



## Therapeutics and Phytochemicals Activity Alterations Due to the NOD2-G908R Point Mutation *In Vitro*

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Received: March 05, 2018; Published: March 29, 2018

### Abstract

The nature of several of the genetic mutations in Inflammatory bowel disease (IBD) indicate that compounds that might be expected to have an anti-inflammatory effect might be altered due to some mutational impacts. Also, there is an increasing trend towards the use of complementary and alternative therapies by IBD patients, especially in pediatric cohorts, and many of these therapies contain phytochemicals and nutraceuticals. A reduction in colonic inflammation, whether through the use of a conventional pharmaceutical or a nutraceutical, and the differential impacts due to key mutations will help in understanding the therapeutic potential of drugs and phytochemicals in aiding IBD subjects with and without vital mutations. Therefore, we have utilized an *in vitro* screening assay that considers an anti-inflammatory response of the chemicals towards a pair of mammalian cell lines, one of which is functional in a key genetic region associated with IBD susceptibility and one of which carries the genetic variant. A representative range of pharmaceuticals and phytochemicals has been selected for comparative study. Results from this study indicated the effects of NOD2 mutation G908R in altering the impacts of some drugs and phytochemicals *in vitro*. The effects of drugs Cyclosporin A, Methotrexate and phytochemicals Arctigenin, Glabridin, AKBA, Ellagic acid and Z-Guggulsterone were differentiated due to the NOD2-G908R mutation in this *in vitro* analysis, while effects of other phytochemicals Triptolide, PEITC, Curcumin, Epigallocatechin-3-gallate and drugs prednisolone and Mesalamine were not significantly altered due to the NOD2-G908R mutation in this *in vitro* study.

**Keywords:** NOD2-G908R; Methotrexate; Mutation; Phytochemicals; Mesalamine; Drugs

### Introduction

NOD2 (nucleotide binding oligomerization domain 2) is a constituent of the NLR (NOD, leucine-rich repeat (LRR) containing protein) family, which are sensors of intracellular pathogen/microbe associated molecular patterns. It is a vital pattern recognition receptor that recognizes the bacterial cell wall peptidoglycan, muramyl dipeptide (MDP) and plays a crucial role in regulating innate immune responses [1-7]. For this reason NOD2-G908R, which is one of the NOD2-LRR region mutations with an impaired NOD2 signaling activity [8] is of considerable interest as genetic polymorphisms affecting the *CARD15* gene on chromosome 16, that codes for the NOD2 protein are known to pose a significant risk for the development of Inflammatory bowel diseases (IBD), especially Crohn's disease (CD) [9-12]. For instance, studies have shown a 17.1 fold increased risk for the development of the disease in individuals with homozygous *CARD15* gene polymorphisms and a 2.4 fold increased risk in heterozygous carriers [9]. The vital role of NOD2 in regulating host defense mechanism in intracellular infections and modulation of the microflora of the gastrointestinal tract, explains the influence of NOD2 polymorphisms on CD risk

[3,11,13]. Also, NOD2 gene variants are known to cause increased disease activity and stricturing lesions in the intestines [14], evaluating drugs and phytochemicals that could modulate pathways involved in intestinal inflammation instead of having a systemic action, could have enhanced therapeutic benefits for IBD as targeting pathways responsible for NF- $\kappa$ B signaling has been found to be beneficial to reduce inflammation and help re-establish intestinal homeostasis [15].

In addition, many Inflammatory bowel disease (IBD) patients with the diseases exploit complementary and alternative therapies (CAM), often without consultation with health professionals. It is unclear as to whether these could augment, antagonise or even replace conventional therapies. For this reason, certain phytochemicals that have been associated with CAM and drug development in IBD are evaluated in this study beside the conventionally used drugs in IBD management.

The aim of this study was to investigate the effects of both conventional drugs and a range of phytochemicals on cell lines with different pattern recognition receptors (PRRs) to analyze the activity of these chemicals, and consider whether these effects still

occur in the presence of a key NOD2 variant. The methodology of this study is similar to that established by Philpott et al in a previous study [16]. HEK293T cells transfected with NOD2 (WT and G908R), TLR2, or TLR4 and a SEAP reporter were employed to identify these effects.

## Materials and Methods

### Chemicals

The compounds Methotrexate, Mesalamine, Prednisolone and Cyclosporin A, Glabridin, (-)-Epigallocatechin gallate, (Z)-Guggulsterone, Phenethyl isothiocyanate, Curcumin and Ellagic acid and Triptolide were purchased from Sigma Aldrich. Arctigenin and Acetyl-11-keto- $\beta$ -Boswellic Acid was purchased from Santa cruz biotechnology and Merck Millipore respectively. Hypericin was purchased from Tocris.

