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Thionamides inhibit the transcription factor NF-kappaB by suppression of the small GTPase Rac1 and IKKalpha

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Running title: Anti-inflammatory potential of thioureylenes

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Non-standard abbreviations: CD, cluster of differentiation; GEF, guanine nucleotide exchange factor; GTPase, guanosine triphosphatases; IKK, inhibitor of kappaB kinase; IκB, inhibitor of kappaB; LDH, lactate dehydrogenase; MLK-3, mixed lineage kinase 3; NEMO, NF-kappaB essential modulator; Pak-PBD, p21 activated kinase 1-p21 binding domain; ZAP, zeta-associated protein.

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Abstract

Thionamides, inhibitors of the thyroid peroxidase-mediated iodination, are clinically used in the treatment of hyperthyroidism. However, the use of anti-thyroid drugs is associated with immunmodulatory effects and recent studies with thionamide related heterocyclic thio-derivates demonstrated direct anti-inflammatory and immunosuppressive properties. Using primary human T lymphocytes, we show that the heterocyclic thionamides carbimazole and propylthiouracil inhibit synthesis of the pro-inflammatory cytokines TNFα and IFNγ. In addition, DNA-binding of NF-κB, a pro-inflammatory transcription factor that regulates both TNFα and IFNγ synthesis and NF-κB dependent reporter gene expression were reduced. Abrogation of NF-κB activity was accompanied by reduced phosphorylation and proteolytic degradation of IκBα, the inhibitory subunit of the NF-κB complex. Carbimazole inhibited NF-κB via the small GTPase Rac-1, whereas propylthiouracil inhibited the phosphorylation of IκBα by its kinase IKKα. Methimazole had no effect on NF-κB induction, demonstrating that drug potency correlated with the chemical reactivity of the thionamide associated sulfur group. Taken together, our data demonstrate that thioureylenes with a common, heterocyclic structure inhibit inflammation and immune function via the NF-κB pathway. Our results may explain the observed remission of pro-inflammatory diseases upon anti-thyroid therapy in hyperthyroid patients. The use of related thioureylenes may provide a new therapeutic basis for the development and application of anti-inflammatory compounds.

Introduction

Thioureylenes, containing a thionamide group are the most widely used drugs to treat hyperthyroidism, a wide spread disease commonly caused by raised circulating levels of thyroid peroxidase antibodies and increased levels of serum thyroid stimulating hormone (Farwell and Braverman, 1996). Anti-thyroid thionamide treatment inhibits the thyroid peroxidase and reduces excessive thyroid hormone synthesis (Farwell and Braverman, 1996). Furthermore, the application of thionamides has been implicated with immune suppression characterized by lactoperoxidase inhibition, diminished antigen presentation, reduced release of pro-inflammatory mediators, T-cell abnormalities, and decreased IL-2 receptor expression (Bandyopadhyay, et al., 2002;Pearce, 2004;Volpe, 2001). In hyperthyroid patients, thionamide treatment has been associated with protection from chronic and pathological inflammation (Dagia, et al., 2004;Elias, 2004), autoimmune disorders (Mozes, et al., 1998;Singer, et al., 1994), and hypertrophic or inflammatory heart disease (Hardiman, et al., 1997).

The aberrant activation of the transcription factor nuclear factor-kappaB (NF-κB) is involved in chronic and pathological inflammation, autoimmunity, sepsis, and heart disease (Kumar, et al., 2004), arguing that this transcription factor might participate in thionamide mediated side effects. In most cell types, NF-κB is sequestered in the cytoplasm by the IκB family of proteins (Karin and Delhase, 2000). Following Rac-1 and IκB-kinase (IKK) activation, IκB proteins become phosphorylated, ubiquitinilated, and degraded by the proteasome (Karin and Delhase, 2000;Marinari, et al., 2002;Piccolella, et al., 2003). Subsequently, NF-κB proteins translocate to the nucleus to bind their cognate DNA sequences and to initiate transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, enzymes, and anti-microbial peptides.

Recently, we described thio-barbiturates as potent inhibitors of IKKs, NF-κB, and pro-inflammatory cytokine production (Loop, et al., 2002;Loop, et al., 2003). Although thio-barbiturates and thionamides have different clinical utilities, both are thioureylenes with a related structure and overlapping cellular activities. Thus, thio-barbiturates reduce thyroid function (Farwell and Braverman, 1996) and inhibit adaptive and cellular immune responses (Correa-Sales, et al., 1997;Nishina, et al., 1998).

In the present study we investigated whether thionamides, comparable to the structurally related barbiturates, impede immune functions by a general, structure dependent mechanism. Here we demonstrated that thionamides inhibited the activation of the transcription factor NF-κB, which might be responsible for the remission of some immune-related diseases observed during anti-thyroid treatment of patients. Comparable to thio-barbiturates, we found that propylthiouracil inhibited the IKK-dependent phosphorylation of IκBα, whereas carbimazole repressed the proximal GTPase Rac-1. The biological effects were mediated by redox-active sulphur of thionamides. Because thioureylenes affect ubiquitously expressed proteins that regulate important cellular responses in health and disease, the development of new pharmaceutical compounds on a mononuclear heterocyclic thioureylene basis as key building blocks in medicinal chemistry may evolve.

Methods

Isolation and treatment of human primary T lymphocytes

Peripheral blood mononuclear cells were purified by Ficoll-Paque™ PLUS (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) centrifugation of whole blood. T-cells were enriched by immunomagnetic cell sorting with anti-CD3 microbeads using LS^+ selection columns (Miltenyi Biotech, Auburn, CA). Isolated cells were suspended in RPMI 1640, supplemented with 10 mM Hepes/pH 7.3, 50 μM β-mercaptoethanol, and 2 mM L-glutamine. Cell suspensions were pretreated with methimazole (Sigma, St. Louis, MO), propylthiouracil (Sigma, St. Louis, MO) or carbimazole (LKT Laboratories, St. Paul, MN) at indicated concentrations. T lymphocytes were activated by CD3/CD28 crosslinking with T cell Expander Dynabeads® (Dynal Biotech, Lake Success, NY) or 15 ng/ml phorbol 12-myristate 13-acetate (Sigma).

Cytokine ELISA

Cytokine concentrations in supernatants of 2 x 10^6 primary human T lymphocytes were analyzed using the human TNF-α or IFNγ Quantikine® Immunoassay (R&D Systems, Minneapolis, MN). Cells were seeded at 10^7 T-cells/ml and pre-incubated with various concentrations (0.1 – 5 mM) of thionamides in different wells of a 96–well round bottom plate (Greiner Bio-One GmbH, Frickenhausen, Germany). T-cells were induced by 10^6 T-cell Expander Dynabeads® 2 h after the onset of thionamide treatment. After 15 h supernatants were collected, diluted 1:5 in Assay Diluent, and the TNF-α or IFNγ concentration determined according to manufacturer's instructions.

