# Vitamin $D_3$ analogues ZK159222 and ZK191784 have anti-inflammatory properties in human adipocytes

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## Abstract

The release of the proinflammatory factors is enhanced with increased fat mass, linking adipose tissue inflammation to metabolic dysfunction. Vitamin $D_3$ is a pleiotropic hormone with a range of functions in addition to calcium and bone metabolism. Low vitamin $D_3$ status has been linked to a number of diseases including obesity and metabolic syndrome. Previous studies from our group and others have shown the anti-inflammatory property of 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) in adipose tissue in obesity. However, administration of $1,25(OH)_2D_3$ at a supraphysiological dose can lead to hypercalcemia and this hampers its use as a safe course of treatment. The development of the $1,25(OH)_2D_3$ analogues that have immunomodulatory activity with minimal calcemic effects could be beneficial in clinical practice. ZK159222 and ZK191784 are vitamin $D_3$ analogues known to act on the vitamin D receptors but their effects on adipose tissue particularly the local inflammation are not known. This study investigated whether ZK159222 and ZK191784 are able to modulate the inflammatory response and the signalling pathways involved in human adipocytes. Like $1,25(OH)_2D_3$, treatment with ZK159222 and ZK191784 significantly reduced macrophage-stimulated activation of NF-κB signalling, upregulating IκBα expression and reducing p65 phosphorylation. The phosphorylation of p38 mitogen-activated protein kinase (MAPK) stimulated by macrophage-derived factors was also inhibited by the two compounds. Additionally, macrophage-induced release of the key proinflammatory cytokine/chemokines (IL-6, IL-8, MCP-1 and RANTES) was markedly decreased by the treatment with ZK159222 and ZK191784. Taken together, both $1,25(OH)_2D_3$ analogues have potent anti-inflammatory effects in human adipocytes. These actions are probably mediated by preventing the activation of the NF-κB and MAPK signalling pathways. The data suggests that the two $1,25(OH)_2D_3$ analogues may serve as a potential therapeutic option to have anti-inflammatory effects in adipose tissue in obesity.

## Introduction

1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), the bioactive form of vitamin $D_3$, is a pleiotropic hormone with a wide range of functions in addition to its classical role in bone and calcium metabolism (Boyle, Gray et al. 1972; Mellanby 1976). It is estimated that vitamin $D_3$ deficiency affects approximately one billion people worldwide (Holick 2007). vitamin $D_3$ deficiency has also been linked to the metabolic disorders, including obesity and insulin resistance (Chiu, Chu et al. 2004; Barchetta, Angelico et al. 2011; Olson, Maalouf et al. 2012). Data from clinical studies have shown that low vitamin $d_3$ status a**r**e common in obese patients (Goldner, Stoner et al. 2008; Brock, Huang et al. 2010; Fish, Beverstein et al. 2010). obesity is now known to be associated with a low-grade chronic inflammation, characterised by a significant increase in accumulation of macrophages and other immune cells in adipose tissue, the activation of inflammatory signalling (i.e. NF-κB and MAPK) pathways and the release of proinflammatory cytokine/chemokines (i.e. TNFα, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1)). Adipose tissue inflammation in obesity is considered as a driving force in the pathogenesis of insulin resistance and type 2 diabetes (Fontana, Eagon et al. 2007; Bourlier and Bouloumie 2009; Lolmede, Duffaut et al. 2011; Gao, Madi et al. 2014). Recent studies from our group and others have demonstrated that $1,25(OH)_2D_3$ has potent anti-inflammatory effects in adipose tissue, such as inhibiting the production of proinflammatory cytokines/chemokines by preadipocytes and adipocytes, and reducing macrophage infiltration (Lorente-Cebrian, Eriksson et al. 2012; Marcotorchino, Gouranton et al. 2012; Ding, Wilding et al. 2013; Gao, Trayhurn et al. 2013). A main concern of using $1,25(OH)_2D_3$ for treatment is the possible disruption of calcium homeostasis inducing hypercalcemia (Smith, Johnson et al. 1999; Slatopolsky and Brown 2002). Furthermore, circulating levels of $1,25(OH)_2D_3$ has been shown to be negatively correlated with coronary calcification (Watson, Abrolat et al. 1997). $1,25(OH)_2D_3$ is known to exert a wide range of effects, which can be classified as genomic and non-genomic. The genomic effects of $1,25(OH)_2D_3$ is characterised by its binding to the vitamin D receptor (VDR) in the cytoplasm, nuclear translocation of the liganded receptor and interaction with vitamin D response elements in the promoter region of genes, leading to changes in gene expression within hours (Haussler, Jurutka et al. 2011). Its non-genomic effects include rapid changes in intracellular calcium concentrations, membrane phospholipid metabolism and activation of protein kinases, which is involved in several signal transduction cascades, and the non-genomic effects of $1,25(OH)_2D_3$ may be VDR independent (Kajikawa, Ishida et al. 1999; Mizwicki and Norman 2009; Menegaz, Barrientos-Duran et al. 2010; Hii and Ferrante 2016). Despite the potential therapeutic effects of $1,25(OH)_2D_3$ *in vitro*, its clinical use could be limited mostly due to unwanted hypercalcemic effects.

The development of $1,25(OH)_2D_3$ analogues, with low calcemic effects but selective actions on VDR-mediated gene transcription, may offer an attractive solution in clinical practice. ZK159222 and ZK191784 are the $1,25(OH)_2D_3$ analogues, both of which act on the vitamin D receptors

(Carlberg 2003; Nijenhuis, van der Eerden et al. 2006). ZK159222 is a partial antagonist of VDR, characteristised by a long butyl ester at the C25 position of the side chain (Carlberg 2003). In contrast to the natural hormone, ZK159222 appears to prevent the interaction of VDR with coactivators (Toell, Gonzalez et al. 2001). However, like $1,25(OH)_2D_3$, the binding of ZK159222 to the VDR can induce a dissociation of the majority of the VDR-corepressor complexes (Polly, Herdick et al. 2000). These observations suggest that the antagonistic quality of ZK159222 is achieved by stabilising the VDR in a conformation that blocks coactivator to interact with vitamin D response elements (VDREs), without affecting the normal VDR-corepressor dissociation (Toell, Gonzalez et al. 2001). It has been shown that ZK159222 is not a complete antagonist as it still retains approximately 20% of the agonistic ability of $1,25(OH)_2D_3$ (Toell, Gonzalez et al. 2001; Vaisanen, Perakyla et al. 2002). However, whether ZH159222 has immunomodulatory activity is largely unknown.