### HEK-NOD2-WT and HEK-NOD2-G908R modulations

HEK293T cells over expressing NOD2 wildtype (WT) and NOD2-G908R mutant genes were created in our lab for studying the genotype specific effects [16]. The HEK293T cells were seeded and grown devoid of antibiotics in 100mm tissue culture plates (BD biosciences, USA). The transfection of the cells was done by Lipofectamine 2000 (Invitrogen, NZ) with pSV- $\beta$ -Galactosidase vector (2 $\mu$ g) (transfection control) (Promega, USA), pNifty2-SEAP vector (2  $\mu$ g) (NF- $\kappa$ B reporter) and 12 ng of pUNO-hNOD2a to form the NOD2 wild-type cell line, or pUNO-hNOD2a G908R to form the NOD2-G908R mutant cell line. The cell lines were maintained and sub-cultured in complete DMEM (10% FCS + 1% Pen/Strep) supplemented with blasticidin (330 $\mu$ l in 550ml media) and zeocin (550 $\mu$ l in 550ml media). The cells were seeded at 4 x 10<sup>5</sup> cells/ml into 96 well plates, grown for 24 hours and then treated with each of the drugs over a 5 fold dilution range. After 24hr incubation with the drugs, the cells in the 96 well plate were stimulated alternatively by the addition of MDP (final concentration 10  $\mu$ g/ $\mu$ l) or an equivalent volume of DMEM media for a non MDP interaction. After a 16 hour incubation, 30 $\mu$ l of cell supernatant was transferred to fresh 96-well plates containing 150  $\mu$ l of QUANTI-Blue™, reagent (Invivogen, USA) for the detection of secreted embryonic alkaline phosphatase (SEAP) production and 15  $\mu$ l of WST-1 reagent (Roche

Applied Science, Germany) added to the wells of the original 96-well plate to assess cell proliferation. QUANTI-Blue™ plate absorbencies were read at 635 nm, and the WST-1 plates were read at 450 nm in a spectrophotometer (Thermo Electron, USA).

### Statistical analysis

The effects on cell proliferation of the drug or nutraceutical at each concentration in each cell line used is calculated by the percentage of cell survival in comparison to the survival of the respective untreated cells:

$$\text{Survival \%} = \frac{\text{Treated Cells}}{\text{Untreated Cells}} \times 100 \quad (\text{Equation 1})$$

The NF- $\kappa$ B activity score at each concentration was calculated by normalizing the quanti blue score of the solvent control from the quanti blue score of the drugs or nutraceuticals at each concentration:

$$\text{NF-}\kappa\text{B activity score} = \text{Solvent control QB score} - \text{Drug QB score} \quad (\text{Equation 2})$$

The NF- $\kappa$ B activity score obtained as above at each viable concentration was tested for significance of the P value against untreated cells or cells at zero concentration of the drug or nutraceutical, and graphically illustrated using the Graphpad PRISM program, with p values of significance represented as \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

## Results

### Comparative analysis of IBD therapeutics in modulating NOD2 wildtype and NOD2-G908R mutant activity *in vitro*

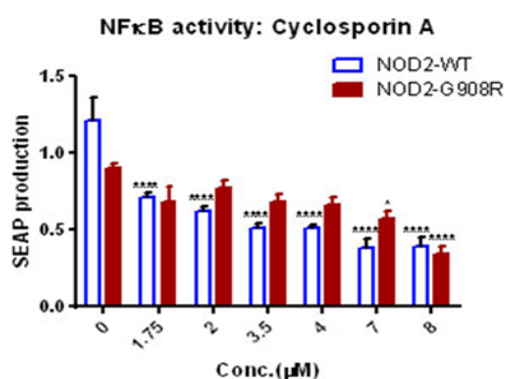
The effects of Cyclosporin A, Prednisolone, Methotrexate and Mesalamine were tested on HEK293 cells over expressing NOD2 wild type and NOD2-G908R mutant genes to investigate any differential impacts due to the mutation at 5 different concentrations of each drug, with the highest concentration causing cell viabilities of at least 50%. The impacts of the drugs on the NOD2 wild type and mutant genes were analyzed in the presence and absence of its stimulant MDP for any anti-inflammatory effects.

Drug	NOD2-Wild type			NOD2-G908R		
	CONC.	NF- $\kappa$ B activity score (Mean +/- SE)	Survival % (Mean +/- SE)	CONC.	NF- $\kappa$ B activity score (Mean +/- SE)	Survival % (Mean +/- SE)
Cyclosporin A (n = 3)	4 $\mu$ M	0.50+/-0.02	71.7+/-1.10	8 $\mu$ M	0.33+/-0.05	81.5+/- 4.12
Prednisolone (n = 3)	150 $\mu$ M	1.08+/- 0.07	76.5+/-5.49	150 $\mu$ M	1.18+/- 0.19	75.3+/- 4.61
Methotrexate (n = 3)	18.8 nM	0.58+/-0.07	64.22+/-4.69	25 nM	0.71+/-0.06	75.38+/-5.64
Mesalamine (n = 3)	3 mM	0.99+/-0.19	79.01+/-11.27	3 mM	1.55+/-0.09	77.68+/-1.63
Control: Ibuprofen (n = 4)	1.07 mM	0.30+/-0.008	68.09+/-2.71	1.07 mM	0.58+/-0.04	69.55+/-1.86

**Table 1:** Drug concentrations at which cell proliferation was closer to ~70% in HEK cells overexpressing NOD2 or NOD2-G908R mutant. CONC: concentration was expressed as nanomol/L (nM), micromol/L ( $\mu$ M), or millimol/L (mM).