NF-κB dependent luciferase reporter gene expression

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Jurkat cells were transiently transfected with 2 µg pNF-κB-Luc (Clontech, Mountain View, CA), using Lipofectamine^{TM}2000 (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Transfection reactions were pooled, redistributed at 10^5 cells per well, and incubated with thionamides and 15 ng/ml phorbol 12-myristate 13-acetate for 15 h. Luciferase reporter gene expression was measured by harvesting cells in 100 µl luciferase reporter lysis buffer (Promega, Madison, WI) and assaying in a Microluminat Plus LB 96P luminometer (Berthold Technologies, Bad Wildbach, Germany). Protein levels were normalized by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

ELISA based NF-κB transcription factor activity assay

Nuclear extracts were analyzed by the TransAM™ NF-κB family transcription factor activation assay according to the manufacturer's recommendations (Active Motif, Carlsbad, CA). NF-κB complexes were captured by binding to a consensus 5'-GGGACTTTCC-3' oligonucleotide immobilized on a 96-well plate. The contend of bound NF-κB was determined by different primary antibodies, detecting p50 or p65 proteins followed by a secondary horseradish peroxidase conjugated goat anti-rabbit IgG for spectrophotometric detection at OD_{450} nm. Data were expressed as percentage of NF-κB/DNA binding compared to activated cells (=100%).

Nuclear protein extraction and electrophoretic mobility shift assays (EMSA)

Preparation of nuclear cell extracts and EMSAs were performed as described previously (Loop, et al., 2002). For DNA-binding, the NF-κB motif 5'-AGTTGAGGGGACTTTCCCAGGC-3' was used as a probe. Binding reactions were carried out at room temperature for 30 min in a volume of 20 µl containing 20 µg nuclear cell extract, 22 mM Hepes/pH 7.9, 70 mM KCl, 50 µM EDTA, 2.2 mM dithiothreitol, 2% glycerol, 4%

Ficoll, 0.1% Nonidet P40, 30 µM phenylmethylsulfonyl fuoride, 20 µg of bovine serum albumin, 2 µg of poly(dI-dC) and 1.75 pmol of ^{32}P end-labeled oligonucleotides (5 µCi/pmol). The resulting DNA-complexes were displayed by electrophoresis on 4% nondenaturating polyacrylamide gels and subsequent autoradiography.

IκBα - phosphorylation and degradation

Primary human $CD3^+$ T lymphocytes were pre-incubated at 2 x 10^6 T-cells per well with various concentrations (0.1 – 5 mM) of thionamides for 4 h. Additionally, lymphocytes were exposed to 10 µM MG132 (Calbiochem, San Diego, CA) for measurement of IκBα phosphorylation, and 5 µg/ml actinomycin D (Sigma) for measurement of IκBα degradation. CD3/CD28 receptor stimulation was induced by 10^6 T-cell Expander Dynabeads® for the last 20 min (IκBα phosphorylation) or 10 min (IκBα degradation) of the experiment. Reactions were terminated by the addition of 30 µl of 3 x SDS sample buffer and boiling. Proteins were separated by 10% SDS-PAGE gels. Immunoblots were analyzed with 0.1 µg/ml of a phosphospecific IκBα(Ser32) antibody (New England Biolabs, Ipswich, MA) or 0.1 µg/ml of an IκBα(total) antibody (New England Biolabs). β-actin was recognized by 20 ng/ml of a β-actin antibody (New England Biolabs). Specific bands were visualized using secondary horseradish peroxidase conjugated antibodies and enhanced chemiluminescence reagents (GE Healthcare).

In vitro kinase assay

T-lymphocytes were lysed for 20 min at 4°C in a volume of 600 µl kinase buffer (20 mM HEPES, 2 mM dithiothreitol, 10 mM NaF, 1 mM Na_3VO_4, 10 mM ß-glycerophosphate, 0.5% Nonidet P-40, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.2 µg/ml pefabloc™). For the kinase reaction 100 µl of cell lysate, 2 µg of GST-IκBα sepharose

beads (Santa Cruz Biotechnology, Santa Cruz, CA), and 0.5 µmol ATP were incubated for 2 h at 30°C. Subsequently, GST-IκBα sepharose beads were repeatedly washed by kinase buffer and analyzed for phospho-IκBα (Ser32) or total IκBα by immunoblotting.

Alternatively, IKKα proteins were immunoprecipitated from cell lysates with 2 µg of anti-IKKα antibody-sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C under constant rotation. The precipitates were washed four times in kinase buffer. Kinase reactions were performed for 20 min at 22°C in 20 µl of kinase buffer containing 10 µM ATP, 5 µCi of (γ-^{32}P)ATP, and 4 µg of GST-IκBα (Santa Cruz Biotechnology) for each immunoprecipitate. Reactions were terminated by addition of 5 x SDS loading buffer and separated by 10% SDS-PAGE. Gels were fixed, dried and analyzed by autoradiography.

Analysis of G-proteins

Rac-1 activity was determined by the Rac activation assay kit (Cytoskeleton, Denver, CO). Briefly, 2 x 10^7 T-cells were harvested and lysed in 300 µl of ice-cold extraction buffer (50 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fuoride, 1% Nonidet P-40, 10% glycerol, 1 µg/ml leupeptin, and 2 µg/ml aprotinin). Where indicated, cell lysate supernatants were induced by 100 µM GTPγs plus 1 mM EDTA on a rocker platform for 30 min at room temperature. Reactions were stopped by the addition of 50 mM $MgCl_2$. For Rac-1 precipitation, cell lysate supernatants were incubated with 20 µl of GST-PAK PBD protein beads for 40 min at 4°C under constant rotation. Protein beads were repeatedly washed with 1 ml of extraction buffer, then resuspended and boiled in 40 µl of 2 x SDS sample buffer. Proteins were separated by 12% SDS-PAGE and analyzed for Rac-1 precipitation by immunoblotting using 0.25 µg/ml anti-Rac-1 (clone 102; BD Biosciences, San Jose, CA). Total cell extracts were analyzed by immunoblotting using an anti-Rac-1 antibody (Cytoskeleton) and an anti-phosphotyrosine antibody (clone 4G10; Upstate, Lake Placid, NY).

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Immunoblotting

Proteins from cell lysates were separated by SDS-PAGE and electroblotted to a PVDF membrane (Millipore Corporation, Billerica, MA). For immunodetection antibodies were used according to manufacturer's recommendations. Antibodies were directed against IκBα(Ser32) (New England Biolabs), IκBα (New England Biolabs), β-actin (New England Biolabs), IκBα(Ser32/36) (New England Biolabs), GST (Rockland Immunochemicals, Gilbertsville, PA), IKKα (Santa Cruz Biotechnology), phosphotyrosines (PharMingen, San Diego, CA), ZAP 70(Tyr318)/Syk(Tyr352) (New England Biolabs), VAV-1(Tyr160) (Sigma), Rac-1(Ser71) (New England Biolabs), Rac-1 (Cytoskeleton), ZAP-70 (New England Biolabs), and VAV (New England Biolabs).