ZK191784 is characterised by an altered side chain structure containing a 22,23-double bond, 24R-hydroxy group, 25-cyclopropyl ring, and 5-butyloxazole-group (Nijenhuis, van der Eerden et al. 2006). ZK191784 has been reported to competitively binds to the VDR with a similar affinity as $1,25(OH)_2D_3$ (Zugel, Steinmeyer et al. 2002). Unlike $1,25(OH)_2D_3$, ZK191784 dose not stimulate intestinal $Ca^{2+}$ absorption and reduces renal $Ca^{2+}$ excretion that it has less calcemic effects than $1,25(OH)_2D_3$ (Nijenhuis, van der Eerden et al. 2006). Studies *in vivo* and *in vitro* have suggested that ZK191784 exhibit immunomodulatory activity. ZK191784 has been shown to have therapeutic potential in T cell-mediated immune disorders with a 100-fold lower hypercalcemic effect in comparison with $1,25(OH)_2D_3$ in rats (Zugel, Steinmeyer et al. 2002). In experimental colitis in mice, ZK191784 treatment ameliorated acute and chronic intestinal inflammation probably by its immunosuppressive effects on mucosal dendritic cells without exhibiting calcemic effects (Strauch, Obermeier et al. 2007). Moreover, ZK191784 downregulated adhesion molecules and MMPs production in colonic biopsies of patient with inflammatory bowel diseases (Martinesi, Ambrosini et al. 2014).

Although ZK159222 and ZK191784 have congruent effects on the VDR, their roles in adipose tissue particularly the local inflammation which often occurs in obesity are not known. This study was therefore to investigate whether the two $1,25(OH)_2D_3$ analogues modulate the inflammatory responses and the signalling pathways involved in human adipocytes. Their effects were compared to the action of the natural ligand, $1,25(OH)_2D_3$.

## Materials and methods

### *Culture of adipocytes*

Human preadipocytes derived from subcutaneous adipose tissue of a female Caucasian subject (BMI 21 $kg/m^2$; age 44 yr) were obtained from PromoCell (Heidelberg, Germany). Cells were

seeded at 40,000/cm$^2$ and grown in 24-well plates in preadipocyte growth medium, containing DMEM-Ham's F-12 (1:1, vol/vol) and supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Lonza, Twekesbury, UK), at 37°C in a humidified atmosphere of $O_2$:$CO_2$ (95:5%). At confluence, cells were induced to differentiate at day 0 by incubation for 3 days in Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (1:1 ratio) medium which contained 32 µM biotin, 1µM dexamethasone, 200 µM 3-isobutyl-1-methyl-xanthine, 100 nM insulin, 11 nM L-Thyroxine (all from Sigma, Poole, Dorset, UK), 8 µM rosiglitazone (GlaxoSmithKline, Uxbridge, UK), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. After induction, cells were cultured in maintenance medium containing 3 % foetal calf serum (FCS; Sigma), 100 nM insulin, 32 µM biotin and 1 µM dexamethasone until fully differentiated. Differentiation into mature adipocytes was confirmed by observing the accumulation of lipid droplets under the microscope.

***Culture of THP-1 macrophages***

Human THP-1 myelomonocytic cell line (Health Protection Agency Culture Collections, Porton Down, Salisbury UK) was used. THP-1 monocytes ($1\times10^6$ cells/ml) were cultured in a 150 cm$^2$ flask in Roswell Park Memorial Institute (RPMI-1640) medium, containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C in a humidified atmosphere of $O^2$:$CO^2$ (95:5%). THP-1 monocytes were differentiated into macrophages with 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 h. The medium was replaced with PMA-free and FCS-free RPMI-1640 medium for 24 h. This medium as macrophage-conditioned (MC) medium was collected, filtered through a 0.22µm filter and stored at -80°C for later use.

***Cell treatment***

ZK159222 and ZK191784, VDR ligands (Herdick, Steinmeyer et al. 2000; Herdick, Steinmeyer et al. 2000; Zugel, Steinmeyer et al. 2002; Schmitz, Hackethal et al. 2015), were a generous gift from Bayer Pharma AG (Berlin, Germany). Differentiated adipocytes (Day 11) were grouped and incubated with adipocytes maintenance media, $10^{-8}$M of 1,25$(OH)_2D_3$ (Sigma) , ZK159222 ($10^{-8}$M or $10^{-6}$M) and ZK191784 ($10^{-8}$M or $10^{-6}$M) for 48 h. The cell culture medium was replaced with RPMI (control), MC medium (MC), MC medium with 1,25$(OH)_2D_3$ ($10^{-8}$M), MC medium with ZK159222 ($10^{-8}$M or $10^{-6}$M) and MC medium with ZK191784 ($10^{-8}$M or $10^{-6}$M) for 24 h. The culture medium and cell lysates were collected and stored at -80°C until later analyses.

***Western blotting***

Cell lysates were prepared by lysing cells in lysis buffer (50 mM Tris-HCl pH 6.7, 10% glycerol, 4% SDS, 2% 2-mer-capoethanol) with protease inhibitor cocktail and phosphatase inhibitors (Sigma). Protein concentrations of the lysates were then measured by the bicinchoninic acid method. Protein samples were separated on 10% Tricine-SDS polyacrylamide gels (Mini Protean Tetra, Bio-Rad, Hemel Hempstead, UK) and transferred to nitrocellulose membranes at 100v for 1 h. Protein marker

was used on one lane for identification of target bands. The membranes were blocked with 5% BSA in Tris-buffered saline (TBS) and 0.1% Tween-20 for 1 h at room temperature. The membranes were then incubated with the primary antibody, including Iκβα, phosphorylated NFκB p65 and phosphorylated p38 MAPK (all from New England Biolabs, Hitchin, UK) overnight at 1:1000 dilution at 4°C. The membranes were washed in phosphate-buffered saline with 0.1% Tween-20 and subsequently incubated with a HRP-conjugated secondary antibody (New England Biolabs). Chemiluminescent signals were evaluated and quantified using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, US) using the ChemiDoc XRS+ System (Bio-Rad). The membranes were further probed for vinculin or GAPDH (both from Abcam, Cambridge, UK) as a loading control.

### *Measurement of cytokine/chemokine release*

The release of IL-6, IL-8, MCP-1 and RANTES by adipocytes and THP-1 macrophages was measured as protein concentrations in culture medium, using the DuoSet ELISA Development kits (R&D Systems, Abingdon, UK).

### *Cytotoxicity evaluation*

Adipocyte viability after treatment with various compounds was assessed as release of lactate dehydrogenase (LDH) into the cell culture medium, using a colourimetric cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, Germany). LDH levels were measured at room temperature by a spectrophotometer at 492 nm with a reference wavelength of 620 nm.

### *Statistical analysis*

All results are presented as means ± standard error of mean (SEM). Statistical differences among groups were assessed by one-way ANOVA coupled with Bonferroni's *t*-test. Differences were considered as statistically significant when P value < 0.05. Statistical tests were performed using Graphpad Prism version 5.03 and SPSS(PASW), release version 19.0.

## Results

### *ZK 159222 and ZK191784 inhibit macrophage-induced activation of NF-κB in human adipocytes*

This set of experiments examined whether ZK159222 and ZK191784 have similar effects to 1,25-dihydroxyvitamin $D_3$ on macrophage-induced NF-κB activation in adipocytes. As shown by Figure.1, treatment with $1,25(OH)_2D_3$ led to an increase in IκBα expression (P = 0.003) in adipocytes stimulated with MC medium. ZK159222 treatment reversed MC medium-induced downregulation of IκBα in a dose-dependent manner, inducing a 3.9-fold at $10^{-8}$M although this was not statistically significant (P = 0.148) and a 7.4-fold increase at $10^{-6}$M (P = 0.002). Compared to $1,25(OH)_2D_3$, the effect of ZK159222 at $10^{-8}$M was less potent but it had a similar effect to $10^{-8}$M $1,25(OH)_2D_3$ at $10^{-6}$M.