## Cyclosporin A

Cyclosporin A was observed to cause significant suppression of the NF- $\kappa$ B activity in this study. The wild type cell lines show an overall higher sensitivity to the action of Cyclosporin A than the NOD2-G908R mutant cell lines at all concentrations in the absence of MDP stimulation. With the inducement of MDP-stimulated inflammatory effect, the NF- $\kappa$ B suppressing impacts of Cyclosporin A is seen to be reduced. However, the NOD2 wild type cells responded better to Cyclosporin A than the NOD2-G908R mutant cells as seen in figure 1.



**Figure 1:** Cyclosporin A in suppressing NF- $\kappa$ B activity in HEK-NOD2 wild type and HEK-NOD2-G908R cell lines in comparison to controls.

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$

Furthermore, Cyclosporin A is observed to influence the cell proliferation differently in the HEK-NOD2 wild type and HEK-G908R mutant *in vitro*. The G908R mutant cells are seen to have a higher cell proliferation than the wildtype cells in the presence of cyclosporin A. A ~70% cell viability is seen at a concentration of ~4  $\mu$ M in the wild type, while a similar effect on proliferation is observed at ~8  $\mu$ M in the NOD2-G908R mutant cell lines.

## Prednisolone

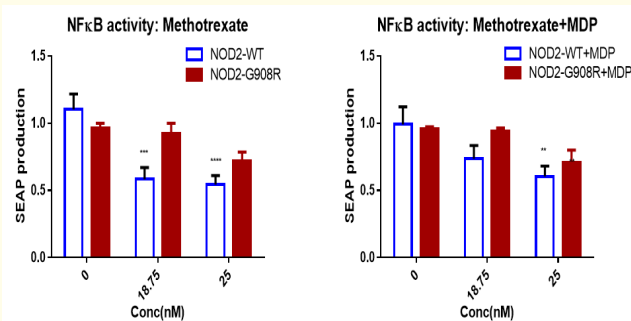
The inhibition of the NF- $\kappa$ B activity by Prednisolone in the HEK-NOD2 cell lines *in vitro* in this study occurred only at its highest concentration, however, due to the lower ~50% cell survival rate observed at ~400  $\mu$ M, this activity is uncertain. Despite causing almost equal survival rates of ~50% in the wildtype and mutant cell lines at the highest concentration of ~400  $\mu$ M used in this study, the inhibition of the NF- $\kappa$ B activity in NOD2 wild type cells was observed to be significantly greater in comparison to the NOD2-G908R mutant cells at this highest concentration point in the presence of MDP stimulation.

The cell viabilities increased with lower concentrations of prednisolone, with a concentration of ~150  $\mu$ M causing ~70% viability in the wild type and the mutant cell lines, However, Prednisolone did not cause any anti-inflammatory effect by NF- $\kappa$ B inhibition at decreased concentrations. These data indicate that the interaction

of Prednisolone in the HEK-NOD2 cell lines could be reduced *in vitro* as it only occurs at the high concentrations, with negligible effects at lower doses.

## Methotrexate

Methotrexate was observed to strongly inhibit the NF- $\kappa$ B activity in the HEK-NOD2 cell line in this study, showing a significant inhibitory effect at all concentrations in the wild type cells. The NF- $\kappa$ B inhibition is also seen to occur in the presence of the MDP stimulation at concentrations of methotrexate over ~25 nM in wildtype cells as seen in figure 2. However, the inhibition of the NF- $\kappa$ B activity in the HEK-NOD2-G908R mutant cell lines was seen to be weaker than in the HEK-NOD2 wild type cell lines. Methotrexate was found to influence the cell proliferation differently in the HEK-NOD2 wild type and HEK-NOD2-G908R mutant cell lines, however this difference is statistically insignificant in this study.



**Figure 2:** Methotrexate in suppressing NF- $\kappa$ B activity in HEK-NOD2 wild type and HEK-NOD2-G908R cell lines.

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$

## Mesalamine

Mesalamine did not show any differential modulating effects on the NF- $\kappa$ B activity in the HEK-NOD2 wild type and HEK-G908R mutant cell lines, in the presence and absence of MDP stimulation. The cell proliferation of the HEK-NOD2 wild type and HEK-NOD2-G908R mutant cells also remained unaffected in the presence of mesalamine in this study, as there was no significant viability changes.