Statistical analysis

Data are shown as the median ± SEM. Statistical analysis was performed using a one-way ANOVA followed by a Holm Sidak post hoc test. P values less than 0.05 were considered significant.

Results

Thionamides inhibited the pro-inflammatory cytokine synthesis

Because clinical observations suggest anti-inflammatory properties, the heterocyclic thioureylenes methimazole, propylthiouracil and carbimazole were analyzed for their ability to inhibit cytokine production (Figure 1). Secretion of the proinflammatory cytokines IL-1, TNFα, and IFNγ was measured in supernatants of primary human T-lymphocytes by ELISA. CD3/CD28 receptor crosslinking induced TNFα and IFNγ secretion (Figure 2A/B). In contrast, IL-1, a cytokine primarily secreted by mononuclear phagocytes, could not be detected in human T lymphocyte supernatants (data not shown). Pre-incubation with either propylthiouracil or carbimazole significantly reduced TNFα or IFNγ synthesis, whereas methimazole showed no effect on cytokine production (Figure 2A/B).

Several transcription factors participate in the transcription of TNFα and IFNγ. To demonstrate that the central mediator of proinflammatory cytokine synthesis is NF-κB, specific inhibitors of the proinflammatory NF-κB pathway were used (Figure 2C). The proteasome inhibitors MG132 and lactacystin, which block IκBα degradation, significantly suppressed cytokine synthesis in a dose dependent manner (Figure 2C). In addition, 25 mM of the antioxidant N-acetylcysteine (NAC), an inhibitor of the IκB kinases, repressed cytokine synthesis but was associated with cytotoxicity (data not shown). We observed that NF-κB inhibitors were more potent inhibitors of TNFα than of IFNγ production. Correspondingly, propylthiouracil repressed TNFα secretion at lower doses than IFNγ (Figure 2A/B).

Thionamides inhibited the activation of the transcription factor NF-κB.

Previously we described that structurally related thio-barbiturates inhibit NF-κB (Loop, et al., 2002). To evaluate whether thionamides influence the proinflammatory transcription

factor NF-κB by a similar mechanism, NF-κB dependent reporter gene expression studies (Figure 3) and NF-κB DNA-binding assays were performed (Figure 4).

To determine NF-κB dependent gene expression, Jurkat T-cells were transiently transfected with a reporter construct containing the firefly luciferase gene under the control of NF-κB (Figure 3). NF-κB activity was induced by phorbol 12-myristate 13-acetate because CD3/CD28 signaling was defective in Jurkat cells (Matjaz Humar, unpublished observation). Upon activation, we observed a dose-dependent inhibition of NF-κB controlled reporter gene expression in Jurkat cells when they were pre-incubated with thionamides (Figure 3). Pre-incubation with either propylthiouracil or carbimazole significantly reduced reporter gene activity, whereas methimazole treatment resulted in only a minor and statistically insignificant decline of luciferase expression.

NF-κB is composed of homo- and heterodimeric complexes of various NF-κB family members. However, the p50/p65 heterodimers are the most common dimers found in the NF-κB signaling pathways. Therefore, DNA binding of p50 and p65(RelA) was analyzed by EMSA using nuclear extracts of CD3/CD28 activated T-cells (Figure 4A). The position and specificity of p50 and p65(RelA) was identified by supershift and competition experiments on independent blots (data not shown). The DNA-binding activity of NF-κB was significantly impaired by pre-incubation with either propylthiouracil or carbimazole. In contrast, methimazole did not affect p50 or p65(RelA) binding to its corresponding DNA consensus sequence.

For quantification of p50 or p65(RelA) DNA-binding, an ELISA based DNA-binding assay was used. Results in Figure 4B displayed a minor DNA-binding activity of p50 and p65(RelA) to a (5´GGGACTTTCG3') DNA consensus sequence in resting T-cells. This was induced 2 to 4 times upon CD3/CD28 T-cell receptor stimulation. The presence of methimazole did not significantly alter DNA-binding. In contrast, pre-incubation with either

propylthiouracil or carbimazole dose-dependently inhibited binding of p50 or p65 to its correspondent DNA consensus sequence.

Repression of NF-κB was due to inhibition of IκB phosphorylation and proteolytic degradation by reduced IκB- kinase activity

The reason for reduced NF-κB/DNA-binding was analyzed. In most cell types, NF-κB exists in an inactive form in the cytoplasm, bound to inhibitory IκB proteins. Phosphorylation and proteolytic cleavage of IκB result in the release and nuclear translocation of NF-κB and in specific gene activation (Li and Verma, 2002). Therefore, we investigated whether thionamides impair the phosphorylation (Figure 5A) and degradation (Figure 5B) of IκBα proteins. Because the phosphorylated form of IκBα is highly transient and difficult to detect due to rapid proteasomal degradation, the proteasome inhibitor MG132 was added (Figure 5A). CD3/CD28 receptor stimulation of T-lymphocytes induced the phosphorylation of IκBα proteins as determined by immunoblotting (Figure 5A). Pre-incubation with propylthiouracil or carbimazole prevented the CD3/CD28-induced phosphorylation of IκBα, whereas methimazole had no effect. Reduced levels of IκBα phosphorylation were not attributed to proteolysis because MG132 efficiently blocked proteasomal degradation. Equal amounts of total IκBα proteins could be detected in each reaction (Figure 5A, lower blots).

In the absence of MG132, IκBα was readily degraded upon CD3/CD28 T-cell receptor stimulation (Figure 5B). Pre-incubation with propylthiouracil or carbimazole abolished IκBα proteolysis. These results demonstrated that thionamides inhibit the phosphorylation and degradation of IκB, preserving NF-κB in an inactive state. The low amount of IκBα protein detected in quiescent cells (Figure 5B, lane 1) has been observed before (Henkel, et al., 1992).

Previously, we have shown that thio-barbiturates inhibit the phosphorylation and the proteasomal degradation of IκBα by a repression of IκB kinases (IKKs) (Loop, et al., 2003).

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To analyze whether thionamides mediate similar effects due to their related structure, IKK activity was examined in the presence of methimazole, propylthiouracil, and carbimazole. T-cells contain multiple different IKK-complexes (Kupfer and Scheinman, 2002). Therefore, the overall IKK activity was determined by a kinase activity assay using total cytosolic extracts of activated T-cells and immobilized recombinant GST-IκBα as a substrate. IKK-specific phosphorylation was measured by an IκBα (Ser32/Ser36) phospho-specific antibody and by immunoblotting (Figure 6A). A small amount of phospho-IκBα was detected when lysates, derived from quiescent T-lymphocytes, were used. CD3/CD28 activation, however, resulted in a marked increase in phosphorylated GST-IκBα. Pretreatment of T-cells with propylthiouracil or carbimazole prevented IκBα Ser32 and Ser36 phosphorylation indicating a reduced IKK activity, whereas methimazole treatment had no effect. Carbimazole was more potent in inhibiting IKKs than propylthiouracil (Figure 6A).