ZK159222 also downregulated the induction of phosphorylated NF-κB p65 expression by MC medium. While 1,25$(OH)_2D_3$ ($10^{-8}$M) decreased phosphorylated NF-κB p65 by 67% (P = 0.011), ZK159222 significantly reversed MC medium-induced upregulation of phosphorylated NF-κB p65 by 90% at $10^{-8}$M (P = 0.002) and 91% at $10^{-6}$M (P = 0.001) compared to adipocytes that received treatment with MC medium only. ZK159222 appeared to have a greater effect in reducing phosphorylated NF-κB p65 compared with 1,25$(OH)_2D_3$, although there were no statistically significant differences among three groups that received treatment with 1,25$(OH)_2D_3$ or its analogues (Figure 2).

As shown by Figure 3, ZK191784, another analogue of 1,25$(OH)_2D_3$, displayed similar effects as 1,25$(OH)_2D_3$. Like 1,25$(OH)_2D_3$ which elicited a 9.1-fold increase in IκBα expression (P = 0.004), ZK191784 treatment dose-dependently reversed macrophage-induced downregulation of IκBα, leading to a 5.4-fold increase at $10^{-8}$M although this was not statistically significant (P = 0.11), and a 10-fold increment at $10^{-6}$M (P = 0.002). There were no statistically significant differences among three groups that received treatment with vitamin $D_3$ or its analogues.

Similar to 1,25$(OH)_2D_3$, ZK191784 also downregulated protein expression of phosphorylated NF-κB p65 in adipocytes stimulated with MC medium (Figure 4). While 1,25$(OH)_2D_3$ decreased phosphorylated NF-κB p65 by 41% (P < 0.001), ZK191784 reduced phosphorylated NF-κB p65 by 57% at $10^{-8}$M (P < 0.001) and 59% at $10^{-6}$M (P < 0.001) compared with adipocytes that received treatment with MC medium only. Compared to 1,25$(OH)_2D_3$ ($10^{-8}$M), ZK191784 showed a greater potency in reducing phosphorylated NF-κB p65 at both concentrations (P = 0.02 for $10^{-8}$M, P = 0.009 for $10^{-6}$M).

### *ZK 159222 and ZK191784 inhibit macrophage-induced expression of p38 MAPK in human adipocytes*

Since both vitamin $D_3$ compounds modulate the activation of NF-κB, we next examined whether ZK159222 and ZK191784 have effects on the MAPK signalling pathway in adipocytes. As shown by Figure 5A, similar to 1,25$(OH)_2D_3$ which caused a 42% decrease in phosphorylated p38 MAPK (P = 0.002), ZK159222 partially reversed macrophage-induced upregulation of phosphorylated p38 MAPK in a dose-dependent manner, with a 27% decrease at $10^{-8}$M (P = 0.03) and a 77% decrease at $10^{-6}$M (P < 0.001). Compared to 1,25-dihydroxyvitamin $D_3$, the level of reduction in phosphorylated p38 MAPK by ZK159222 was not significantly different at $10^{-8}$M (P = 0.45) but the inhibitory effect was enhanced at $10^{-6}$M (P=0.006).

Similar to 1,25$(OH)_2D_3$, ZK191784 downregulated phosphorylated p38 MAPK expression in adipocytes stimulated with MC medium (Figure 5B). While 1,25$(OH)_2D_3$ decreased phosphorylated p38 MAPK by 57% (P < 0.001), ZK191784 dose-dependently reversed upregulation of phosphorylated p38 MAPK by MC medium, inducing a 55% decrease at $10^{-8}$M (P < 0.001) and a 94% at $10^{-6}$M (P < 0.001). At similar concentrations ($10^{-8}$M), there was no significant difference in

the potency of inhibition on phosphorylated p38 MAPK between ZK191784 and $1,25(OH)_2D_3$ ($P > 0.05$) but at higher dose ($10^{-6}$M) the inhibitory effect of ZK191784 was greater ($P = 0.002$).

### *ZK159222 and ZK191784 inhibit macrophage-induced secretion of IL-6, MCP-1, IL-8 and RANTES by human adipocytes*

To further study the downstream effects of inhibiting NF-κB and MAPK signalling by ZK159222 and ZK191784, we examined the release of some key cytokine and chemokines in adipocytes. As shown by Figure 6, MC medium-induced IL-6 secretion by adipocytes was reduced 36% following the treatment with $1,25(OH)_2D_3$ compared to adipocytes incubated in MC medium alone ($P < 0.001$). $10^{-8}$M and $10^{-6}$M of ZK159222 reduced macrophage-induced IL-6 release by 52% and 71% respectively (both $P < 0.001$). $10^{-8}$M and $10^{-6}$M of ZK191784 reduced IL-6 release from adipocytes by 35% and 54% respectively (both $P < 0.001$). Furthermore, the inhibitory effect of $10^{-6}$M ZK159222 on IL-6 production was stronger compared with $10^{-8}$M of $1,25(OH)_2D_3$ (54% decrease, $P < 0.001$).

In Figure 6, MC medium-induced MCP-1 secretion by adipocytes was reduced 47% with the treatment of $1,25(OH)_2D_3$ compared to adipocytes incubated in MC medium alone ($P = 0.001$). MCP-1 release was reduced by 78% and 77% by adipocytes treated with $10^{-8}$M and $10^{-6}$M of ZK159222 (both $P < 0.001$). Similarly, MCP-1 release by adipocytes treated with $10^{-8}$M and $10^{-6}$M of ZK191784 was reduced by 77% and 88% respectively (both $P < 0.001$). Of the compounds tested, only $10^{-6}$M of ZK191784 showed a greater effect in lowering MCP-1 secretion compared with $10^{-8}$M $1,25(OH)_2D_3$ ($P < 0.01$).

Furthermore, MC medium-stimulated IL-8 secretion by adipocytes was reduced by 31% with the treatment of $1,25(OH)_2D_3$ ($P < 0.001$). Treatment with $10^{-8}$M and $10^{-6}$M of ZK159222 reduced IL-8 release by 53% and 64% respectively (both $P < 0.001$). Treatment with $10^{-8}$M and $10^{-6}$M of ZK191784 reduced IL-8 release by 49% and 65% in a dose-dependent manner (both $P < 0.001$). Compared with $10^{-8}$M of $1,25(OH)_2D_3$, IL-8 secretion was significantly lower from adipocytes treated with ZK159222 and ZK191784 at all doses ($P < 0.001$).

Finally, MC medium-induced RANTES secretion by adipocytes treated with $1,25(OH)_2D_3$ was attenuated (by 96%, $P < 0.001$). Treatment with $10^{-8}$M and $10^{-6}$M of ZK159222 markedly reduced macrophage-induced release of RANTES (by 85% and 91% respectively, both $P = 0.002$). Similarly, treatment with $10^{-8}$M and $10^{-6}$M of ZK191784 significantly reduced RANTES release by adipocytes by 82% ($P = 0.004$) and 93% ($P = 0.005$), respectively. Compared with $10^{-8}$M of $1,25(OH)_2D_3$, there were no significant differences in RANTES secretion by ZK159222 and ZK191784 treatment at all doses.