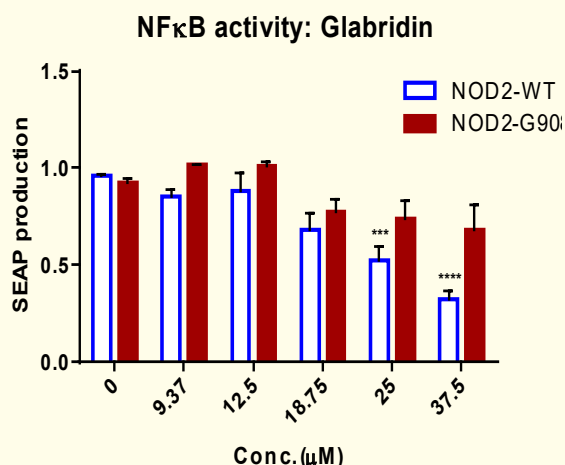
## Comparative analysis of phytochemicals used in IBD management in modulating NF- $\kappa$ B activity in HEK-NOD2 wildtype and HEK-NOD2-G908R cell lines

The effects of some phytochemicals are investigated in this phase of this study, to scrutinize their effect on the NF- $\kappa$ B activity in the HEK cells overexpressing NOD2 wild type, or NOD2-G908R mutant to evaluate any differential impacts of the phytochemicals due to the mutation in modulating the NF- $\kappa$ B activity.

## Glabridin

Glabridin was found to significantly suppress NF- $\kappa$ B activity in HEK-NOD2 wild type cells. The NF- $\kappa$ B suppression was observed to be significant above a concentration of ~25  $\mu$ M in HEK-NOD2 wild type cells. While the difference in the impacts of Glabridin

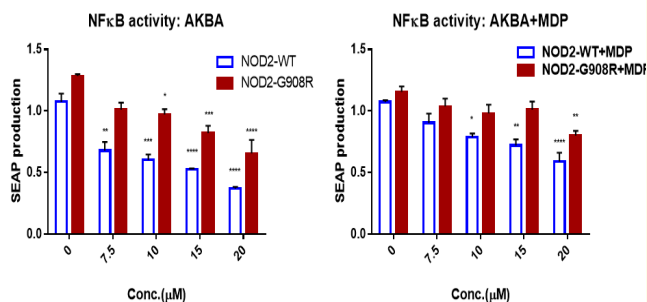
between the wild type and mutant cells is not statistically significant at all the concentrations used here, the difference in effects of Glabridin was found to vary between the wildtype and mutant cell lines, with the mutant cells requiring higher concentrations of Glabridin in comparison to the wildtype cells to have similar effects of NF-κB suppression. Furthermore, the cell viability of HEK-NOD2-G908R cells was found to be less effected by Glabridin in comparison to the NOD2 wild type cells.



**Figure 3:** Glabridin in suppressing NF-κB activity in HEK-NOD2 wild type and HEK-NOD2G908R cell lines. [ $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ]

**Acetyl-keto-β-Boswellic acid (AKBA)**

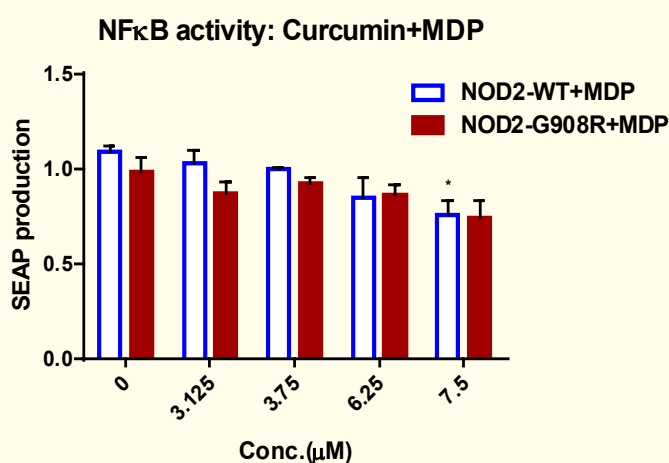
AKBA significantly suppressed NF-κB activity in HEK-NOD2 wild type and HEK-NOD2G908R mutant cells. The suppression in NF-κB activity by AKBA was observed to be more prominent in the wild type cells than the mutant cells, due to a lower concentration of AKBA required in wildtype cells in comparison to the mutant cells. P values for the comparison of treated cells versus untreated cells was found to become significant from concentrations of ~7.5 μM in wild type cells and ~10 μM in mutant cells. In the presence of MDP stimulation the significance values were observed from ~10 μM in wild type cells and 20 μM in mutant cells as seen in figure 4.



**Figure 4:** AKBA in suppressing NF-κB activity in HEK-NOD2 wild type and HEK-NOD2-G908R cell lines. [ $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ]

**Curcumin**

The NF-κB inhibition was equal in HEK-NOD2 wildtype and HEK-NOD2-G908R mutant cell lines. The P values of comparison between cell lines was observed to be significant over a concentration of ~12.5 μM in the wild type and mutant cell lines, however this is debatable due to a lower cell survival of ~60%. The difference of curcumin activity between wildtype and mutant cell lines was observed to be insignificant indicating the potential of curcumin in having almost equal NF-κB inhibiting anti-inflammatory impact in the NOD2 wild type and NOD2G908R mutant cell lines in the absence of any stimulation. However, with the induction of an MDP stimulation, a somewhat significant NF-κB suppression was noticed at ~7.5 μM in wild type cell lines, while the NOD2-G908R mutant cell lines were not found to be influenced as seen in the figure 5.