To determine the specific IκB-kinaseα activity, IKKα was immunoprecipitated from cytosolic extracts. Subsequently, immunoprecipitates were incubated with GST-IκBα and (^{32}P)ATP. Because methimazole had no major effect on the CD3/CD28-induced increase in total IKK activity, only the effects of carbimazole and propylthiouracil were tested. Basal IKKα activity was low in quiescent $CD3^+$ T-cells, whereas stimulation of the CD3/CD28 receptor caused an increase in IKK activity (Figure 6B). However, IKKα activity remained at the level of the non-stimulated controls when cells were pretreated with propylthiouracil (5 mM) or carbimazole (1 mM) prior to CD3/CD28 crosslinking. This observation indicated that thionamides inhibited the activity of IKKα. The amount of recovered IKKα protein was comparable under all conditions (Figure 6B, lower blots).

Carbimazole but not propylthiouracil mediated repression of IKK-activity was due to inhibition of the small G-protein Rac-1

T-cell receptor mediated T-cell activation is induced by a supramolecular activation complex referred to as the immunological synapse (Schmitz, et al., 2003). Because activation of the immunological synapse leads to the induction of NF-κB, a suppression of signal transmission via the T-cell receptor might explain the inhibition of IKKs. However, anti-phosphotyrosine immunoblots of total cell lysates displayed no fundamental decrease in activating tyrosine phosphorylation following CD3/CD28 receptor stimulation and thionamide treatment (Figure 7A). Actually, carbimazole induced a marked increase in tyrosin phosphorylation of proteins at 70 kDa (Figure 7A, arrowed). Subsequent experiments revealed that ZAP-70/Syk protein tyrosine kinase was a target of carbimazole mediated tyrosine phosphorylation. Increased phosphorylation of ZAP-70/Syk was accompanied by an increased kinase activity. Vav-1, a direct substrate of ZAP-70, was also hyper-phosphorylated at tyrosine 160 (Figure 7B) in the presence of carbimazole.

Vav-1 acts as a GDP/GTP exchange factor (GEF) for the small GTPase Rac-1 (Crespo, et al., 1997) and thus integrates T-cell receptor and CD28-derived signals with the activation of NF-κB via a Rac-1/MLK3/IKK or Rac-1/MEKK1/IKK-dependent pathway. To analyze whether thionamides block Rac-1 and thereby mediate the repression of IKKs and NF-κB, the activity of Rac-1 was determined in the cytosol of activated cells using immobilized Pak-PBD as a binding partner for pull down experiments. Upon CD3/CD28 T-cell receptor stimulation inactive Rac-1(GDP) was converted to active Rac-1(GTP) as demonstrated by its ability to complex with its binding partner Pak (Figure 7C). Only carbimazole, but not methimazole or propylthiouracil, repressed the ability of Rac-1 to bind the serine/threonine kinase Pak.

The carbimazole mediated inhibition of Rac-1 was more closely investigated. Activation of Rac-1 by GTPγs is independent of regulatory events by associated signaling cascades. Consequently, inhibition of Rac-1/Pak binding must be direct. Indeed, GTPγs activated cell lysates showed a direct repression of Rac-1/Pak binding upon carbimazole treatment (Figure 7C, lower blot). In contrast, phosphorylation of Rac-1 at Ser71, that both

inhibits GTP-binding and activation of the small G protein (Kwon, et al., 2000), was not responsible for the inhibitory potential of carbimazole (Figure 7B). Also, isoprenylation that is essential for membrane association and biological function of Rac-1 (Didsbury, et al., 1990) was not involved in carbimazole mediated effects, because GTPγs-dependent Rac-1 activation does not require a specific subcellular protein localization (Figure 7C, lower blot). In contrast, both methimazole and propylthiouracil did not influence the GTPγs dependent activation of Rac-1 as observed in pull down experiments before, using CD3/CD28 activated T-cells (Figure 7C). In summary, carbimazole directly inhibits Rac-1 and thus demonstrates another inhibitory profile than heterocyclic thio-derivates with a pyrimidine like nucleus (Figure 8).

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Discussion

NF-κB serves as an ubiquitous regulator of the host immune and inflammatory response and its participation in apoptosis, proliferation, and differentiation has recently been demonstrated (Karin, 1998;Karin, 2006;Li and Verma, 2002). However, excessive activation or deregulation of NF-κB is thought to play a fundamental role in the pathogenesis of several immunologic and inflammatory disorders such as morbus Alzheimer, atherosclerosis, adult respiratory distress syndrome, asthma, rheumatoid arthritis, cardiovascular disease, inflammatory bowel disease, cystic fibrosis, and multiple sclerosis (Kumar, et al., 2004;Wright and Christman, 2003).

Interestingly, single case studies with hyperthyroid patients suffering from inflammatory myopathy described a complete clinical and pathologic resolution upon anti-thyroid treatment with carbimazole (Hardiman, et al., 1997). Thionamide therapy is also beneficial to some chronic inflammatory disorders such as psoriasis (Elias, 2004). Additionally, it protects from autoimmune diseases in experimental animal models (Mozes, et al., 1998;Singer, et al., 1994) or diminishes aberrant leukocyte adhesion as observed in pathological inflammation (Dagia, et al., 2004). These observations may now be explained by the thionamide mediated inhibition of NF-κB.

We observed that the mechanism of thionamide mediated immunosuppression depended on the chemical structure of the individual reagent. Thioureylenes with a pyrimidine like nucleus such as the anti-thyroid drug propylthiouracil (presented data) or the barbiturates thiopental or thiamylal (Loop, et al., 2002;Loop, et al., 2003) showed high resemblance in dose response and both inhibited the IκBα-kinase complex. In contrast, carbimazole, a sulfur containing imidazole derivate, repressed the proximal activation of Rac-1, necessary for MLK3 or MEKK1 dependent activation of IKKα/β.

Redox-active sulfur was central for the NF-κB inhibitory potential of carbimazole, propylthiouracil, and thio-barbiturates as demonstrated by comparison with methimazole or

the oxy-analogues of barbiturates. Thio-barbiturates inhibited NF-κB but their oxy-analogues showed only marginal effects on their activation (Loop, et al., 2002;Loop, et al., 2003). Similarly, carbimazole and methimazole demonstrated diametric properties depending on the biochemical activity of the associated sulfur group. Methimazole, due to its stable aromatic structure, shows no anti-inflammatory activity whereas carbimazole, although it contains an analog imidazole nucleus, is not an aromatic compound but includes redox-active sulfur. Carbohydrate side chains (ethylcarbamate) and components of the heterocycle (thiourea, malonic acid, imidazole) had no immunoregulatory effects, because these reagents did not significantly inhibit NF-κB-dependent reporter gene expression (Matjaz Humar, unpublished observations).

