SD with *P values < 0.05

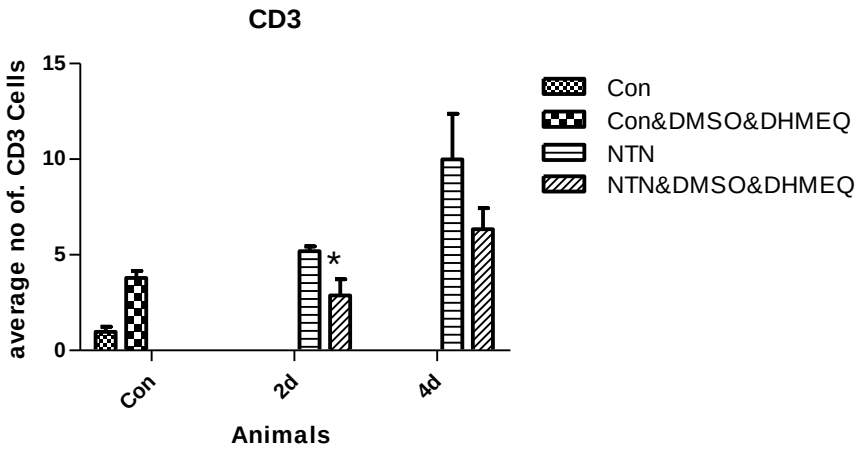


FIGURE 10: Histological scoring CD-3 cells after 2 days treated animals and data are expressed as mean ± SD with *P values < 0.05 and 4 days treated animals P value > 0.05

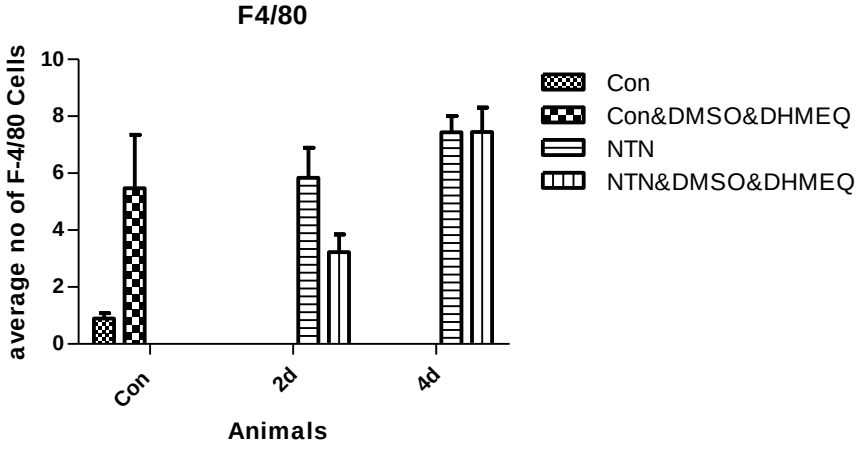


FIGURE 11: Histological scoring F4/80 cells after 2 days and 4 days treated animals and data are expressed as mean ± SD with P values > 0.05

3.1.1.1. Result of RT-PCR

As chemokines play a pivotal role in the regulation of inflammatory cells infiltrating kidneys after NTN-induction next the expression of the chemokines CCL2, CCL5 and CXCL10 was determined by RT-PCR.

The expression of the chemokines was significantly increased in mice 2 and 4 days after NTN-induction when compared with controls (p values < 0.05).

Expression of the chemokines CCL2 (*FIGURE 13*), CCL5 (*FIGURE 14*) and CXCL10 (*FIGURE 12*) was significantly (p values 0.049, 0.038 and 0.034 respectively) reduced in NTN-mice treated with DHMEQ 2 days after disease induction.

However, at day 4 chemokine expression demonstrated reduced CXCL10 (*FIGURE 12*), unchanged CCL5 (*FIGURE 14*) or even increased CCL2 (*FIGURE 13*) expression in DHMEQ-treated mice when compared with non-treated animals after NTN-induction. Along with the increased chemokine expression in DHMEQ treated controls the results further demonstrate that the solvent DMSO might be toxic to some extend for kidneys as the duration of the treatment period is increased.

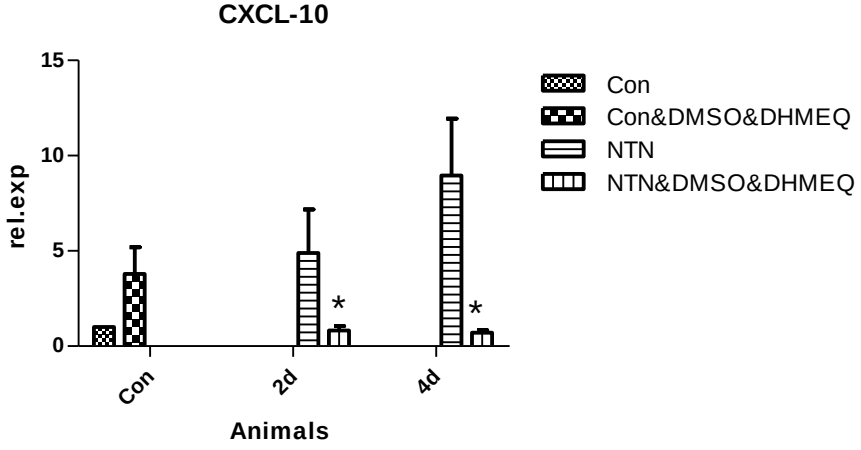


FIGURE 12: Expression level of CXCL-10 after 2 days and 4 days treated animals and data are expressed as mean ± SD *P values < 0.05

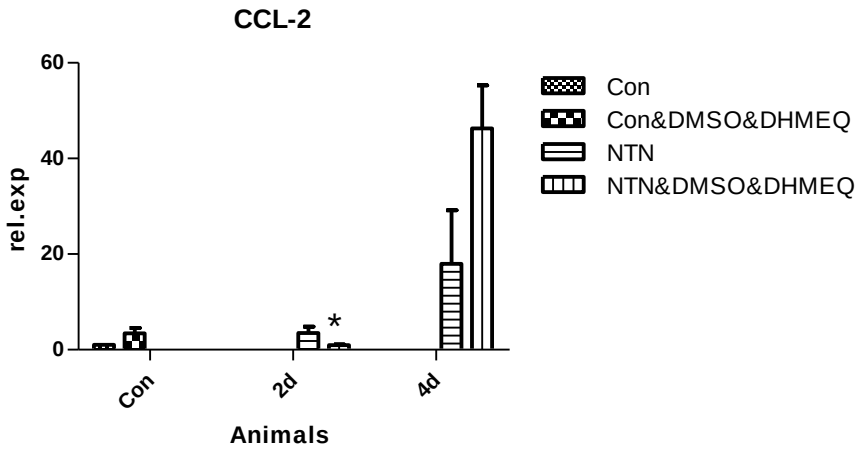


FIGURE 13: Expression level of CCL-2 after 2 days treated animals and data are expressed as mean ± SD *P value < 0.05 and 4 days treated animals P value > 0.05