### *Effects of MC medium, 1,25-dihydroxyvitamin $D_3$, ZK159222 and ZK191784 on cell viability*

Cytotoxicity of the compounds (MC medium, $1,25(OH)_2D_3$, ZK159222 and ZK191784) used in this study were examined by the measurement of LDH release into cell culture media. As shown by Figure 7, there was no significant difference between the control and the treatment groups.

## Discussion

Chronic inflammation has an important role in the pathogenesis of obesity and its related metabolic disorders (Gustafson, Hammarstedt et al. 2007; Gregor and Hotamisligil 2011). Adipose tissue as a major endocrine organ releases a wide range of protein factors, including cytokines/chemokines (i.e. IL-1, IL-6, IL-8 and MCP-1) (Fain 2006). The release of the proinflammatory factors is enhanced with increased fat mass, linking adipose tissue inflammation to metabolic dysfunction (Hotamisligil 2006). Previous studies from our group and others have provided evidence for the anti-inflammatory property of $1,25(OH)_2D_3$ in adipose tissue, which was demonstrated by reduced proinflammatory response in adipocytes stimulated by macrophage-derived factors, cytokines and LPS as well as in adipose tissue of obese mice induced by high-fat diet (Lira, Rosa et al. 2011; Lorente-Cebrian, Eriksson et al. 2012; Mutt, Karhu et al. 2012; Ding, Wilding et al. 2013; Gao, Trayhurn et al. 2013). However, these studies suggest that supraphysiological doses of $1,25(OH)_2D_3$ are required to have a significant effect in adipose tissue. As using high doses of $1,25(OH)_2D_3$ has been linked to the increased risk of hypercalcemic side effects, (Huckins, Felson et al. 1990; Nijenhuis, van der Eerden et al. 2006), it is unlikely that $1,25(OH)_2D_3$ can be used as a sustainable treatment particularly for those with renal insufficiency. The analogues of $1,25(OH)_2D_3$ with low calcemic effects may offer an alternative solution in clinical practice. A number of $1,25(OH)_2D_3$ analogues have been developed with the purpose of improving the biological profile of the natural hormone for potential therapeutic value (Carlberg 2003). The current study therefore investigated whether the two $1,25(OH)_2D_3$ analogues, ZK159222 and ZK191784, have modulatory effects on adipose tissue inflammation *in vitro*.

In the present study, we demonstrate for the first time the anti-inflammatory actions of the two $1,25(OH)_2D_3$ analogues in human adipocytes. Our study initially examined the effects of ZK159222 and ZK191784 on the NF-κB signalling pathway. The transcription factor NF-κB is a key regulator of inflammatory gene expression and its activity is enhanced in adipose tissue during obesity (Chiang, Bazuine et al. 2009; Ding, Gao et al. 2012). NF-κB is activated by the degradation of IκBα protein, allowing nuclear translocation of the p65 subunit of NF-κB (Tak and Firestein 2001). In the current study, ZK159222 was able to reverse IκBα expression suppressed by MC medium in adipocytes although to a lesser extent when compared to $1,25(OH)_2D_3$ in equimolar concentrations. In addition, both doses of ZK159222 ($10^{-8}$M and $10^{-6}$M) inhibited macrophage-induced phosphorylation of NF-κB p65 and this effect appeared to be stronger in comparison with $1,25(OH)_2D_3$ (Figure. 2). Although ZK159222 is an antagonist of VDR being used to study the effect of full VDR activation by $1,25(OH)_2D_3$, it still has some agonistic potential of $1,25(OH)_2D_3$ (Herdick, Steinmeyer et al. 2000; Vaisanen, Perakyla et al. 2002). However, little is known whether ZK159222

has immunomodulatory property. A previous study has shown that ZK159222 inhibited $1,25(OH)_2D_3$-induced NO production in porcine aortic endothelial cells (Molinari, Rizzi et al. 2013). In the present study, ZK159222 enhanced IκBα stability supporting the notion that VDR binding stabilises IκBα in adipocytes. Furthermore, NF-κB p65 phosphorylation has been shown to be mediated by intracellular calcium levels (Sun and Carpenter 1998; Thuringer, Hammann et al. 2011) and $1,25(OH)_2D_3$ is also a non-genomic enhancer. As ZK159222 is not known to have non-genomic effect, it may have greater potency in preventing the phosphorylation of NF-κB p65. In the present study, we have also shown that ZK191784 can reverse IκBα expression inhibited by macrophage-derived factors in adipocytes. Moreover, ZK191784 at both doses ($10^{-8}$M and $10^{-6}$M) attenuated upregulation of phosphorylated NF-κB p65 stimulated by MC medium, and this effect was stronger than $1,25(OH)_2D_3$ (Figure. 4). Taken together, our data suggest that ZK159222 and ZK191784 have strong inhibitory effects on macrophage-induced activation of NF-κB signalling in human adipocytes.

The activation of the MAPK signalling is also involved in the signal transduction of inflammatory mediators leading to inflammatory response in several cell types (Zhang, Leung et al. 2012) including preadipocytes and adipocytes (Gao, Trayhurn et al. 2010; Ding, Wilding et al. 2013). In this study, we have shown that ZK159222 and ZK191784 are able to inhibit the activation of the MAPK signalling in human adipocytes. Both $1,25(OH)_2D_3$ analogues inhibited phosphorylation of p38 MAPK in a dose-dependent manner. Moreover, their inhibitory effect was comparable to that of $1,25(OH)_2D_3$ (Figure. 5). Therefore, the MAPK signalling pathway could be potential target of the $1,25(OH)_2D_3$ analogues for reducing inflammatory response in adipose tissue.

To further study the downstream effects of ZK159222 and ZK191784 on adipose tissue inflammation, we have examined the release of the key proinflammatory cytokine and chemokines by adipocytes upon macrophage stimulation. IL-6, a major cytokine and expressed by adipocytes, is critically involved in obesity related inflammation and insulin resistance (Rotter, Nagaev et al. 2003; Kristiansen and Mandrup-Poulsen 2005). In the present study, ZK159222 and ZK191784 markedly reduced protein release of IL-6 by adipocytes as the natural hormone $1,25(OH)_2D_3$ (Figure. 6). Although the effect of the two compounds on IL-6 release was unknown previously, ZK191784 has been shown to inhibit LPS-induced secretion of cytokine IL-12 and TNF-α by human PBMCs, with a similar potency as $1,25(OH)_2D_3$ (Zugel, Steinmeyer et al. 2002). MCP-1 is a chemokine with a chemotactic activity for monocytes and its overexpression enhances macrophage infiltration and insulin resistance (Kamei, Tobe et al. 2006; Kanda, Tateya et al. 2006). The current study has shown that ZK159222 and ZK191784 markedly reduced MCP-1 secretion (77-88%) elicited by MC medium to an extent similar to the treatment with $1,25(OH)_2D_3$. Moreover, the higher dose ($10^{-6}$M) of ZK191784 had strongest inhibitory effect on MCP-1 release by adipocytes (Figure. 6). IL-8 as a neutrophil chemotactic factor has been shown to induce chemotaxis in other cell types including macrophages (Waugh and Wilson 2008), and it has been reported that lack of IL-8

receptor reduced macrophage infiltration in adipose tissue (Neels, Badeanlou et al. 2009). In the present study, ZK159222 and ZK191784 decreased macrophage-induced IL-8 release (up to 65%), with a greater efficacy compared to $1,25(OH)_2D_3$. Chemokine RANTES is another key player in obesity-associated inflammation in adipose tissue. RANTES stimulates monocyte migration and macrophage survival in human adipose tissue; its expression in adipose tissue is increased in obese patients (Keophiphath, Rouault et al. 2010). Like $1,25(OH)_2D_3$, both analogues significantly reduced macrophage-induced RANTES secretion (82-93%) by adipocytes. Taken together, our results show that ZK159222 and ZK191784 produced comparable effects to $1,25(OH)_2D_3$ in reducing the production of the cytokine/chemokines by adipocytes. It is suggested that both compounds have anti-inflammatory actions similar to the natural ligand in adipose tissue.