Several publications confirm that molecular targets of thioureylenes are susceptible to sulfur. The structural analysis of IKK subunits suggests that cysteine residues are present in the activation loop within the kinase domain at sites critical for enzymatic activity (Byun, et al., 2006). These sites might serve as molecular targets for heterocyclic thio-derivates. Additionally, it has been described that thiol-reactive agents block IKK activity and prevent the subsequent activation of NF-κB (Jeon, et al., 2000;Loop, et al., 2003). For Rac-1 activation, a redox reactive cysteine within the P-loop motif is crucially involved in the guanine nucleotide exchange (Heo and Campbell, 2005). The x-ray crystal structure indicates that this Cys18 thiol is solvent-accessible (Heo and Campbell, 2005) and thus might represent a direct target for carbimazole. Most probably, active posttranslational modifications do not participate in carbimazole mediated Rac-1 inhibition because the interaction of Rac-1 and Pak was repressed in cellular lysates despite of GTPγs stimulation.

NF-κB is critically involved in the pathogenesis of diseases independent from inflammation (Kumar, et al., 2004). In these cases, heterocyclic thio-derivates might also be useful for molecular intervention and medical treatment. Screening methods and molecular modeling indicate that heterocyclic agents containing thioamides and thiourea are potent

inhibitors of protein function (Buchholz, et al., 2006). To evaluate the application of heterocyclic thioureylenes in NF-κB related diseases, more case observations and clinical studies are necessary. We suggest the analysis of hyperthyroid patients with secondary clinical symptoms associated with NF-κB deregulation and ongoing therapy with thionamides. Following treatment, differences in the clinical manifestation of NF-κB associated symptoms might be related to our observations. Additionally, the inhibitory potential of heterocyclic thioureylenes might be optimized by chemical modifications.

In summary, our work demonstrates a molecular mechanism of immunosuppression by mononuclear heterocyclic thioureylenes. Due to the fact that thionamides inhibit proteins that are involved in numerous aspects of cellular responses and are ubiquitously expressed in most tissues, extensive side effects of treatment might be expected. At the same time new therapeutic applications may evolve using thioureylenes for the development of new molecules in medical chemistry.

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Footnotes

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Legends for Figures

Figure 1. Structure of heterocyclic thio-derivates in clinical use. The thioureylenes used in this study include the anti-thyroid drugs methimazole, propylthiouracil, and carbimazole.

Figure 2. Thionamides inhibit TNFα and IFNγ secretion. Cytokine ELISAs of supernatants from peripheral human T lymphocytes are shown. T cells were either untreated or pre-incubated with increasing doses of propylthiouracil, carbimazole, methimazole, MG132 or lactacystin for 15 h. Cytokine synthesis was induced by 10^6 CD3/CD28 T cell Expander Dynabeads® (0.5 beads per cell) for the final 13 h of culture. **(A)** Propylthiouracil significantly inhibited TNFα synthesis at 1 mM, carbimazole at 100 µM, whereas methimazole treatment showed no effect. **(B)** Carbimazole inhibited IFNγ production stronger than propylthiouracil, methimazole showed no effect. **(C)** The NF-κB inhibitors MG132 and lactacystin repress synthesis of TNFα and IFNγ demonstrating NF-κB dependent transcription. Error bars indicate the median ± SEM of four independent experiments. *, $P < 0.05$ versus positive controls was considered as significant.

Figure 3. Propylthiouracil and carbimazole inhibit NF-κB-dependent reporter gene expression. Jurkat cells were transfected with 2 µg of pNF-κB-Luc and incubated with 0 to 5 mM methimazole, propylthiouracil, or carbimazole for 15 h. Cells were stimulated with 15 ng/ml phorbol 12-myristate 13-acetate to induce NF-κB for the final 13 h of culture. Lysates were analyzed for luciferase reporter gene activity, and the results were normalized to protein levels. Results are displayed as percentage of relative light units (RLUs) compared to stimulated, transfected cells in the absence of thionamides. Statistics represent the median ± S.E.M. of four independent experiments. *, $P < 0.05$ versus positive control (stimulation in the absence of thionamides) was considered as significant.

Figure 4. Carbimazole and propylthiouracil inhibit DNA binding of NF-κB. $CD3^+$ T-cells were treated for 3 h with thionamides at the indicated concentrations and subsequently stimulated by CD3/CD28 T-cell receptor crosslinking for 30 min (10^7 T-cell Expander Dynabeads® per assay). Nuclear extracts were prepared and analyzed for DNA binding of p50 or p65(RelA) by an electrophoretic mobility shift assay **(A).** NF-κB DNA complexes, the non specific binding activity of the probe (*), and unbound oligonucleotides (>) are indicated.

For quantification of DNA-bound NF-κB, a plate-immobilized oligonucleotide containing a 5´-GGGACTTTTTCC-3´ NF-κB binding site was used **(B)**. NF-κB members were recognized by specific anti-p50 or anti-p65(RelA) antibodies and secondary HRP-immune complexes for colorimetric readout. Spectrophotometric data were expressed as percentage of induction by absorbance compared to activated cells (=100%). Error bars indicate the median ± SEM of six independent experiments. *, $P < 0.05$ versus positive controls was considered as significant. Propylthiouracil and carbimazole inhibited binding of p50 and p65(RelA) NF-κB subunits.

Figure 5. Carbimazole and propylthiouracil inhibit the phosphorylation and degradation of IκBα. $CD3^+$ T-cells were treated for 3 h with thionamides at the indicated concentrations. CD3/CD28 T-cell receptor crosslinking was induced for 20 min to detect phosphorylation or 10 min to detect IκBα degradation (10^6 T-cell Expander Dynabeads® per assay). Total cellular lysates were analyzed by immunoblotting. **(A)** Both, propylthiouracil and carbimazole inhibited IκBα phosphorylation in a dose dependent manner, whereas methimazole had no effect. Analysis of total IκBα protein demonstrated equal amounts of protein in each lane, because IκBα degradation was inhibited during thionamide treatment and T-cell activation by addition of 10 µM of the proteasome inhibitor MG132. **(B)** Effects of

thionamides on IκBα degradation are demonstrated (upper blot). To inhibit protein synthesis, thionamide treatment and CD3CD28 T-cell receptor stimulation were performed in the presence of 5 µg/ml actinomycin D but absence of MG132. Total cell lysate supernatants were analyzed for β-actin to demonstrate that equal amounts of protein were included (lower blot). Representatives of three independent experiments are shown.