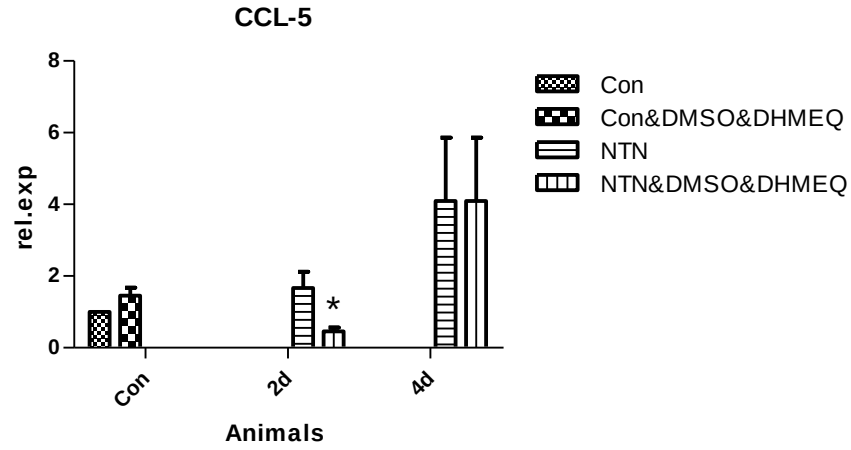


FIGURE 14: Expression level of CCL-5 after 2 days treated animals and data are expressed as mean ± SD *P value < 0.05 and 4 days treated animals P value > 0.05

3.1.2. Effect of DHMEQ with vehicle DMSO and cremaphor in LPS treated animals

Having seen the results that DMSO might have toxic effects on the kidneys to some extend we decided to compare the effect of two different solvents of DHMEQ on renal morphology and immune-histochemistry and also on the expression of chemokines.

The first solvent used was 0.5% carboxymethylcellulose and dimethyl sulfoxid (DMSO) as established. The second solvent we tested was Cremaphor.

There is lot of evidences about DHMEQ dissolved in 0.5% carboxymethylcellulose with DMSO in different studies, but there was no evidence yet in which Cremaphor as a vehicle for DHMEQ was demonstrated. However, as DMSO itself is toxic for mice in higher concentration cremaphor was tested to circumvent this problem.

For the experiments to test the different solvents for DHMEQ the LPS (lipopolysaccharide)-model of nephritis was used.

LPS was injected intraperitoneally and at the same time mice were injected subcutaneously with DHMEQ dissolved either in 0.5% carboxymethylcellulose with DMSO or in Cremaphor as a vehicle.

3.1.1.2. Result of Histology and Immunhistology

The results of glomerular morphology score and immune-histochemistry examinations using Cremaphore as vehicle for DHMEQ were very promising.

Glomerular morphology score was significantly reduced in DHEMQ-Cremaphore treated animals after LPS-injection when compared with non-treated LPS-injected mice ($p<0.019$) (*FIGURE 16*).

Even more promising were the results of immune-histochemistry demonstrating a significant ($p<0.016$ and $p<0.006$ respectively) reduction of CD3-positive and F4/80-positive renal cells in animals treated with DHMEQ-Cremaphore after LPS-injection when compared with DEMEQ-DMSO treated mice after LPS-injection (*FIGURES 15 and 17)*.

Also DHMEQ-Cremaphore did not increase the number of CD3-positive and F4/80-positive renal cells in control mice which is in contrast to control animals treated with DHMEQ-DMSO in which the number of infiltrating cells was significantly increased ($p<0.05$) (*FIGURES 15 and 17*).

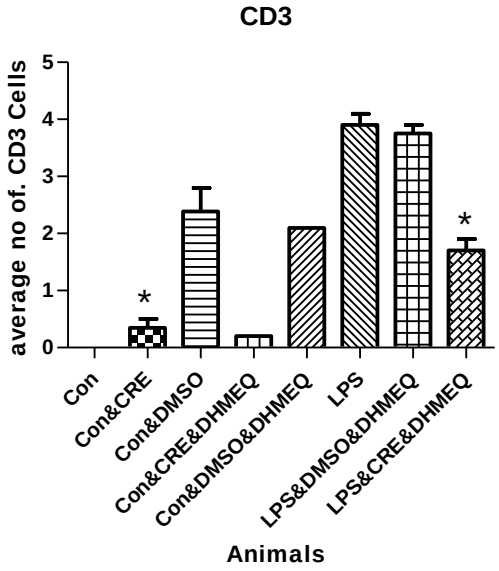


FIGURE 15: Histological scoring of CD-3 cells after 24 hours treated animals and data are expressed as mean ± SD with *P values < 0.05

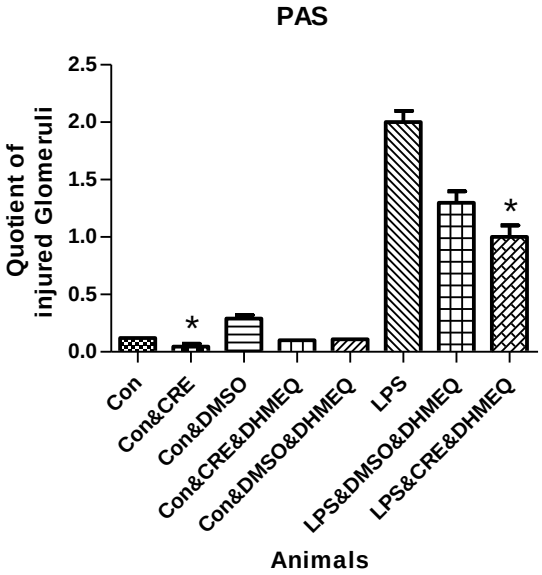


FIGURE 16: Histological scoring of injured glomeruli after 24 hours treated animals and data are expressed as mean ± SD with *P values < 0.05

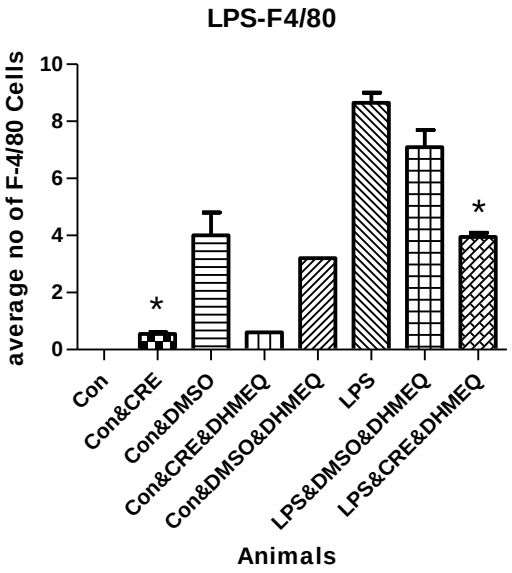


FIGURE 17: Histological scoring of F4/80 cells after 24 hours treated animals and data are expressed as mean ± SD with *P values < 0.05

3.1.1.1 Result of RT-PCR

We have seen a high relative expression of CCL2 and CCL5 in mice treated with LPS. Control mice treated with vehicle cremaphore only did not show a significant expression of these cytokines, however there is a significant difference between vehicle cremaphor and methylcellulose/DMSO treated mice (*P value < 0.05). Animals treated with DHMEQ dissolved in vehicle Cremaphor or DHMEQ did show a significant decrease of relative expression of CCL2 (*P value 0.026) (*FIGURE 19*) and CCL5 (*P value 0.031) (*FIGURE 18*) 24 hours after LPS-injection.