In conclusion, we have demonstrated that ZK159222 and ZK191784 possess anti-inflammatory properties in adipose tissue. Both $1,25(OH)_2D_3$ analogues powerfully inhibit macrophage-induced secretion of the proinflammatory cytokine and chemokines by adipocytes. This effect is probably via preventing the activation of the NF-κB and MAPK signalling pathways. Our study suggests that the $1,25(OH)_2D_3$ analogues may serve as a potential option to enhance VDR-mediated anti-inflammatory effects in adipose tissue in obesity.

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We would like to thank Dr Arndt Schmitz (Bayer Pharma AG) for providing ZK159222 and ZK191784. This study was financially supported by the University of Liverpool.

## Legends

**Figure 1. ZK159222 reverses macrophage-inhibited IκBα expression by adipocytes.** Differentiated adipocytes were incubated with maintenance medium, 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK159222 ($10^{-8}$M and $10^{-6}$M) for 48 h. Adipocyte medium was then replaced with RPMI-1640 medium (control), MC medium or MC medium with 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK159222 ($10^{-8}$M and $10^{-6}$M) for 24 h. IκBα protein content was determined by western blotting and quantified by densitometry. Data are means ± SEM, normalised to vinculin levels, n=3 per group. $^{**}P < 0.01$ vs MC group.

**Figure 2. ZK159222 inhibits macrophage-induced expression of NF-κB p65 by adipocytes.** Differentiated adipocytes were incubated with maintenance medium, 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK159222 ($10^{-8}$M and $10^{-6}$M) for 48 h. Adipocyte medium was then replaced with RPMI-1640 medium (control), MC medium or MC medium with 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK159222 ($10^{-8}$M and $10^{-6}$M) for 24 h. Phos-NF-κB p65 protein content was analysed by western blotting and quantified by densitometry. Data are means ± SEM, normalised to vinculin levels, n=3 per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs MC group .

**Figure 3. Effects of ZK191784 on macrophage-inhibited expression of IκBα by adipocytes.** Differentiated adipocytes were incubated with maintenance medium, 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK191784 ($10^{-8}$M and $10^{-6}$M) for 48 h. Adipocyte medium was replaced with RPMI-1640 medium (control), MC medium or MC medium with 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK191784 ($10^{-8}$M and $10^{-6}$M) for 24 h. IκBα protein content was analysed by western blotting and quantified by densitometry. Data are means ± SEM, normalised to vinculin levels, n=3 per group. $^{**}P < 0.01$ vs MC group.

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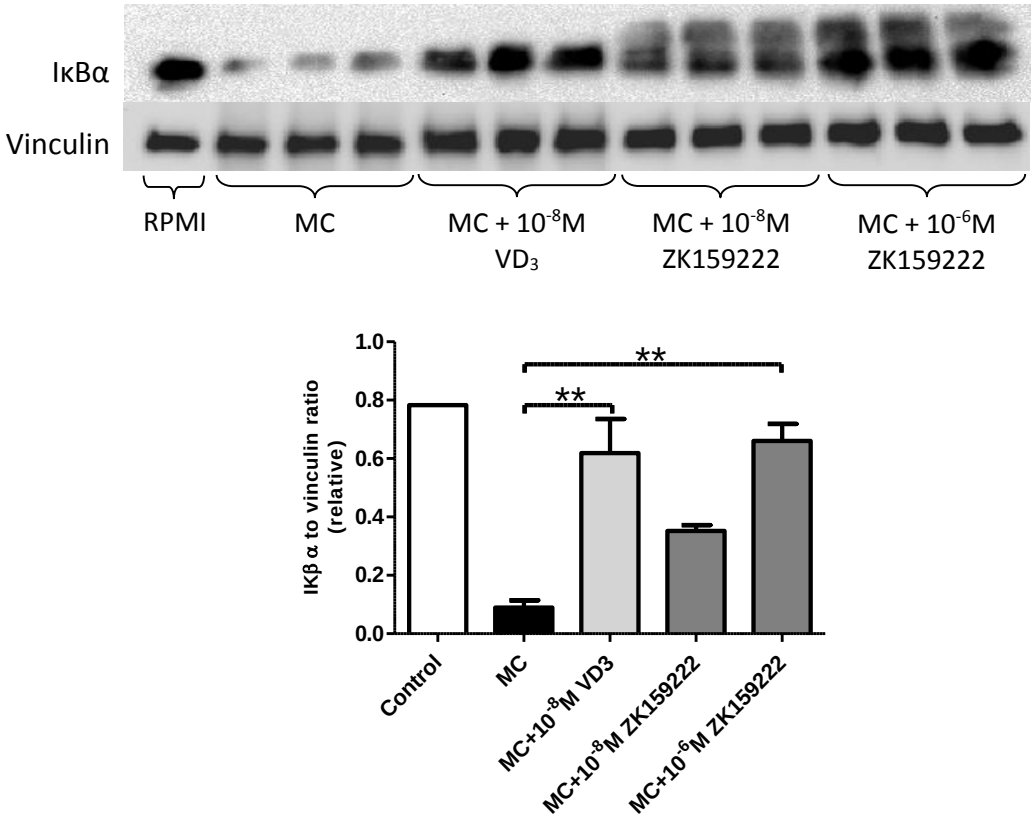
**Figure 5. ZK159222 and ZK191784 inhibit macrophage-induced expression of Phos-p38 MAPK by adipocytes.** Differentiated adipocytes were incubated with maintenance medium, 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK191784 ($10^{-8}$M and $10^{-6}$M) for 48 h. Adipocyte medium was replaced with RPMI-1640 medium (control), MC medium or MC medium with 1,25-$(OH)_2D_3$ ($10^{-8}$M), ZK159222 ($10^{-8}$M and $10^{-6}$M), ZK191784 ($10^{-8}$M and $10^{-6}$M) for 24 h. Effect of ZK159222 (A) and ZK191784 (B) on phos-NF-κB p65 protein content was analysed using western blotting and quantified by

densitometry. Data are means ± SEM, normalised to vinculin levels, n=3 per group. $^{**}P < 0.01$, $^{***}P < 0.001$ vs MC group.