Figure 6. Carbimazole and propylthiouracil inhibit the IκB-kinase activity. Kinase activity assays are shown. Primary human T-lymphocytes (2×10^7) were pretreated with thionamides for 2 h and stimulated with 0.5 CD3/CD28 T cell Expander Dynabeads per cell for 10 min. Total cell lysates were prepared and incubated with 2 µg of GST-IκBα sepharose beads plus 0.5 µmol ATP to perform an IκBα kinase reaction at 30°C for 2 h **(A)**. Posphorylation of IκBα at Ser32 and Ser36 were visualized by immunoblotting (upper blot). Reprobed blots using an anti-GST specific antibody demonstrated equal amounts of GST-IκBα substrate in each reaction (input control, middle blot). Total cell lysates contained equal amounts of IKKα (lower blot). In **(B)** IKKα-specific IκBα phosphorylation is demonstrated. IKKα was immunoprecipitated from total cell lystes by 2 µg of antibody-sepharose beads. Immunoprecipitates were incubated with 4 µg GST-IκBα kinase substrate, 10 µM ATP, and 5 µCi (γ-^{32}P)ATP for 20 min at 22°C. Carbimazole or propylthiouracil treated cells demonstrated a dose dependent decline of IKKα specific kinase activity (upper blots). Western blot analysis of the immunoprecipitates demonstrated equal amounts of IKKα in each kinase reaction (input control, lower blots).

Figure 7. Carbimazole induces phosphorylation of ZAP70/Syk and VAV-1 but inhibits activation of the small GTPase Rac-1. Immunoblots are shown. T cells were pretreated with thionamides for 3 h and stimulated with 0.5 CD3/CD28 T cell Expander Dynabeads per cell

for 10 min as indicated. **(A)** Protein phosphorylation was detected by an anti-phosphotyrosine (4G10) HRP-conjugated antibody using whole cell lysates. < indicates the position of increased tyrosine phosphorylation in the presence of carbimazole. Alternatively, tyrosine phosphorylation of ZAP-70/Syk, Rac-1, and VAV-1 were analyzed by protein specific antibodies **(B)**. As a loading control, antibodies directed against total Rac-1, ZAP-70, and VAV-1 were used to demonstrate equal amounts of protein in each lane. In **(C)** the effect of thionamides on Rac-1 activity was determined by a PAK pull down assay. Active Rac was pulled down from whole cellular lysates with GST-PAK PRB fusion proteins. Precipitated proteins (upper panels) show the active fraction of the GTPase, whereas the lower panels show the total amount of Rac-1 in each cell lysate fraction. GTPγs was used for direct activation of Rac-1 in cellular lysates, in the absence of CD3/CD28 T cell receptor co-activation (final blots). Representatives of three independent experiments are shown.

Figure 8. Effects of heterocyclic thio-derivates on TCR-dependent NF-κB activation are shown. T-cell activation involves a supramolecular activation complex (immunological synapse) and is mediated by tyrosine phosphorylation and protein-protein interactions. VAV-1, a direct substrate of ZAP-70 and a GDP/GTP exchange factor of the small GTPase Rac-1, is induced by tyrosine phosphorylation, leading to the activation of the transcription factor NF-κB by a Rac-1/MLK-3/IKK or a Rac-1/MEKK1/IKK-dependent pathway. Carbimazole, a sulfur-derivate of imidazole, directly inhibits the small G-protein Rac-1, whereas thio-derivates with a pyrimidine like nucleus inhibit the IκB-kinase activity.