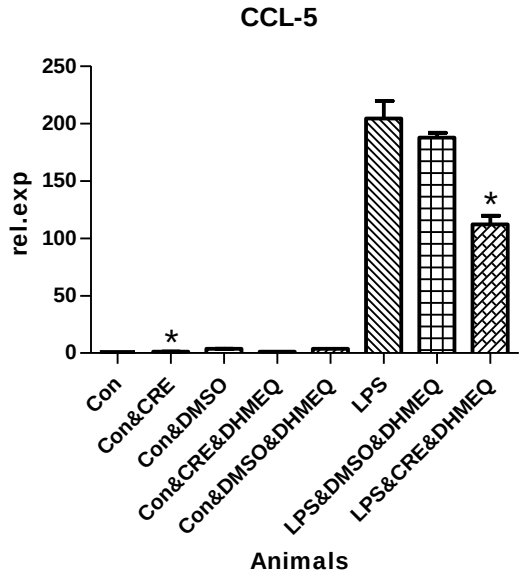


FIGURE 18: Expression level of CCL-5 after 24 hours treated animals and data are expressed as mean ± SD *P values < 0.05

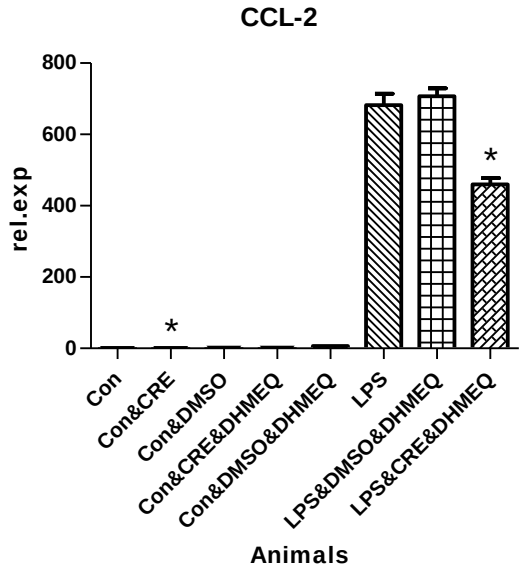


FIGURE 19: Expression level of CCL-2 after 24 hours treated animals and data are expressed as mean ± SD *P values < 0.05

According to these results we have demonstrated that DHMEQ can be dissolve in Cremaphor and the results demonstrate significantly improved morphological and chemokine expression data when Cremaphor is used as vehicle when compared to DMSO as a vehicle.

3.1.3. Effect of DHMEQ with a vehicle Cremaphor in NTN treated animals

After these finding we used cremaphor as a vehicle for our further experiments only. Now we examined another group of animals using Cremaphor as a vehicle. Therefore the following groups were examined: *1)* control animals not treated *2)* control animals treated with DHMEQ for 10 days, *3)* NTN animals not treated and *4)* NTN animals treated with DHMEQ dissolved in cremaphor. The following time points were examined after NTN induction: 2 days, 4 days, 10 days and 14 days.

3.1.3.2. Kidney function and proteinuria

Serum creatinine was significantly reduced in DHMEQ-treated mice when compared with non-treated animals after NTN-induction at all time points examined during the 14 days observation period (*p value<0.05) (*FIGURE 20*).

The urine albumin / creatinine quotient was also significantly reduced ($p < 0.01$) in treated when compared with non-treated nephritic animals at all time points examined (*FIGURE 21*).

These data demonstrate that kidney function and proteinuria significantly improved in DHMEQ treated nephritic animals at all time points examined.

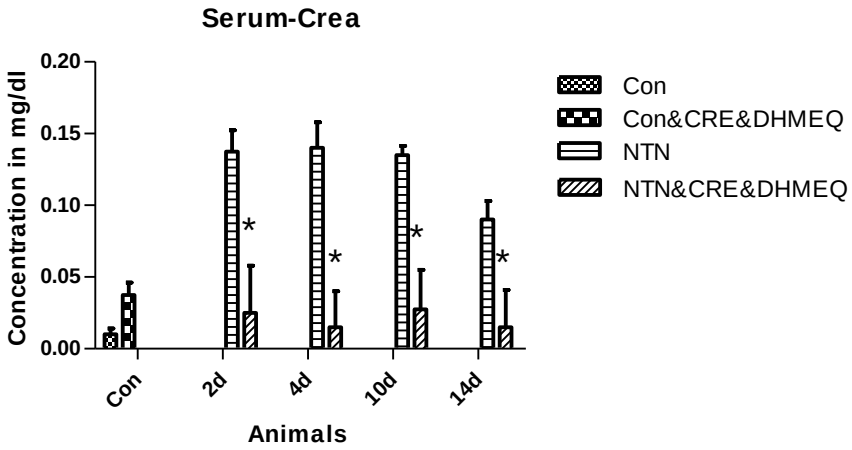


FIGURE20: Serum level of creatinine after 2 days (mean SD= 0.1125 ± 0.03603 and *P value 0.0205), 4 days (mean SD= 0.1250 ± 0.03069 and *P value 0.0066), 10 days (mean SD= 0.1075 ± 0.02825 and *P value 0.0089) and 14 days (mean SD= 0.0750 ± 0.02901 and *P value 0.0415) treated animals. Data are expressed as mean ± SD *P values < 0.05

