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Effects of 2-Benzoylbenzoic Acid on Viability and Nitric Oxide Production in HIG-82 Synovial Fibroblasts

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Rheumatoid arthritis (RA) is characterized by persistent synovial inflammation, with synovial fibroblasts playing a key role in producing inflammatory mediators. 2-Benzoylbenzoic acid, known for its inhibitory activity against Aldo-keto reductase 1C3 (AKR1C3), was investigated for its effects on rabbit synovial fibroblasts (HIG-82) cell viability and nitric oxide (NO) production, both at rest and following stimulation with phorbol-12-myristate 13-acetate (PMA). Cells were treated with 2-benzoylbenzoic acid (3.125–300 μM) for 24, 48, or 72 hours, with or without PMA (10 nM). Reference NSAIDs, diclofenac and ibuprofen (100 μM), served as controls. The Griess reaction was used to evaluate nitrite concentrations in culture supernatants, and the MTT assay was used to assess cell viability. PMA significantly reduced cell viability over time compared to non-stimulated controls (24 h: 80.82 ± 8.70%; 48 h: 59.70 ± 12.50%; 72 h: 44.94 ± 8.40%). In non-stimulated cells, viability was generally maintained at concentrations =200 μM, whereas 300 μM reduced viability at 24 hours (67.75 ± 14.42%). In PMA-stimulated cells, viability at 72 hours was higher with 2-benzoylbenzoic acid (61.87–83.29%) than with PMA alone. Nitrite levels remained low, variable, and were not consistently decreased by the treatments, indicating that 2-benzoylbenzoic acid did not demonstrate an NO-linked anti-inflammatory effect under these conditions. Notably, despite its AKR1C3 inhibitory potential, 2-benzoylbenzoic acid did not suppress nitrite production, and no clear anti-inflammatory activity via NO modulation was observed.

Keywords: 2-benzoylbenzoic acid; Cell viability; Nitrite; Phorbol-12-myristate 13-acetate; Synovial fibroblasts.

Chronic synovitis, increasing joint degeneration, pain, and disability are the hallmarks of rheumatoid arthritis (RA), a systemic inflammatory disease.[1,2] Within the inflamed synovium, fibroblast-like synoviocytes can adopt an activated phenotype that promotes pannus formation and sustains inflammation through the release of cytokines, chemokines, and matrix-degrading enzymes.[3] Despite major therapeutic advances,[4] incomplete responses and adverse effects remain

practical barriers for some patients, supporting continued interest in alternative anti-inflammatory scaffolds. Conventional disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate, sulfasalazine, and hydroxychloroquine, have been widely used; however, they are associated with toxicity, delayed onset of action, and sometimes inadequate efficacy.[5,6] However, they are associated with toxicity, delayed onset of action, and sometimes inadequate efficacy.[6] This ongoing challenge supports continued interest in alternative anti-inflammatory scaffolds. Because there is no definitive cure, current therapies aim to control inflammation, relieve symptoms, and prevent structural damage using DMARDs and adjunct agents such as non-steroidal anti-inflammatory drugs (NSAIDs).[7,8]

2-benzoylbenzoic acid (Figure 1) is a benzoic-acid derivative reported to inhibit aldo-keto reductase 1C3 (AKR1C3), an enzyme involved in prostaglandin and steroid metabolism.[3] Physicochemical information on 2-benzoylbenzoic acid is available in curated chemical databases.[9] Nitric oxide (NO) has been implicated in inflammatory tissue injury, and nitrite accumulation in culture medium is commonly used as a practical proxy for NO production in cell-based assays.[10, 11] In this study, we tested whether 2-benzoylbenzoic acid influences viability and nitrite production in HIG-82 synovial fibroblasts under basal conditions and after PMA stimulation.

MATERIALS AND METHODS

Chemicals and Reagents

Sigma-Aldrich (St. Louis, MO, USA) supplied the 2-benzoylbenzoic acid, PMA, ibuprofen, diclofenac, MTT, and Griess reagents. Dimethyl sulfoxide (DMSO) was used to generate stock solutions, which were then diluted in culture medium until the final DMSO content was less than 0.1% (v/v).

Cell culture

HIG-82 rabbit synovial fibroblasts (ATCC® CRL-1832™; Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO, as previously described.[12] Briefly, HIG-82 synoviocyte cell line was kept in culture medium supplemented with 10% of foetal bovine serum (FBS; Biowest, South America), 100 U/ml penicillin (Biowest) and 100 ìg/ml streptomycin (Biowest). Culture medium was changed every 3-4 days. After entering the confluence, which took 2–3 weeks, cells were subcultured serially using a solution of 0.25% Trypsin-EDTA with a subculture ratio of 1:2 to 1:4 and subjected to experiments below passage 10.