**Figure 6. Effect of ZK159222 and ZK191784 on macrophage-induced secretion of IL-6, MCP-1, IL-8 and RANTES by human adipocytes.** Differentiated adipocytes were incubated with maintenance medium, 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK191784 ($10^{-8}$M and $10^{-6}$M) for 48 h. Adipocyte medium was replaced with RPMI-1640 medium (control), MC medium or MC medium with 1,25-$(OH)_2D_3$ ($10^{-8}$M), ZK159222 ($10^{-8}$M and $10^{-6}$M), ZK191784 ($10^{-8}$M and $10^{-6}$M) for 24 h. Protein release of IL-6, MCP-1, IL-8 and RANTES was measured as protein concentrations in the culture medium using ELISAs. Results are expressed as means ± SEM, n=3 per group. $^{\gamma\gamma\gamma}P < 0.001$ vs RPMI control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs MC control and $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs MC + 1,25-dihydroxyvitamin $D_3$. N.D. – not detected. MC only, macrophage-conditioned medium was used for measuring basal levels of the cytokine/chemokines released by macrophages.

**Figure 7. Cytotoxicity of macrophage-conditioned medium, 1,25-$(OH)_2D_3$, ZK159222 and ZK191784 in adipocytes.** Differentiated adipocytes were incubated with maintenance medium, 1,25-$(OH)_2D_3$ ($10^{-8}$M) or ZK191784 ($10^{-8}$M and $10^{-6}$M) for 48 h. Adipocyte medium was replaced with RPMI-1640 medium (control), MC medium or MC medium with 1,25-$(OH)_2D_3$ ($10^{-8}$M), ZK159222 ($10^{-8}$M and $10^{-6}$M), ZK191784 ($10^{-8}$M and $10^{-6}$M) for 24 h. Cytotoxicity was determined as LDH release into the cell culture medium. Data are expressed as means ± SEM, n=3 per group.

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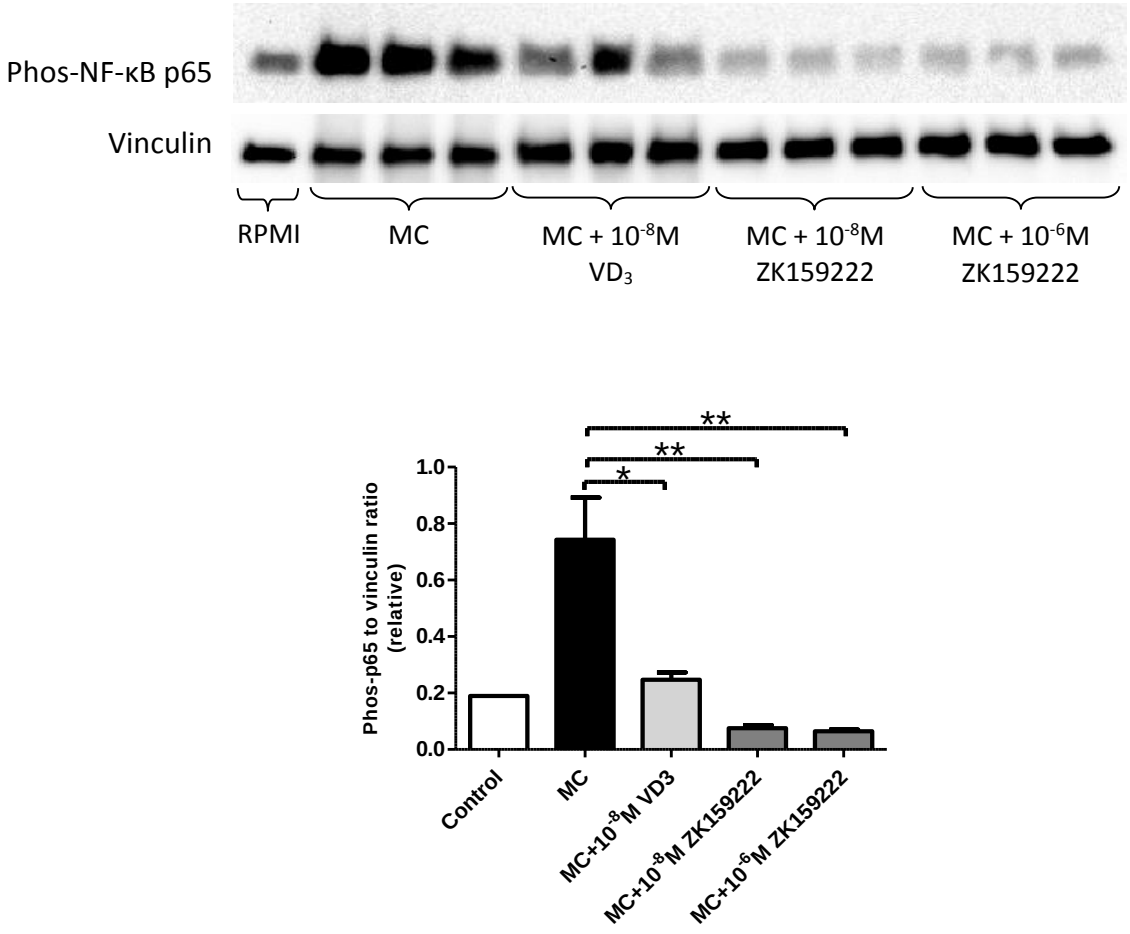


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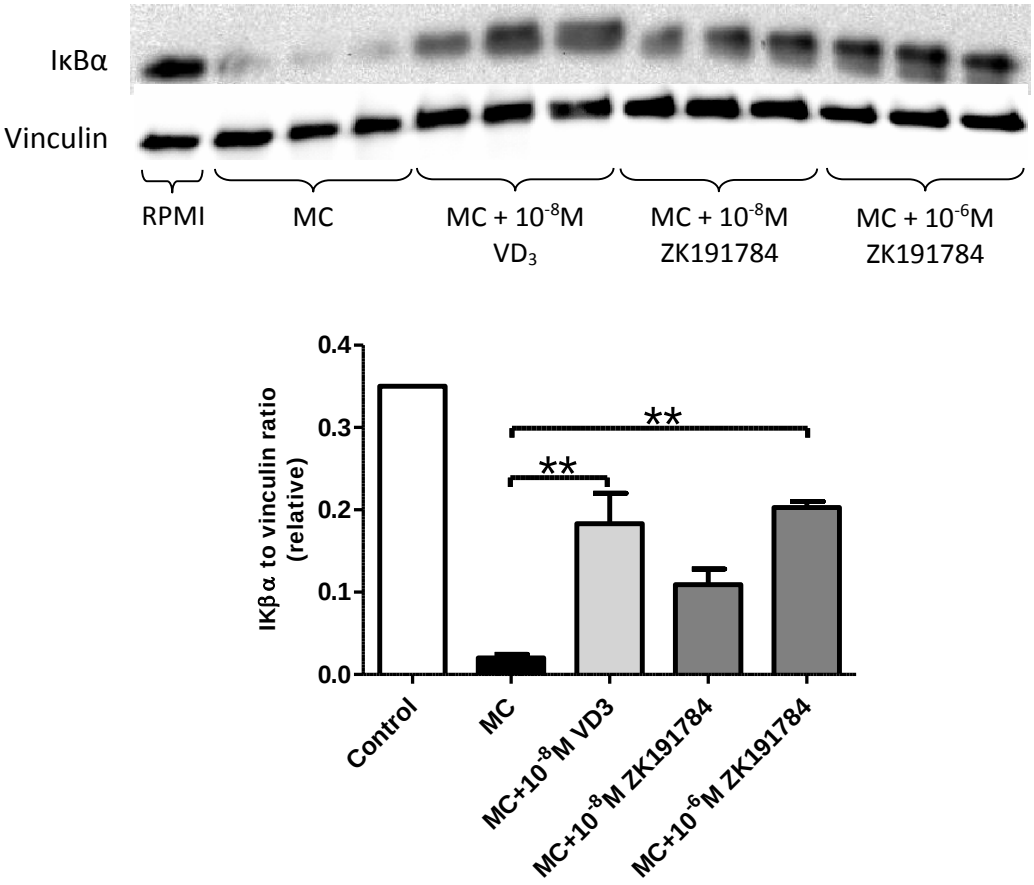