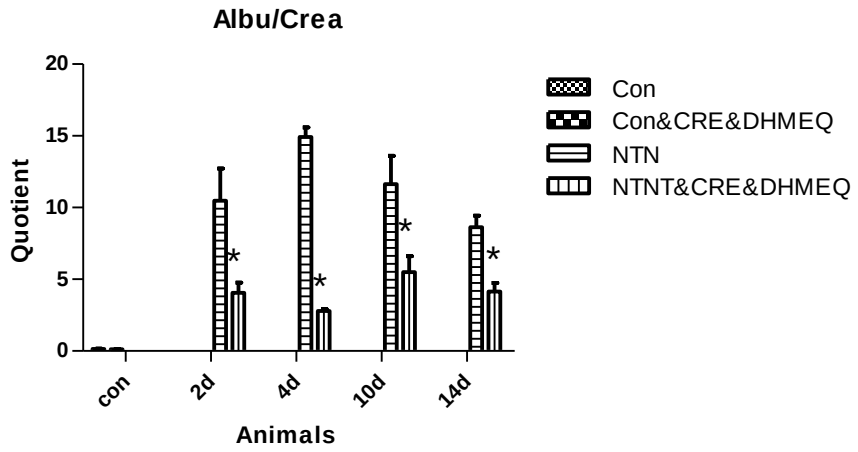


FIGURE21: Serum level of albumin/creatinine quotient after 2 days (mean SD= 6.425 ± 2.340 and *P value 0.0335), 4 days (mean SD= 12.15 ± 0.6695 and *P value < 0.0001), 10 days (mean SD= 6.120 ± 2.273 and *P value 0.0359) and 14 days (mean SD= 4.493 ± 0.9944 and *P value 0.0040) treated animals. Data are expressed as mean ± SD *P values < 0.05

3.1.3.3. Result of Histology and Immunohistology

Additionally, the histology and immunohistology results support the above findings.

In PAS-stained kidney sections the number of damaged glomeruli counted at days 2, 4, 10 and 14 after NTN-induction was significantly reduced in DHMEQ treated mice (*p value at least <0.05) (*FIGURE 22*).

By immunohistology the number of CD-3 and F-4/80 positive cells infiltrating kidneys after NTN-induction was significantly reduced in DHMEQ-treated mice when compared with untreated nephritic animals at all time points examined (*p value at least <0.05) (*FIGURES 23 and 24*).

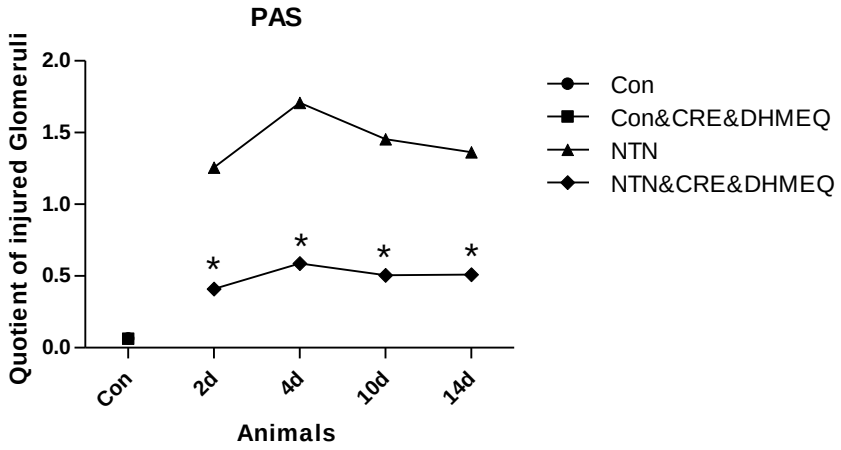


FIGURE22: Histological score of injured glomeruli after 2 days (mean SD= 0.8475 ± 0.2568 and *P value 0.0164), 4 days (mean SD= 1.120 ± 0.08453 and *P value < 0.0001), 10 days (mean SD= 0.9500 ± 0.09460 and *P value < 0.0001) and 14 days (mean SD= 0.8525 ± 0.09393 and *P value 0.0001) treated animals. Data are expressed as mean ± SD *P values < 0.05

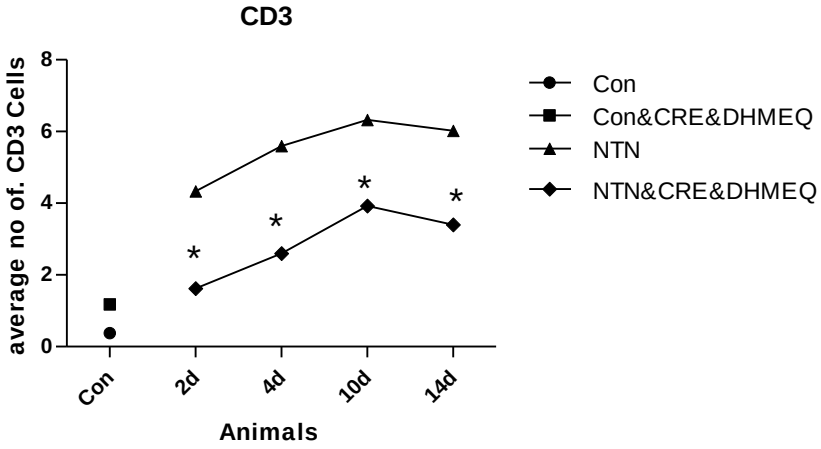


FIGURE23: Histological score of CD-3 cells after 2 days (mean SD= 2.713 ± 0.6093 and *P value 0.0043), 4 days (mean SD= 3.000 ± 0.6137 and *P value 0.0027), 10 days (mean SD= 2.400 ± 0.8404 and *P value 0.0290) and 14 days (mean SD= 2.625 ± 0.4732 and *P value 0.0015) treated animals. Data are expressed as mean ± SD *P values < 0.05

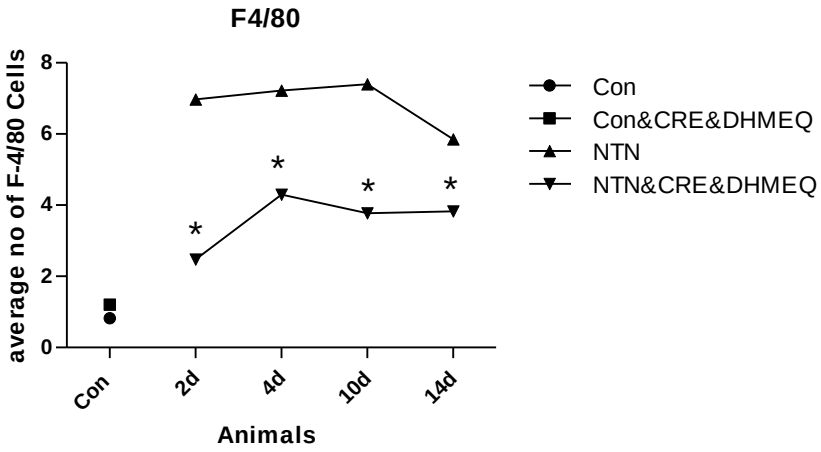


FIGURE24: Histological score of F4/80 cells after 2 days (mean SD= 4.500 ± 0.7797 and *P value 0.0012), 4 days (mean SD= 2.925 ± 0.4939 and *P value 0.0010), 10 days (mean SD= 3.625 ± 0.4479 and *P value 0.0002) and 14 days (mean SD= 2.025 ± 0.5757 and *P value 0.0126) treated animals. Data are expressed as mean ± SD *P values < 0.05