**Figure 5:** Curcumin in suppressing NF-κB activity in HEK-NOD2 wild type and HEK-NOD2-G908R cell lines in the presence of the MDP ligand stimulation. [ $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ]

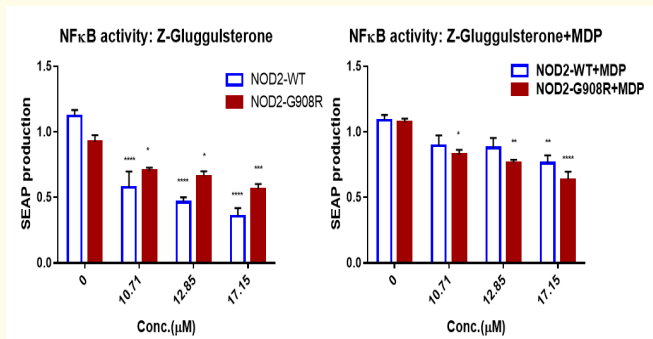
**Z-Gluggulsterone**

Z-Gluggulsterone was found to suppress NF-κB activity significantly in wild type NOD2 cells at all concentrations in comparison to mutant cells. While the NF-κB suppression was substantial in both NOD2 wild type and NOD2-G908R mutant cell lines, the P values of significance in wildtype HEK-NOD2 cell lines was observed to be stronger as indicated in figure 6. However, with the induction of MDP stimulation, Z-Gluggulsterone was observed to have a significant NF-κB suppression at a lower concentration of ~10 μM in the HEK-NOD2-G908R mutant cells and an insignificant but higher concentration of ~17 μM in HEK-NOD2 wild type cells, indicating a better NF-κB suppression in the mutant G908R cells in comparison to NOD2 wild type cells, as indicated in figure 6.

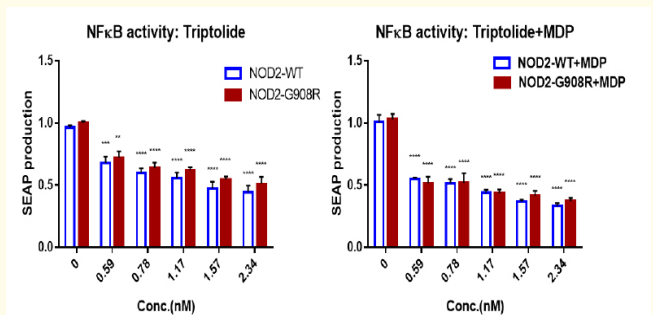
**Triptolide**

Triptolide strongly suppressed NF-κB activity in HEK-NOD2 wildtype and HEK-NOD2-G908R mutant cell lines in the presence and absence of MDP stimulation as shown in figure 7. Even though

Triptolide seemed to have a slightly stronger NF-κB suppression in wildtype cells, this difference was not statistically significant, indicating its almost equivalent interaction in the HEK-NOD2 wild type and HEK-NOD2G908R mutant cells.



**Figure 6:** Z-Gluggulsterone in suppressing NF-κB activity in HEK-NOD2 wild type and HEK-NOD2-G908R mutant cell lines with and without MDP stimulation. [ $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ]



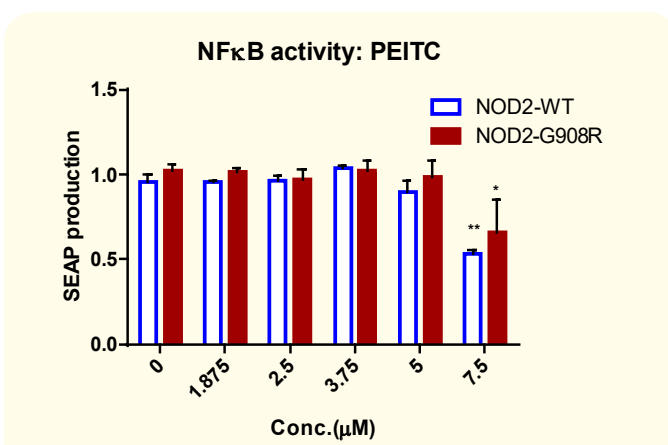
**Figure 7:** Triptolide in suppressing NF-κB activity in HEK-NOD2 wild type and HEK-NOD2-G908R mutant cell lines in the absence and presence of MDP stimulation. [ $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ]