Experimental design

After being seeded onto 96-well plates and given an overnight period to adhere, the cells were treated to PMA (10 nM) as needed. Cells were treated with 2-benzoylbenzoic acid (3.125–300 µM); ibuprofen and diclofenac (100 µM) served as reference NSAIDs. Incubations were conducted for 24, 48, or 72 h. Detailed methods and calculations were published previously.[7,12]

MTT assay

After incubation, culture medium was removed and 20µL of 5mg/ml MTT solution was added to each well.[12] Plates were incubated until formazan crystals formed, the crystals were solubilized in DMSO, and absorbance was read using a microplate reader. Viability was expressed as a percentage of the relevant control.[13]

Griess assay for nitrite

Culture supernatants were collected and nitrite concentrations were determined using the Griess reaction with sodium nitrite standards as previously described.[14]

Statistical analysis

Data from three independent experiments are presented as mean ± standard error of the mean (SEM). Comparisons among groups were performed using one-way ANOVA followed by Tukey's post hoc test.[15] A p-value of less than 0.05 was considered statistically significant.

RESULTS

Summary data for cell viability and nitrite production are shown in Tables 1–4. After 24 h, 2-benzoylbenzoic acid produced minimal cytotoxicity in non-stimulated HIG-82 cells across 3.125–200 µM, with viability remaining close to baseline (81.41% to 101.60%). A more marked reduction was observed at 300 µM (67.75 ± 14.42%; Table 1). In PMA-stimulated cells, PMA alone reduced viability to 80.82 ±

8.70%. Co-treatment with 2-benzoylbenzoic acid at concentrations of 3.125–300 µM resulted in viability values ranging from 67.39% to 92.72%, without a consistent concentration-dependent effect observed at 24 hours (Table 1). At the same time point, the reference drugs (100 µM) produced viabilities of 96.08 ± 22.07% (ibuprofen) and 97.70 ± 21.80% (diclofenac) in PMA-stimulated cells (Table 1).

Nitrite levels measured at 24 h were low and highly variable in both non-stimulated and PMA-stimulated conditions. For 2-benzoylbenzoic acid (50–200 µM), nitrite concentrations fluctuated without a consistent concentration-dependent decrease in either condition (Table 2). In PMA-stimulated cells, several mean values were near zero, indicating no reproducible suppression pattern across concentrations under the present assay conditions (Table 2).

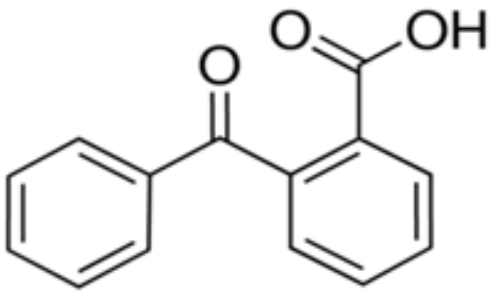

Fig. 1. Chemical structure of 2-Benzoylbenzoic acid ($C_{14}H_{10}O_3$; MW of 226.23 g/mol)

At 48 h, PMA exposure reduced viability to 59.70 ± 12.50% (Table 3). In non-stimulated cells, viability remained stable across 3.125–300 µM 2-benzoylbenzoic acid (83.82% to 91.71%). In PMA-stimulated cells, most concentrations of 2-benzoylbenzoic acid produced viabilities between 58.38% and 67.97%, while the highest concentration (300 µM) substantially reduced viability to 32.65 ± 14.64% (Table 3). Under PMA stimulation, ibuprofen (100 µM) yielded 66.22 ± 14.58%, while diclofenac (100 µM) yielded 74.32 ± 13.65% at 48 h (Table 3).

By 72 h, PMA alone further reduced viability to 44.94 ± 8.40% (Table 4). In PMA-stimulated cells treated with 2-benzoylbenzoic acid, viability ranged from 61.87% to 83.29% across 3.125–300 µM, with the lowest mean viability observed at 300 µM (61.87 ± 20.11%; Table 4). At 72 h under PMA stimulation, ibuprofen and diclofenac produced viabilities of 68.10 ± 11.76% and 73.21 ± 14.92%, respectively (Table 4).