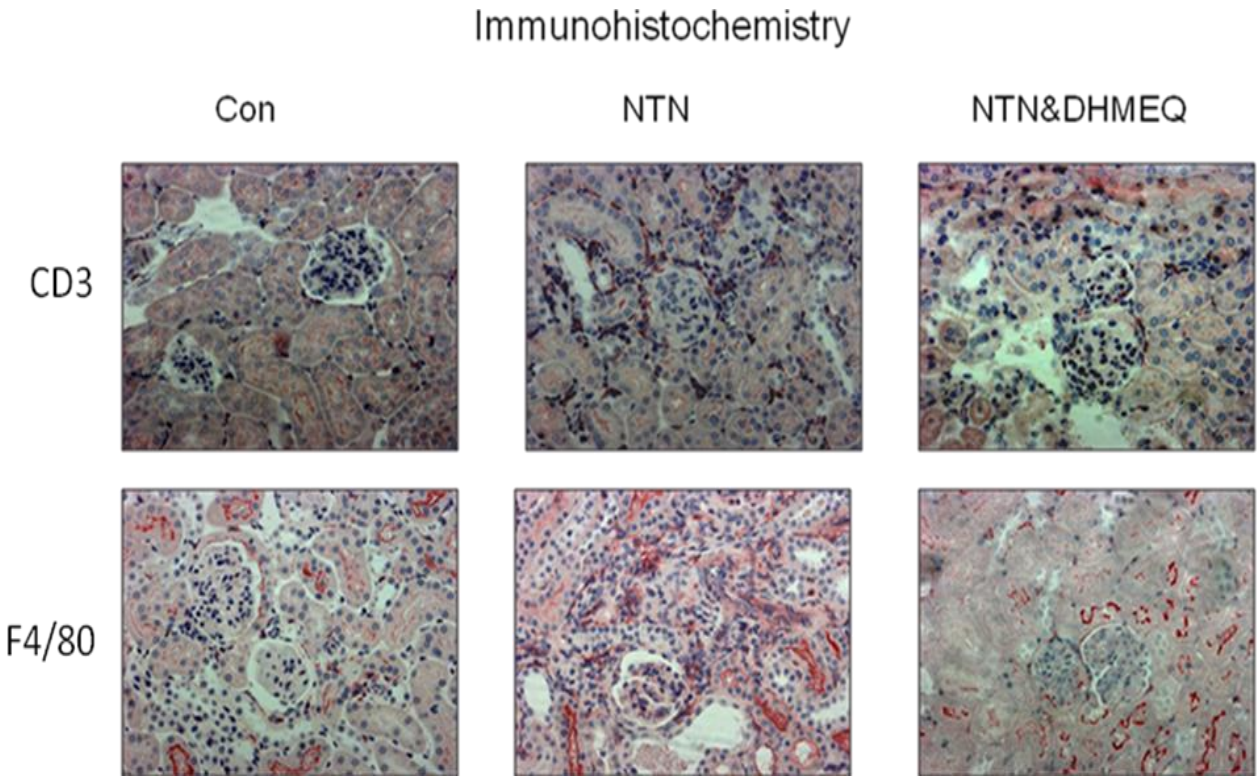


FIGURE25: Immunhistochemistry of control, NTN and DHMEQ treated animal. Glomerular and interstitial leukocytes infiltrates in mice. Leukocyte infiltration was determined by immunostainig for CD3 and F4/80 (Original magnification x200).

3.1.3.1. Result of RT-PCR

In this group of animals using Cremaphor as a vehicle, we observed a highly expression of cytokines, CCL-2 and CCL-5 chemokines after NTN induction.

We next examined the effect of DHMEQ. Treatment with DHMEQ at a concentration of 10 µg/ml known to abrogate NF-κB activation significantly reduced expression of chemokines CCL-2 (*p value <0.05) and CCL-5 (*p value <0.05) at 2 days, 4 days, 10 days and 14 days after NTN-induction, further supporting the results of the protective effect of DHMEQ in inflammatory kidney disease (*FIGURES 26 and 27*).

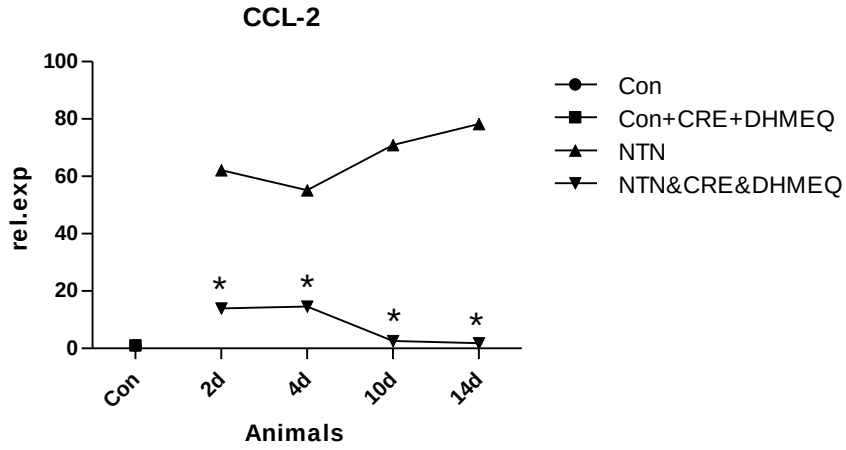


FIGURE26: Expression level of CCL-2 after 2 days (mean SD= 48.15 ± 11.75 and *P value 0.0064), 4 days (mean SD= 40.57 ± 10.88and *P value 0.0098), 10 days (mean SD= 68.39 ± 6.998 and *P value < 0.0001) and 14 days (mean SD= 76.42 ± 11.78 and *P value 0.0006) treated animals. Data are expressed as mean ± SD *P values < 0.05

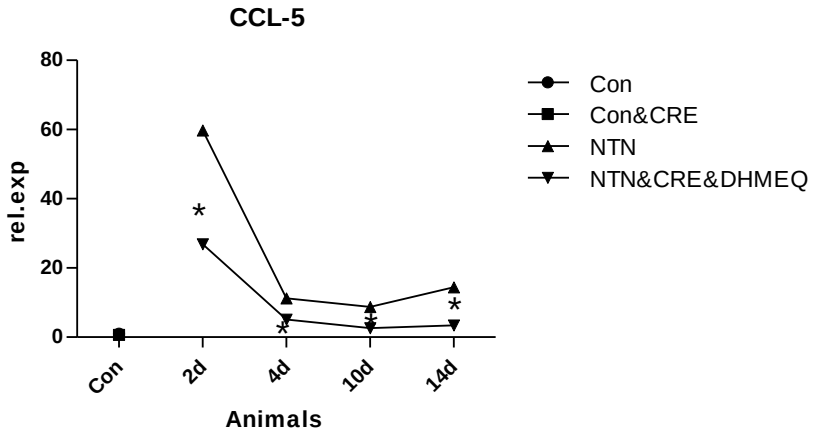


FIGURE27: Expression level of CCL-5 after 2 days (mean SD= 32.88 ± 10.82 and *P value 0.0228), 4 days (mean SD= 6.191 ± 2.260 and *P value 0.0338), 10 days (mean SD= 6.183 ± 1.613 and *P value 0.0086) and 14 days (mean SD= 11.04 ± 1.075 and *P value < 0.0001) treated animals. Data are expressed as mean ± SD *P values < 0.05

DHMEQ inhibited TNF-α and IL-1ß induction in our experiments in 10 days and 14 days treated animal but there is no inhibition of TNF-α and IL-1ß at 4 days after NTN-induction.