**Phenethylisothiocyanate (PEITC)**

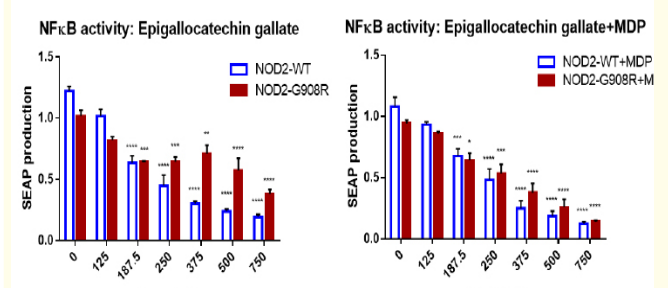
PEITC inhibited NF-κB activity significantly over a concentration range of ~7.5 μM in HEK-NOD2 wild type and HEK-NOD2G908R mutant cell lines as shown in figure 8. Also, the effect of PEITC was also observed to be similar on NF-κB suppression in the NOD2 wild type and mutant cell lines after stimulated with MDP ligand, implicating almost similar impacts in the NOD2 wild type and mutant cells.

**Epigallocatechin gallate**

Epigallocatechin gallate was found to inhibit NF-κB activity at similar concentrations in HEK-NOD2 and HEK-NOD2-G908R mutant cell lines. However, the wild type cell lines showed stronger significance P values with epigallocatechin gallate treatment, as illustrated in figure 9.



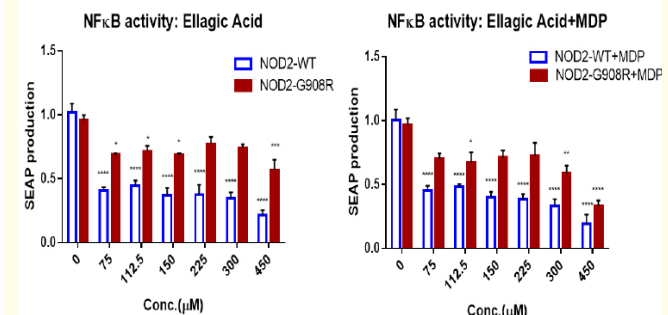
**Figure 8:** PEITC in suppressing NF-κB activity in HEK-NOD2 wild type and HEK-NOD2-G908R cell lines. [ $*p < 0.05$ ,  $**p < 0.01$ ]



**Figure 9:** Epigallocatechin gallate in suppressing NF-κB activity in HEK-NOD2 wild type and HEK-NOD2-G908R cell lines. [ $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ]

**Ellagic acid**

The effect of Ellagic acid in the inhibition of NF-κB activity in the HEK-NOD2 wild type cells was observed to be more significant in comparison to its NF-κB inhibition in HEK-NOD2-G908R mutant cells in the absence and presence of MDP stimulation, as indicated in figure 10.



**Figure 10:** Ellagic acid in suppressing NF-κB activity in HEK-NOD2 wild type and HEK-NOD2-G908R cell. [ $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ]

## Arctigenin

Arctigenin suppressed NF- $\kappa$ B activity significantly at very low concentrations of 0.98  $\mu$ M in HEK-NOD2 wildtype cells, in comparison to a significant higher  $\sim$ 20  $\mu$ M in HEK-NOD2-G908R mutant

cell lines as shown in table 2. Thus, there was a substantial difference in arctigenin concentrations needed to inhibit NF- $\kappa$ B activity in the NOD2 wildtype and NOD2-G908R cell lines.

Phytochemical	NOD2-Wild type			NOD2-G908R		
	CONC.	NF- $\kappa$ B activity score (Mean +/- SE)	Survival % (Mean +/- SE)	CONC.	NF- $\kappa$ B activity score (Mean +/- SE)	Survival % (Mean +/- SE)
Glabridin (n = 3)	25 $\mu$ M	0.52 +/- 0.07	68.32 +/- 5.61	37.5 $\mu$ M	0.67 +/- 0.13	70.66 +/- 4.34
AKBA (n = 3)	15 $\mu$ M	0.52 +/- 0.007	78.03 +/- 6.10	20 $\mu$ M	0.65 +/- 0.10	74.57 +/- 1.93
Curcumin (n = 3)	7.5 $\mu$ M	0.70 +/- 0.03	91.01 +/- 2.02	7.5 $\mu$ M	0.76 +/- 0.09	76.14 +/- 4.69
Z-Gluggulsterone (n = 3)	12.85 $\mu$ M	0.46 +/- 0.03	74.73 +/- 9.43	17.15 $\mu$ M	0.56 +/- 0.04	77.62 +/- 3.70
Triptolide (n = 3)	2.34 nM	0.44 +/- 0.05	71.75 +/- 3.47	2.34 nM	0.51 +/- 0.05	76.87 +/- 6.90
Phenethylisothiocyanate (PEITC) (n = 3)	7.5 $\mu$ M	0.53 +/- 0.02	84.72 +/- 10.22	7.5 $\mu$ M	0.65 +/- 0.19	55.01 +/- 3.51
Epigallocatechin gallate (n = 3)	500 $\mu$ M	0.24 +/- 0.01	Color interference	500 $\mu$ M	0.57 +/- 0.09	Color interference
Ellagic Acid (n = 3)	450 $\mu$ M	0.21 +/- 0.03	Color interference	450 $\mu$ M	0.56 +/- 0.08	Color interference
Arctigenin (n = 3)	0.98 $\mu$ M	0.41 +/- 0.02	71.58 +/- 7.74	20.0 $\mu$ M	0.57 +/- 0.02	73.03 +/- 5.68
Control: Ibuprofen (n = 4)	1.07 mM	0.30 +/- 0.008	68.09 +/- 2.71	1.07 mM	0.58 +/- 0.04	69.55 +/- 1.86