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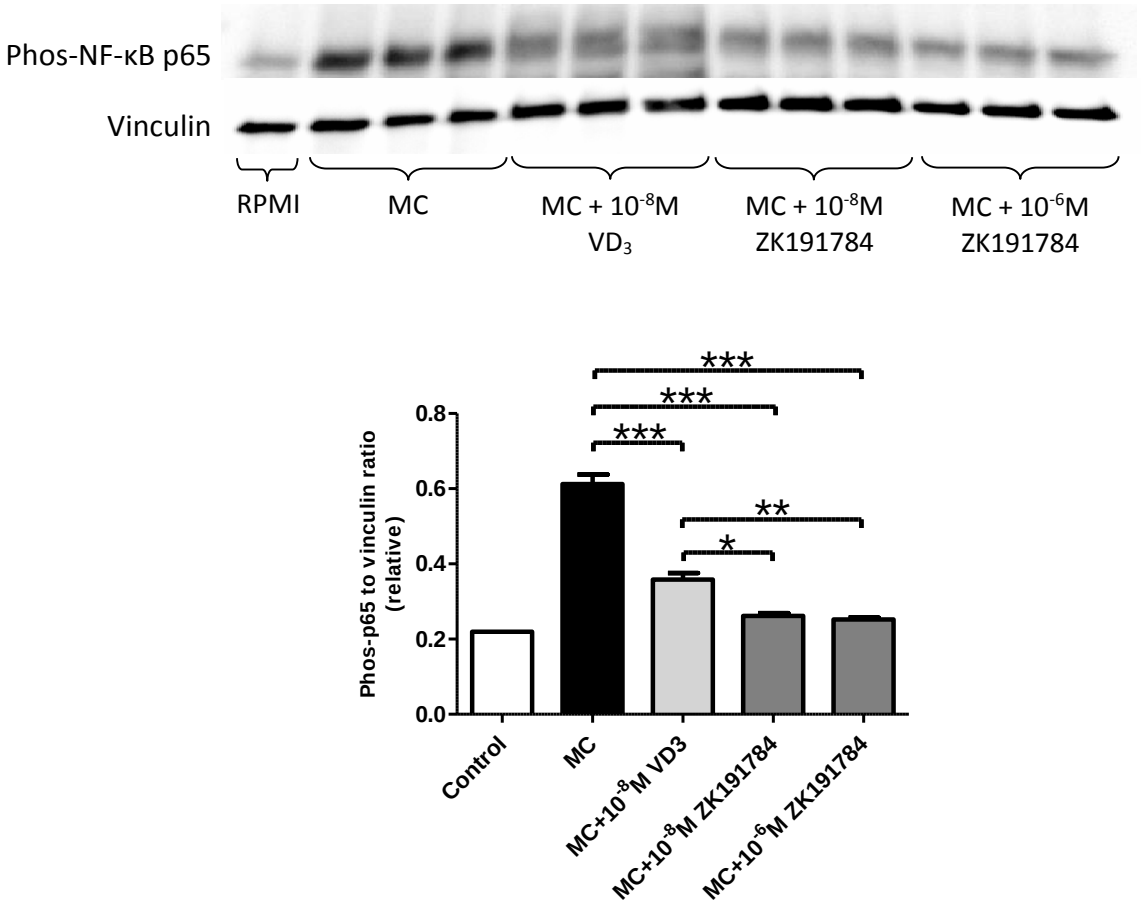


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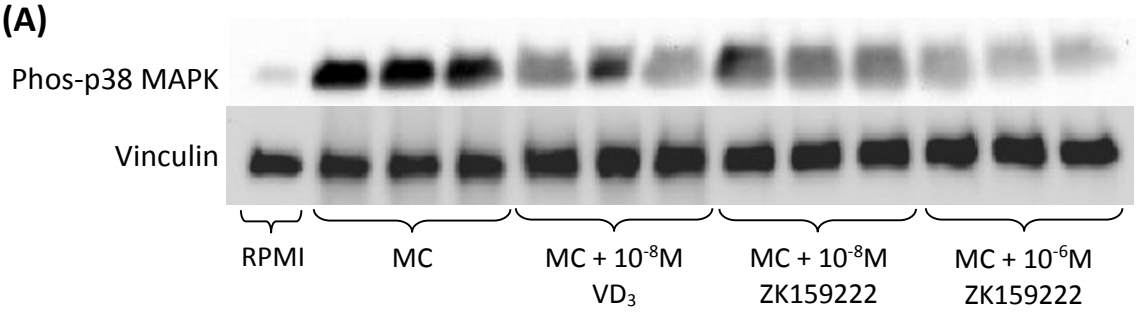
**(A)**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Vinculin
RPMI
MC
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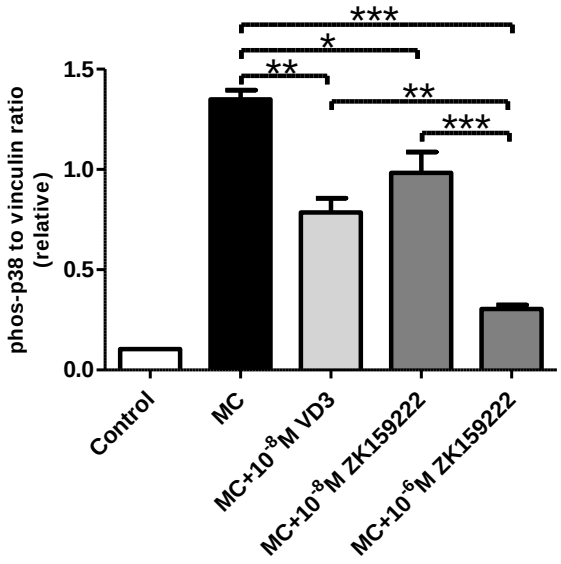
MC + 10-8M ZK159222
MC + 10-6M ZK159222


phos-p38 to vinculin ratio (relative)
1.5
1.0
0.5
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Control
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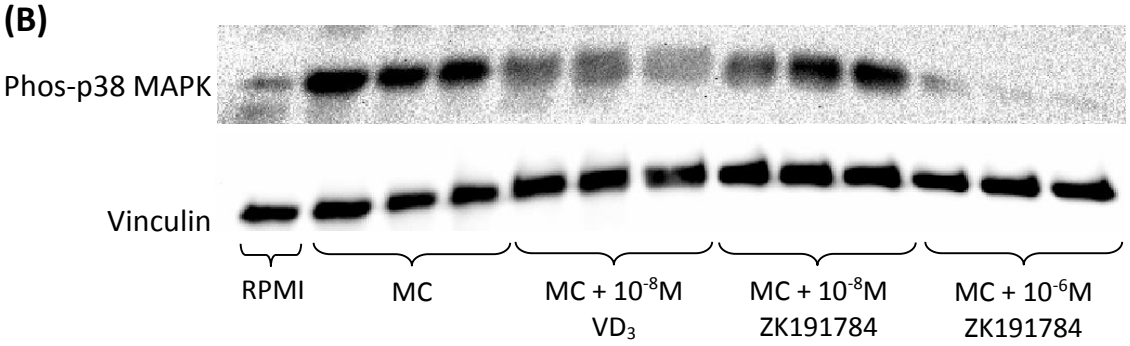
MC+10-8M ZK159222
MC+10-6M ZK159222