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Figure 1

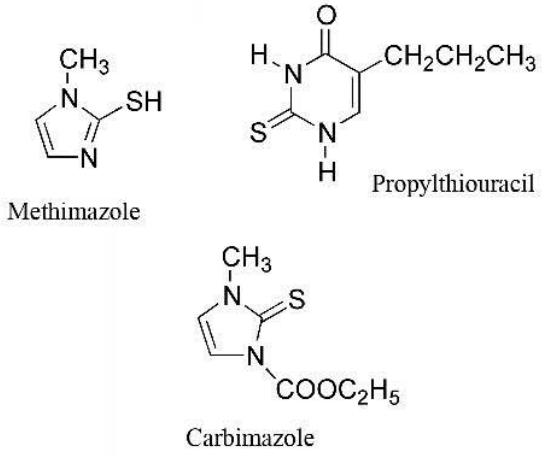

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Figure 2

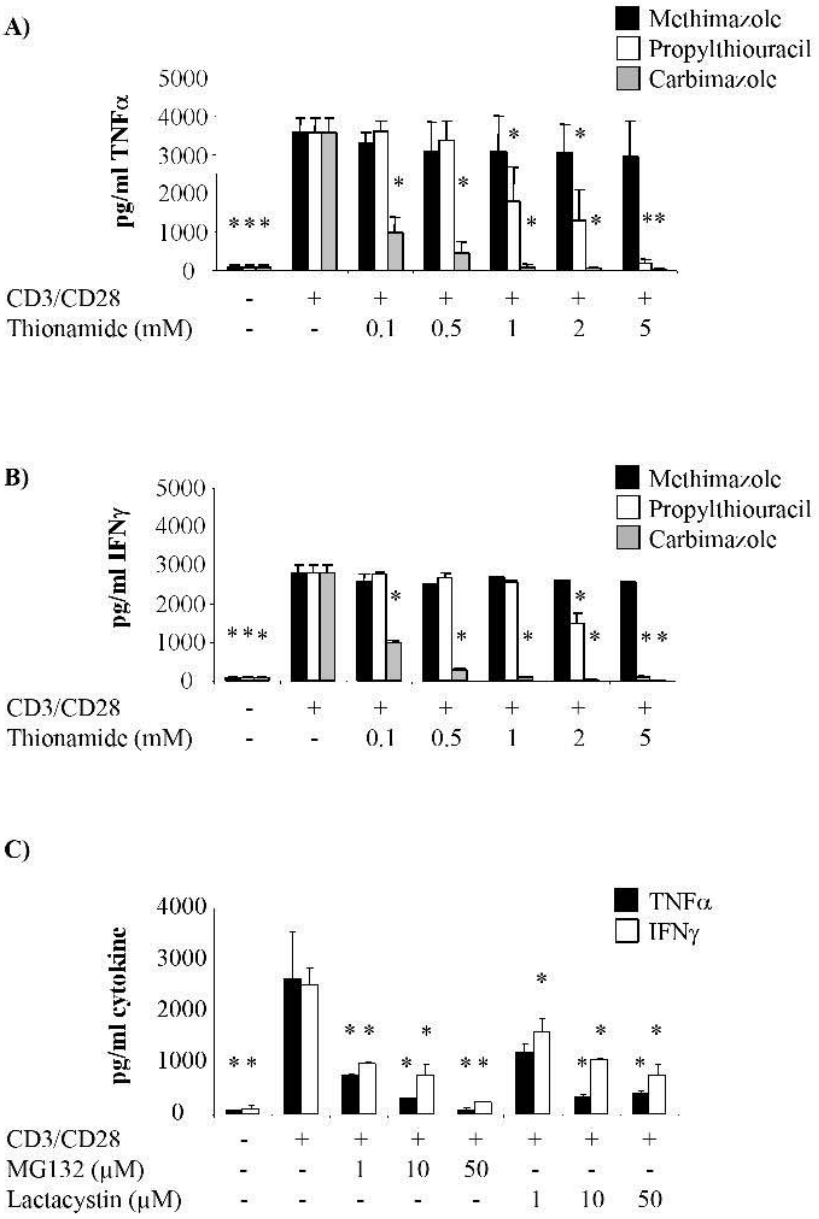

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Figure 3

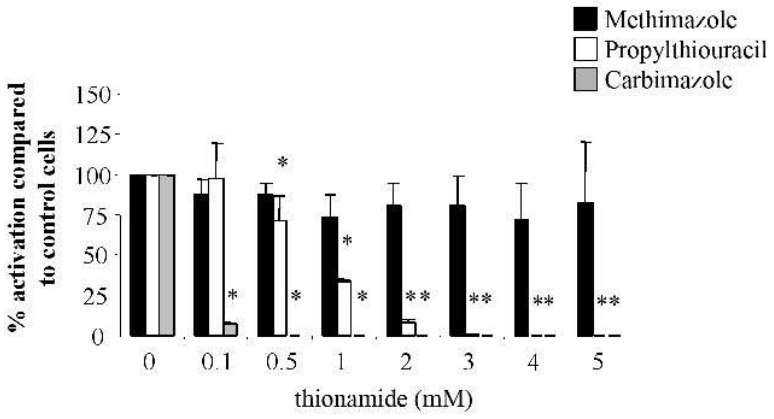

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Figure 4