IP-10 expression has also been suppressed significantly reduced in all 14 days, 10 days and 4 days treated animals.

3.2.4. NF-kappa B activation after NTN induction

In the Blot of cytoplasmic protein with antibody phosphor-IκB-α we have shown high expression of protein with molecular weight of 30kD in NTN and in the animals treated with DHMEQ no significant change in the expression of this protein has been observed (FIGURE 28). The Blot of nuclear protein with antibody p65 we have seen high expression of this protein with molecular weight of 65kD in NTN, but in animals treated with DHMEQ expressed less amount of this protein (FIGURE 29). With these results we have confirmed

that DHMEQ inhibits NF-κB translocation to the nuclear compartment but does not inhibit IkappaBalpha activation in the cytoplasm.

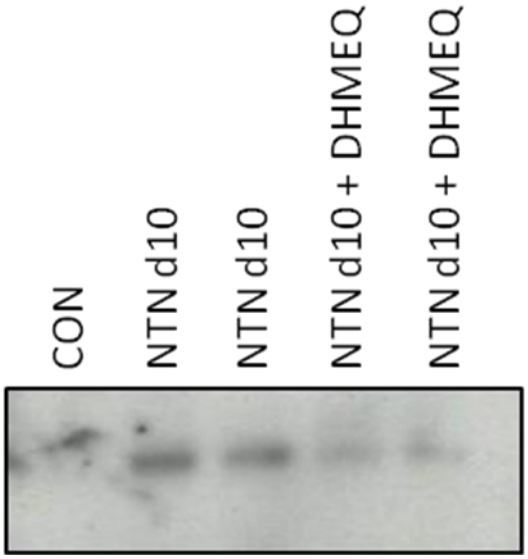


FIGURE29: Nuclear translocation of NF-kappaB p65-protein was significantly reduced in DHMEQ-treated mice 10 days after NTN-Induction when compared with untreated animals.

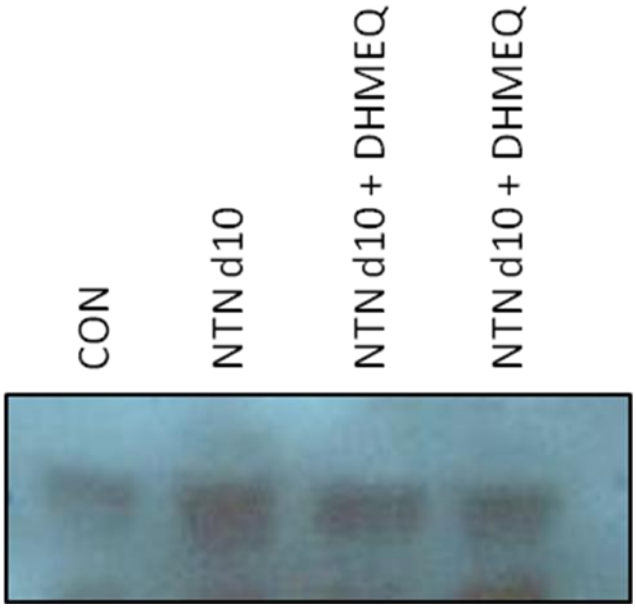


FIGURE28: Phosphorylation of IkappaBalpha is significantly increased after NTN-induction when compared with controls. DHMEQ treatment did not reduce phosphorylation of

IkappaBalpha significantly when compared with untreated NTN-nephritic mice. Results shown are at day 10 after NTN-induction.

4. Discussion

Inflammatory renal diseases such as glomerulonephritis are induced by activation of proinflammatory signaling pathways leading to the production of proinflammatory mediators such as cytokines and chemokines. In the last decades the molecular signaling pathways that initiate these inflammatory reactions have been extensively studied.

NF-kappa B is an inducible hetero- or homodimeric transcription factor composed of members of the Rel family of DNA-binding proteins that recognize a common sequence motif. Five mammalian Rel proteins have been identified so far, NF-kappa B1 (p50 and its precursor p105), NF-kappa B2 (p52 and its precursor p100), c-Rel, RelA (p65), and RelB. NF-kappa B is an important regulator of inflammation and immune responses by mediating pro-inflammatory gene expression including chemokines, cytokines, adhesion molecules and growth factors. Under basal conditions NF-kappa B dimers remain in the cytoplasm as an inactive form. A family of inhibitory proteins, I-kappa-Bs, binds to NF-kappa B and mask its nuclear localization signals, thereby blocking nuclear translocation. Exposure of cells to a variety of proinflammatory stimuli leads to the rapid phosphorylation, ubiquitinylation, and ultimately proteolytic degradation of I-kappa-B, which allows nuclear translocation of NF-kappa B and subsequent gene transcription.

We and others have described the activation of NF-kappa B in the induction phase of several experimental models of glomerulonephritis. Furthermore, it was shown that early NF-kappa B blockade in an experimental model of glomerulonephritis markedly reduced proteinuria, glomerular lesions, and monocytes/macrophages infiltration. Therefore, blocking of NF-kappa B represents an interesting new target for the treatment of renal inflammatory disease.

Numerous studies have shown that NF-κB is a key regulator of inflammation and is activated in experimental models of kidney inflammation including immune complex nephritis, anti-glomerular basement membrane nephritis and mesangial proliferative GN. These findings indicate NF-κB as a potential target for the treatment of renal inflammatory diseases.

Activation of NF-κB takes place in several steps. In numerous studies have illustrated that different pharmacological agents, such as glucocorticoids, aspirin, cyclosporin A and FK-506 inhibits various stages of NF-κB activation although these agents are not specific for NF-kB and have many others effects (T. Kosaka et al. 2008)

To inhibit NF-kappaB activation a recently described, low-molecular-weight NF-kappaB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), which was derived from the antibiotic epoxyquinomicin C was used. It is a highly specific NF-kappaB inhibitor that interferes with the nuclear translocation of activated NF-kappaB. DHMEQ exhibits anti-inflammatory, anti-fibrotic, and antitumor effects. In a macrophage-like cell line, DHMEQ inhibited phagocytic activity and cytokine production, suggesting that it may have immunosuppressive effects. However, the effect of DHMEQ on an experimental model of rapid progressive glomerulonephritis has not been determined. Therefore, we examined the effects of DHMEQ as a possible immunosuppressive agent by investigating whether DHMEQ could regulate NTN-nephritis in mice.