**Table 2:** Concentrations at which cell proliferation was closer to  $\sim$ 70% in HEK cells overexpressing NOD2 wild type or NOD2-G908R mutant.

CONC: concentration was expressed as nanomol/L (nM), micromol/L ( $\mu$ M), or millimol/L (mM).

## Discussion and Conclusion

### Evaluation of the *in vitro* impacts of the IBD drugs on NF- $\kappa$ B activity in the HEK-NOD2 and HEK-NOD2-G908R mutant cell lines

Clinical studies have shown that IBD patients with CARD15/NOD2 gene variants have a greater disease activity than other patients, with early need for surgery and higher rates of surgical recurrence, who might require more intensive treatments to avoid complications. Frequent formation of stricturing lesions is a pathogenic phenomenon observed in the disease progression course in patients with NOD2 gene variants [14,17].

Studies on the clinical response to treatments by the IBD gene variant patients in comparison to controls has been varied [18]. This study was carried out to scrutinize the effects of some commonly used drugs and phytochemicals in IBD therapies to investigate for any differential impacts in modulating NF- $\kappa$ B activity in the HEK-NOD2 wild type and HEK-NOD2-G908R mutant cells *in vitro*, as NOD2-G908R is one of the variants known to cause increased susceptibility to both CD and UC [19].

The drugs Cyclosporin A and Methotrexate, which are potent immune-modulators [20,21], inhibited NF- $\kappa$ B activity in both the NOD2 and NOD2-G908R mutant cell lines in this study. However, the impacts of Cyclosporin A and methotrexate was enhanced in the HEK-NOD2 wildtype cell line in comparison to the HEK-G908R mutant cell line, implicating the effect of the mutation in possibly altering drug activity in this *in vitro* study.

Prednisolone had a negligible impact on the NOD2 cell lines in this study. As the NF- $\kappa$ B inhibition observed in this study was at the highest dose of Prednisolone used, NOD2 mutations could potentially further impair the prednisolone effects due to its possible negligible interaction through the NOD2 pathway. This observation could be the possible cellular level factor attributing to the steroid resistance reported in CD patients with NOD2 mutations [22].

Clinically, Mesalamine is known to be anti-inflammatory at higher concentrations around inflamed tissues [23]. However, in this *in vitro* study, this drug did not have any significant effects on the HEK-NOD2 wild type and HEK-NOD2-G908R mutant cell lines, which might be attributed to negligible *in vitro* interactions of mesalamine in this study.

### Evaluating the *in vitro* impacts of Phytochemicals in the HEK-NOD2 wild type and HEK-NOD2-G908R mutant cell lines

As the studies on the gene specific impacts of phytochemicals for IBD have been limited, some phytochemicals identified to have therapeutic benefits for IBD are evaluated for any gene specific therapeutic features through the HEK-NOD2 and HEK-NOD2-G908R mutant cell lines to investigate for any differential impacts. This study is an initiative to analyze if the therapeutic competence of phytochemicals in assisting patients with an IBD susceptible gene polymorphism could be detected through an *in vitro* research.

Glabridin, which is a polyphenolic flavonoid was known to have therapeutic benefits for IBD [24]. In this study, Glabridin suppressed NF- $\kappa$ B activity, confirming its anti-inflammatory potential established in prior studies [24,25]. However, the results in this *in vitro* study demonstrate the NF- $\kappa$ B inhibiting anti-inflammatory impacts of Glabridin on the cells with the wildtype NOD2 to be greater than on the cells with the NOD2-G908R mutation. However, in the presence of MDP stimulation, the differential impacts of glabridin were not observed between the HEK-NOD2 wild type and HEK-NOD2-G908R mutant cells implicating an equivalent effect. This might indicate that the intensity of the differential impacts of Glabridin in the wild type and mutant cells could be influenced by the environmental conditions and the MDP stimulation induced in the present study and could have altered the interaction or activity of Glabridin in both the NOD2 cell lines.

AKBA in this *in vitro* analysis, inhibited NF- $\kappa$ B activity, affirming its anti-inflammatory impacts reported in prior studies [26,27]. Nevertheless, AKBA showed to have an enhanced NF- $\kappa$ B suppression in the HEK-NOD2 wild type cells, in comparison to HEK-NOD2-G908R mutant cells, and this differential impact was also persistently seen with the induction of MDP stimulation. This observation implicates the G908R mutation in effecting the ability of AKBA in inhibiting NF- $\kappa$ B activity differently in the HEK-NOD2 wild type and the HEK-NOD2-G908R mutant cells.