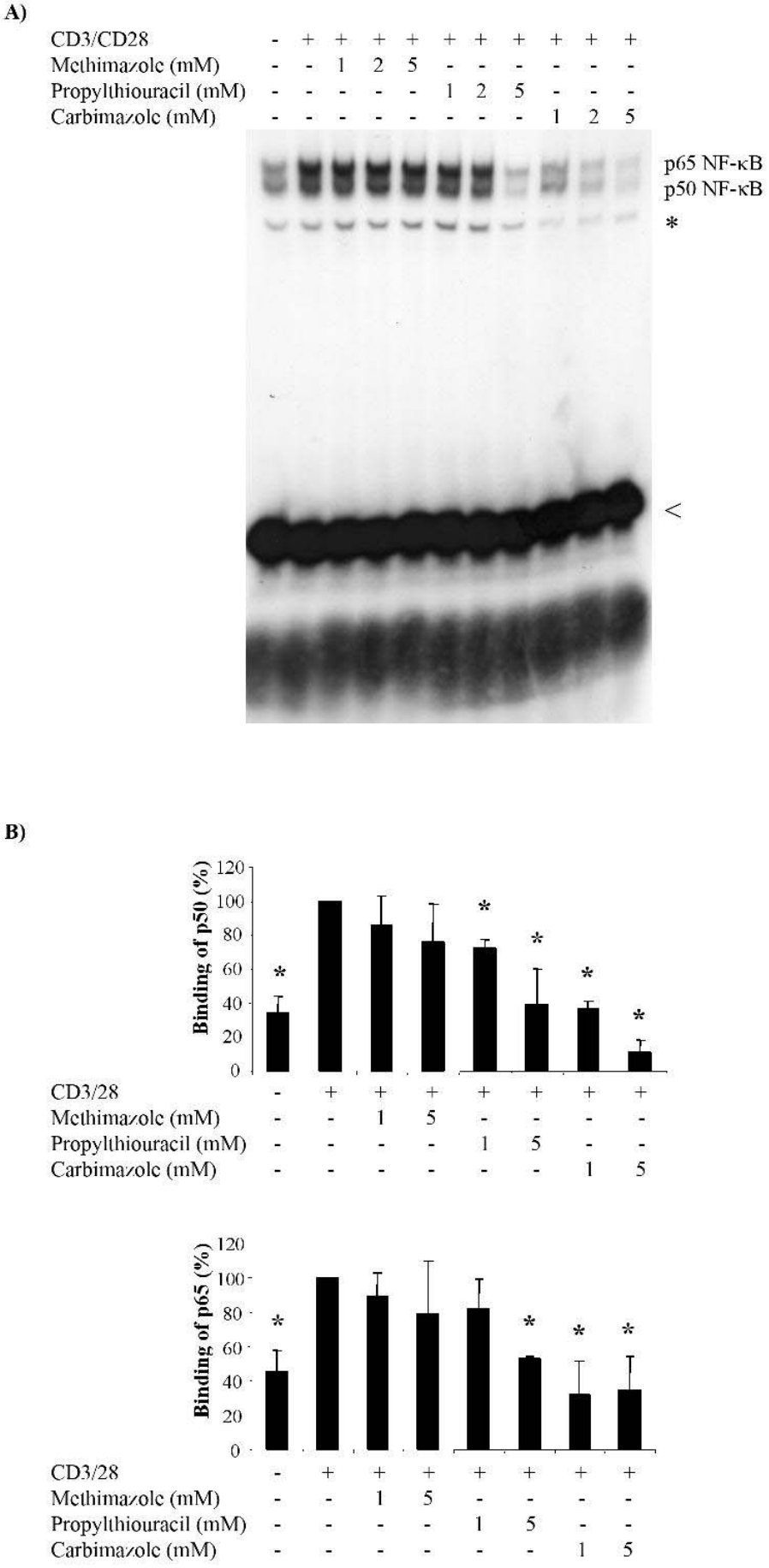

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Figure 5

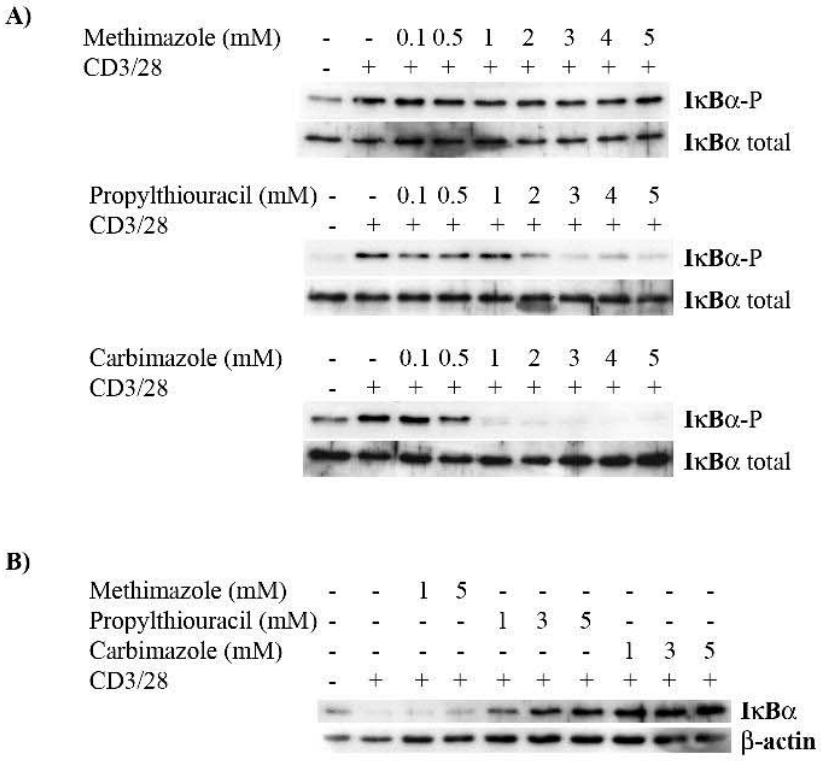

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Figure 6

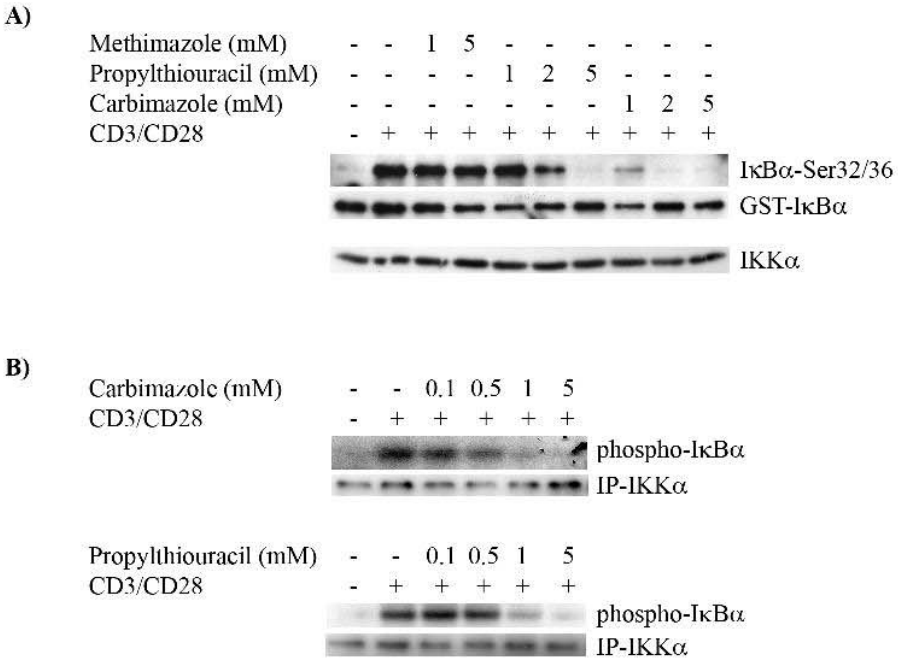

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Figure 7

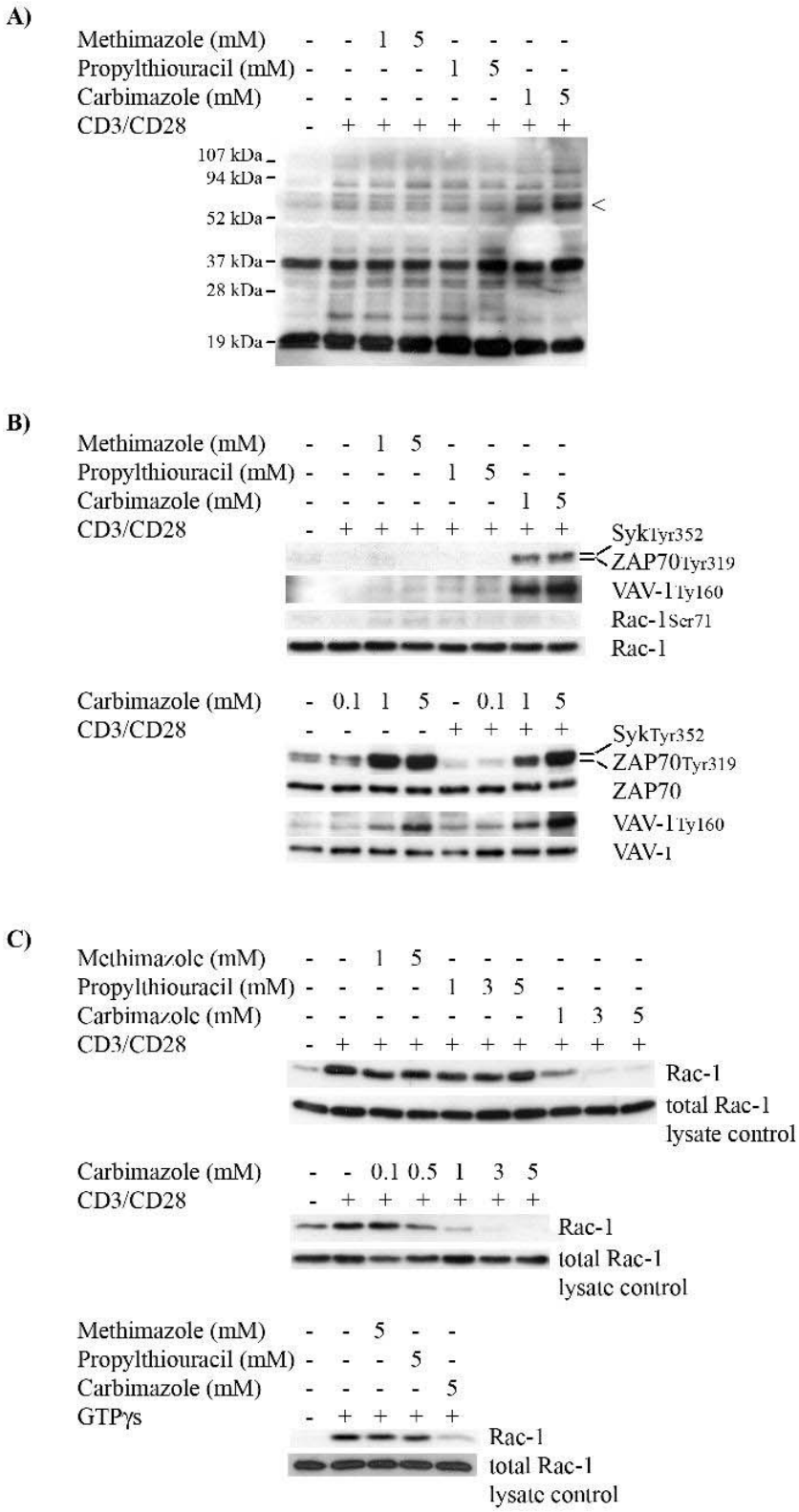

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Figure 8