**(B)**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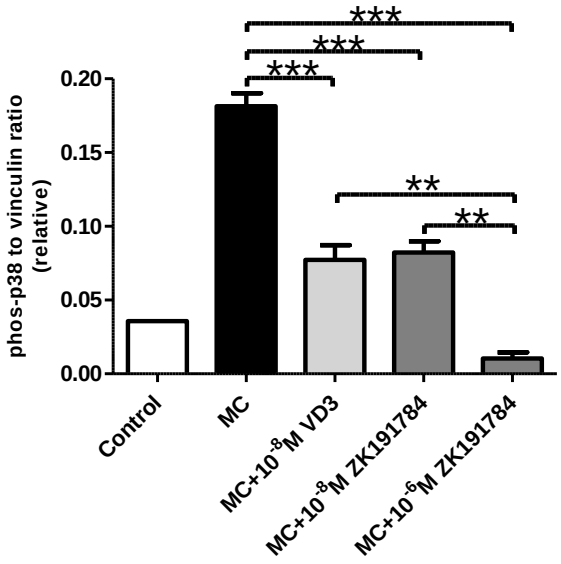
MC + 10-8M VD3
MC + 10-8M ZK191784
MC + 10-6M ZK191784


phos-p38 to vinculin ratio (relative)
0.20
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Control
MC
MC+10-8M VD3
MC+10-8M ZK191784
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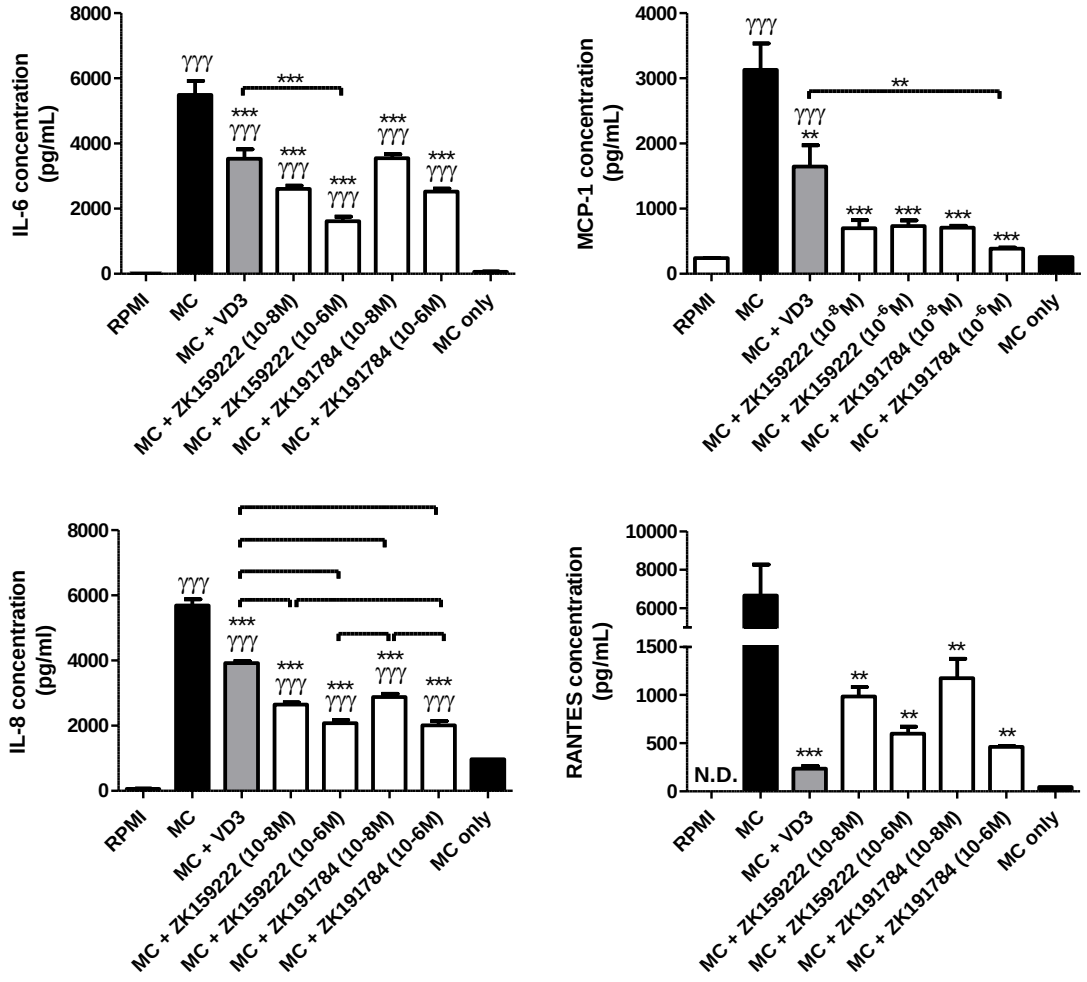


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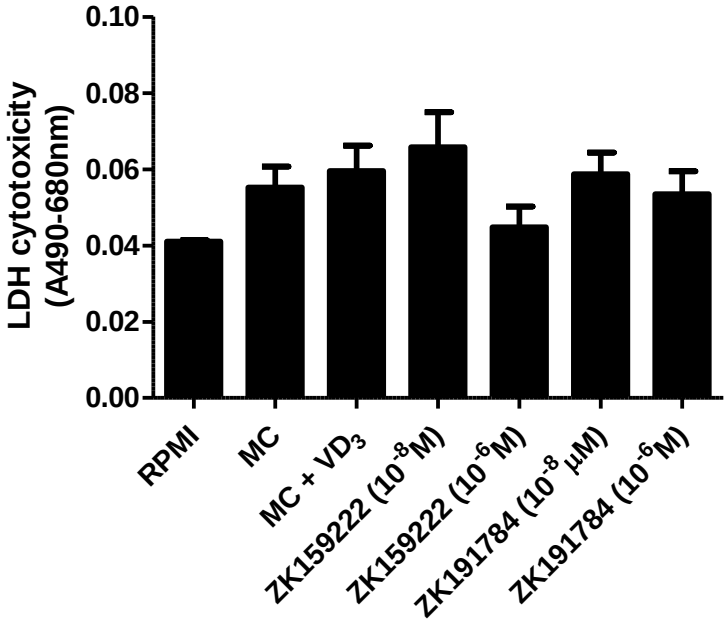


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