The influence of the phytochemical Ellagic acid on the NOD2 wild type cells and NOD2-G908R mutant cells in this *in vitro* study were almost similar to AKBA indicating an enhanced inhibition of NF- $\kappa$ B activity in NOD2 wild type cells in comparison to the NOD2-G908R mutant cells. While the inhibition of NF- $\kappa$ B activity in this study by ellagic acid was an affirmation of its anti-inflammatory potential reported in earlier studies [28], the results of present study could suggest that the NF- $\kappa$ B inhibiting therapeutic potential of ellagic acid could be reduced in the presence of NOD2-G908R mutation, or the altered inflammatory activity caused due to this NOD2 mutation could impair the therapeutic benefits of ellagic acid *in vitro*.

This study on the effects of Z-Guggulsterone (GS) confirms its anti-inflammatory effects reported in prior investigations by suppressing the NF- $\kappa$ B activity [29]. While Z-Guggulsterone showed to have a stronger impact on the NOD2 wild type cells in comparison to the NOD2-G908R mutant cells, implicating the possible effect of the G908R mutation in reducing the therapeutic efficiency of Z-Guggulsterone, nevertheless, with MDP stimulation, Z-Guggulsterone had a stronger NF- $\kappa$ B inhibition in the NOD2-G908R mutant cells in comparison to the NOD2-wild type cells.

Triptolide, which is a potent anti-inflammatory phytochemical inhibited NF- $\kappa$ B activity through the NOD2 pathway in this study affirming its anti-inflammatory properties reported in earlier studies [30]. In the present study, triptolide had similar impacts on NOD2 wild type cells and the NOD2-G908R mutant cells, in all the conditions used, implicating the therapeutic potential of triptolide to be unaffected by the NOD2-G908R mutation. These results indicate the ability of triptolide in suppressing NF- $\kappa$ B activity is strong

to challenge the altered inflammatory activity caused in the NOD2-G908R mutant cells *in vitro*.

PEITC has been reported to be a phytochemical with anti-inflammatory effects [31]. As the effects of PEITC in this *in vitro* study showed to be almost equal in impacting the NOD2 wild type cells and NOD2-G908R cells *in vitro*, present data may suggest the possibility of the NOD2-G908R mutation in having no considerable impact on altering the NF- $\kappa$ B inhibiting therapeutic potential of PEITC.

Curcumin with its potent anti-inflammatory effects in numerous studies [32-35], evaluated in this study was found to have equal impacts on the NOD2 wild type and NOD2-G908R mutant cells. Although, with the *in vitro* stimulation of MDP inflammation, curcumin showed a slightly higher anti-inflammatory impact on NOD2 wild type cells, the overall impact of Curcumin in the NOD2 wild type and NOD2-G908R mutant cells were found to be similar, indicating a potential therapeutic advantage of curcumin in aiding to the treatment of IBD patients with and without this NOD2-G908R polymorphisms similarly.

Epigallocatechin-3-gallate, which is known to be an anti-inflammatory agent, significantly suppressed NF- $\kappa$ B activity in this study, affirming its anti-inflammatory effects reported earlier [36,37]. Also, the action of epigallocatechin-3-gallate *in vitro* showed a non-differential impact on the NOD2 wild type and NOD2-mutant cells, implicating almost equal therapeutic impacts unaltered by the presence of the NOD2-G908R mutation in this *in vitro* analysis.

The therapeutic potential of Arctigenin [38-40], which is a phytochemical from *Arctium lappa* L (Greater burdock), was observed to be the most affected among all the phytochemicals and drugs used in this study, by the NOD2-G908R mutation according to this study. The HEK-NOD2 wild type cells were much sensitive to the impacts of Arctigenin at very low concentrations in comparison to the HEK-NOD2-G908R mutant cells. This implicates, that the NOD2-G908R mutation could be strongly impeding the anti-inflammatory impacts of Arctigenin, indicating a prominent involvement of arctigenin with the NOD2-G908R mutation during its modulation of NF- $\kappa$ B activity in the mutant cell lines.

Previous studies have reported the use of various phytochemicals in CAMs, often without the knowledge of their clinicians by many IBD patients [41-43]. Also, natural products have been associated with drug development in IBD [44-46]. Therefore, examining some significant phytochemicals in lieu of conventional drugs at the molecular and cellular levels on inflammatory reactions associated with IBD will aid in providing an insight to tailor appropriate therapies [16,47-50]. Furthermore, *in vitro* investigations like those of the present study may aid to initiate an understanding of mutation effects that alter drug/phytochemical activity. This understanding can help to initiate the adaptation of anti-inflammatory therapies to the individual genetic profiles of patients, resulting thus, in a more personalized tailored therapeutic approach as this would augment the consequences of the therapeutics used in IBD management.

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