Only a limited number of studies testing the *in vivo* effects of small molecular weight compounds on nephritis have been reported (Sanz AB, Sanchez-Niño MD, et al. 2010). It has been shown previously that DHMEQ inhibited TNF-α-induced nuclear translocation of NF-kappaB, and does not inhibit phosphorylation and degradation of IkappaB, or a c-Jun N-terminal kinase (JNK) and a caspase-activating pathway in Jurkat T cells. Here, we extended these studies to test the therapeutic effect of DHMEQ on NTN, and to test the efficacy on the inhibition of the inflammatory pathway in this model of rapid progressive glomerulonephritis. The results showed that this unique inhibitor of NF-kappaB nuclear translocation may hold promise for treatment of NTN.

The number of known roles of NF-κB is rapidly increasing. There is much evidence that DHMEQ inhibits nuclear translocation. Therefore we examined whether DHMEQ reducing the inflammatory cytokines (M. Yamamoto et al. 2008).

For our study firstly we used two different vehicles to solve DHMEQ. One was Cremaphor and the other was DMSO with Methylcellulose. There is lots of evidence, in which it has been

demonstrated that DHMEQ can solve in DMSO but there is no evidence till yet in which Cremaphor used as a vehicle for DHMEQ. We have seen slightly better result with Cremaphor rather than the DMSO. So we used only cremaphor for our further experiments. In our study we have also demonstrated that the DMSO itself toxic for the animals with high concentration. We have seen an expression of inflammatory cytokines in control animals injected only with vehicle DMSO compare to cremaphor.

DHMEQ was derived from antibiotic epoxyquinomicin C and inhibits NF-κB activation in vitro and in vivo with blockade of the nuclear translocation of NF-κB being a potential mechanism of action. DHMEQ specifically inhibits NF-κB in various disease models in different organs in which NF-κB is involved without evidence of toxicity (T. Kosaka et al. 2008)

Results of DHMEQ treated animals with vehicle DMSO at a concentration of 10ug/ml gave some abrogated constitutive NF-κB activity and at the same time showed expression of chemokines in different days of treatment some disturbing results. Histology and immunhistology results are also correlated to the above results. Again the results of control animal with DMSO as a vehicle control only, supports our above statement of toxic effect of DMSO in mice and showing as same as above a high number of CD-3 and F4/80 cells compare to control animals.

After this we have analyzed the serum of treated animal with DHMEQ dissolved in DMSO. It was showing a less concentration of serum creatinine compare to animal with NTN, however control animals injected with DMSO only as a vehicle showing a high concentration of serum creatinine compare to control one. Again, this result give us a hint that DMSO seemed to be toxic itself for mice.

Now using Cremaphor as a vehicle we next examined the effect of DHMEQ constitutively. Treatment with DHMEQ at a concentration of 10µg/ml abrogated constitutive NF-κB activity. It has been demonstrated a strongly decreased expression of cytokines CCL-2 (*P value <0.05) and CCL-5 (*P value <0.05) in all period of time 2 days, 4 days, 10days and 14days treated animals, showing the positive and protective effect of DHMEQ in inflammation. The

vehicle control of cremaphor in control animals doesn't show any expression. It is showing that the animals endure cremaphor without any problem.

Serum creatinine concentration and the ratio of Urine alb/crea demonstrated very positive results as so far, in all 2 days, 4 days, 10 days and 14 days treated animals with DHMEQ dissolved in cremaphor. It has been showing the animal with less protenurie and more excretion of creatinine in all 2, 4, 10 and 14 days treated animals (*P value <0.05). In other words it said that the kidney function is improving after treatment with DHMEQ in all days treated animals. In addition to this the histology and immunhistology results does support the above results.

Now if we just look at the results of treatment of different time periods. We can see that DHMEQ with a vehicle cremaphor does inhibit the induction of NF-κB in all 2, 4, 10 and 14 days treated animals Results of RT-PCR has been shown less CCL-5, CCL-2, CXCL-10, TNF-α and IL-1b expressions significantly.

Same kind of results observed in histology and immunhistology and correlate to the above results. In Western-Blot it has been showed that the DHMEQ inhibits NF-κB on a translocation level. In the first peak of inflammation, p65/p50 heterodimer protein complex is dominant. P65 protein is normally active in nucleus. . We have seen in Western-Blot less expression of both the protein in the DHMEQ treated animals.

The present study demonstrated the activation of NF-κB in the NTN model at different days. Treatment with DHMEQ significantly reduced NF-κB activation, glomerular proliferation, crescent formation, mesangial matrix deposition and significantly increased the level of glomerular apoptosis. These results suggest that specific inhibition of NF-κB activation by DHMEQ suppresses the inflammatory response in NTN.

In summary, we demonstrated specific inhibition of NF-kappaB by DHMEQ in the NTN nephritis model without evidence of toxicity. The inhibition of NF-kappaB was associated with diminished infiltration of inflammatory cells, reduced glomerular damage index, reduced

chemokine expression and a significant improvement of renal functional parameters. The targeting of NF-kappaB activation by DHMEQ may represent a promising new treatment option for patients with inflammatory renal diseases. So all in general it showing that DHMEQ solved in Cremaphor and on the initial time and in the later stage of disease does influence the expression of inflammatory cytokines and chemokines significantly. It should be carry on in order to find, what factors does influence in it? Is there any other process that plays an important role?

5. Conclusion

We have demonstrated in our experiments that DHMEQ suppressed inflammatory cytokines CCL-2, CCL-5, CXCL-10 and TNF-α at 2 days, 4 days, 10 days and 14 days after the induction of NTN. These results have been supported by morphology, where PAS staining showed significantly less damaged glomeruli and significantly reduced numbers of CD-3 and F4/80 positive cells as demonstrated by immune-histochemistry.

In the present study, we demonstrated that the newly developed NF-kappaB inhibitor DHMEQ markedly improved renal function and morphology and suppressed pro-inflammatory cytokine levels in the mice with NTN.

Therefore targeting of NF-kappaB activation by DHMEQ may represent a promising new treatment option for patients with inflammatory renal diseases.

6. References

1 A. Ariga, J. Namekawa, N. Matsumoto, J. Inoue, K. Umezawa, "Inhibition of Tumor Necrosis Factor-α-induced Nuclear Translocation and activation of NF-κB by Dehydroxymethylepoxyquinomicin" The Journal of Biological Chemistry 277, 24625-24630 (2002)

2 C. Kurts, F. Heymann, V. Lukacs-Korneck, P. Boor, and J. Floege „Role of T cells and dendritic cells in glomerular immunopathology „ Semin Immunopatholog 29, 317-335 (2007)

3 E. Suzuki, Y. Ninomiya, K. Umezawa,"Induction of histidine decarboxylase in macrophages inhibited by the novel NF-κB inhibitor (-)-DHMEQ", Biochemical and Biophysical Research Communications 379, 379–383(2009)

4 F. Wan, M. J Lenardo "The nuclear signaling of NF-κB: current knowledge, new insights, and future perspectives", Cell Research 20:24-33 (2010)

5 K. Umezawa and C. Chaicharoenpong, Molecular Design and Biological Activities of NF-κB Inhibitors", Mol. Cells, 14, 163-167 (2002)

6 K. Wakamatsu, T. Nanki, N. Miyasaka, K. Umezawa and T. Kubota, „Effect of a small molecule inhibitor of nuclear factor-κB nuclear translocation in a murine model of arthritis and cultured human synovial cells „Arthritis Research & Therapy 7, R1348-R1359 (2005)

7 K. Shinoda, K. Nakagawa,T. Kosaka, N. Tanaka, T. Maeda, H. Kono, R. Mizuno, E. Kikuchi, A. Miyajima, K. Umezawa, M. Oya, „Regulation of human dendritic cells by

a novel specific nuclear factor-κB inhibitor, dehydroxymethylepoxyquinomicin, Human Immunology 71, 763-770 (2010)

8 M. S. Hayden and S. Ghosh, „Signaling to NF-kappaB“, Genes Dev 18, 2195-2224 (2004)

9 M. Watanabe, M.Z. Dewan, M. Taira, M. Shoda, M. Honda, T. Sata, M. Higashihara, M. E. Kadin, T. Watanabe, N. Yamamoto, K. Umezawa and R. Horie” IκBa independent induction of NF-kB and its inhibition by DHMEQ in Hodgkin/Reed-Sternberg cells“ Laboratory Investigation 87, 372–382 (2007)

10 M. Yamamoto, R. Horie, M. Takeiri, I. Kozawa, and K. Umezawa, „Inactivation of NF-KB Components by Covalent Binding of (-)-Dehydroxymethylepoxyquinomicin to Specific Cysteine Residues“, Journal of Medicinal Chemistry 51, 5780–5788 (2008)

11 M. Watanabe, M. Nakashima, T. Togano, M. Higashihara, T. Watanabe, K. Umezawa, R. Horie, „Identification of the RelA domain responsible for action of a new NF-kB inhibitor DHMEQ“, Biochemical and Biophysical Research Communications 376, 310–314 (2008)

12 M. Murohashi, K Hinohara1, M Kuroda, T Isagawa, S Tsuji, S Kobayashi, K Umezawa, A Tojo,H Aburatani and N Gotoh, „Gene set enrichment analysis provides insight into novel signalling pathways in breast cancer stem cells“, British Journal of Cancer 102, 206-212 (2010)

13 N. Lampiasi, A. Azzolina, N. D’Alessandro, K. Umezawa, J. A. McCubrey, G. Montalto, and M. Cervello, „Antitumor Effects of Dehydroxymethylepoxyquinomicin, a Novel Nuclear Factor-B Inhibitor, in Human Liver Cancer Cells Are Mediated through a Reactive Oxygen Species-Dependent Mechanism“, Mol Pharmacol 76, 290–300 (2009)

14 N. Dabaghmanesh, A. Matsubara, A. Miyake, K. Nakano, T. Ishida, H. Katano, R. Horie, K. Umezawa and T. Watanabe, „Transient inhibition of NF-κB by DHMEQ induces cell death of primary effusion lymphoma without HHV-8 reactivation“, Cancer Science 100 (4), 737-746 (2009)

15 P. G. Tipping and A. R. Kitching „Glomerulonephritis, Th1 and Th2: What's new?“, Clin Exp Immunol 142, 207-215 (2005)

16 S. Ghosh, M. J. May, and E. B. Kopp „NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses” Annu Rev Immunol 16, 225-260 (1998)

17 S. Segrer, F. Heller, M. T. Lindenmeyer, H. Schmid, C. D. Cohen, D. Draganovici, J. Mandelbaum, P. J. Nelson, H. J. Grone, E. F. Grone, A. M. Figel, E. Nossner, and D. Schlondorff „Compartment specific expression of dendritic cell markers in human glomerulonephritis” Kidney Int 74, 49-62 (2007)

18 Sanz AB, Sanchez-Niño MD, Ramos AM, Moreno JA, Santamaria B, Ruiz-Ortega M, Egido J, Ortiz A, “NF-kappaB in renal inflammation“, J Am Soc Nephrol. 21(8), 1254-62 (2010)

19 T. Chiba, Y. Kondo, S. Shinozaki, E. Kaneko, A. Ishigami, N. Maruyama, K. Umezawa, and K. Shimokado, „ A selective NFκB inhibitor, DHMEQ, reduced atherosclerosis in ApoE-deficient mice“, Journal of Atherosclerosis and Thrombosis 13, 308-313 (2006)

20 T. Kubota, M. Hoshino, K. Aoki, K. Ohya, Y. Komano, T. Nanki, N. Miyasaka and K. Umezawa „NF-κB inhibitor dehydroxymethylepoxyquinomicin suppresses osteoclastogenesis and expression of NFATc1 in mouse arthritis without affecting

expression of RANKL, osteoprotegerin or macrophage colony-stimulating factor", Arthritis Research and Therapy 9, 1186-2298 (2007)

21 T. Kosaka, A. Miyajima, E. Kikuchi, Y. Horiguchi, K. Umezawa, T. Ohigashi, J. Nakashima, T. Asano, M. Oya „The Novel NF-κB Activation Inhibitor Dehyroxymethyl-Epoxyquinomicin Suppresses Anti-Thy1.1-Induced Glomerulonephritis in Rats" Nephron Exp Neprhol 110, 17-24 (2008)

22 T. Suzuki, K. Yamashito, W. Jomen, S. Ueki, T. Aoyagi, M. Fukai, H. Furukawa, K. Umezawa, M. Ozaki, S. Todo "The Novel NF-κB Inhibitor, Dehydroxymethylepoxyquinomicin, prevents local and remote organ injury following intestinal ischemia/reperfusion in rats" Journal of surgical research 149, 69-75 (2008)

23 T. Lawrencea, C. Fonga, „The resolution of inflammation: Anti-inflammatory roles for NF-kB", The International Journal of Biochemistry & Cell Biology 42, 519–523 (2010)

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8. Affidavit

I do assure formally that I compose this work independently and without any help or assistance from others. Other as the sources indicated by me and remedy uses and made would have the places taken out of the used works literally or in substance individually after edition (edition and year of the appearance), volume and side of the used work recognizable. Furthermore I assure that I produced the dissertation not yet a specialty representative at another university for inspection or applied myself other around allowance to the graduation.

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