DISCUSSION

In this study, we investigated the effects of 2-benzoylbenzoic acid on cell viability and

Table 1. Cell viability of non-stimulated and PMA-stimulated HIG-82 cells after 24 h treatment

Compound	Concentration (µM)	Cell Viability (%) Non-induced	Cell Viability (%) PMA-induced
Control (Normal cells)	0	100.01 ± 2.90	-
PMA-Induced (Negative Control)	0	-	80.82 ± 8.70
2-Benzoylbenzoic Acid	3.125	89.64 ± 10.13	85.59 ± 24.95
	6.25	101.60 ± 3.20	87.70 ± 20.35
	12.5	99.45 ± 6.41	89.84 ± 19.44
	25	94.13 ± 21.51	92.72 ± 25.09
	50	88.95 ± 13.42	80.66 ± 12.69
	100	87.92 ± 15.85	72.37 ± 8.18
	200	81.41 ± 14.38	72.85 ± 11.64
	300	67.75± 14.42*	67.39 ± 10.86
Ibuprofen	100	104.09 ± 5.31	96.08 ± 22.07
Diclofenac	100	106.18 ± 13.05	97.70 ± 21.80

*; significant difference compared with positive control drugs, Ibuprofen and Diclofenac. All values are presented as mean ± SEM of 3 separate experiments.

nitric oxide (NO)-related nitrite production in HIG-82 synovial fibroblasts under basal and PMA-stimulated conditions. Our findings demonstrate that PMA induces a significant, time-dependent reduction in cell viability from 24 to 72 hours, consistent with its role as an inflammatory activator that imposes cellular stress, as reported in previous studies.[16] The ability of PMA to activate NF-êB pathways and promote inflammatory mediator production makes it a relevant model for studying synovial inflammation *in vitro*.

Regarding cytotoxicity, 2-benzoylbenzoic acid did not exhibit a clear concentration-dependent reduction in viability at doses d"200 µM under basal conditions, aligning with prior reports that phenolic acids often display low cytotoxicity within this concentration range.[3] Notably, at 300 µM, viability decreased at 24 hours, and under PMA stimulation, there was a marked reduction at 48 hours, indicating potential cytotoxic effects at higher concentrations. Interestingly, several concentrations showed higher MTT signals than PMA alone at 72 hours, which could suggest partial protection against PMA-induced stress. However, as the MTT assay primarily measures mitochondrial metabolic activity, these apparent improvements could reflect altered cellular metabolism rather than true increases in cell number or survival. Similar phenomena have been observed in studies where phenolic compounds modulate mitochondrial

Table 2. Nitrite concentration in culture supernatant of non-stimulated and PMA-stimulated HIG-82 cells after 24 h treatment (Griess assay)

Compound	Concentration (µM)	NO concentration (µM) Non-induced	NO concentration (µM) PMA-induced
2-Benzoylbenzoic Acid	50	0.56 ± 5.55	3.35 ± 11.74
	100	7.95 ± 6.00	0.04 ± 13.06
	200	3.71 ± 3.03	0.00 ± 14.92
Ibuprofen	100	8.92 ± 5.48	0.00
Diclofenac	100	9.55 ± 15.26	0.00

Values are mean ± SEM of three independent experiments.

Table 3. Cell viability of non-stimulated and PMA-stimulated HIG-82 cells after 48 h treatment

Compound	Concentration (µM)	Cell Viability (%) Non-induced	Cell Viability (%) PMA-induced
Control (Normal cells)	0	100.17 ± 4.20	-
PMA-Induced (Negative Control)	0	-	59.70 ± 12.50*
2-Benzoylbenzoic Acid	3.125	87.71 ± 11.54	63.38 ± 14.34*
	6.25	91.71 ± 8.71#	67.97±12.10***
	12.5	84.31 ± 3.08#	67.69±12.79***
	25	87.81 ± 5.78#	67.96±12.94***
	50	84.34 ± 5.42#	64.61 ± 7.32*
	100	84.41 ± 2.26#	58.38 ± 3.73*
	200	85.84 ± 3.64#	61.47 ± 4.86*
	300	83.82 ± 11.48	32.65 ± 14.64**
Ibuprofen	100	85.83 ± 4.04#	66.22 ± 14.58*
Diclofenac	100	92.13 ± 12.17#	74.32 ± 13.65

**;significant difference compared with positive control drugs, Ibuprofen and Diclofenac.
***; significant difference compared with 300µM of 2-Benzoylbenzoic acid in PMA-induced cell line.
*; significant difference compared with normal control.
#; significant difference compared with negative control.
All values are presented as mean ±SEM of 3 separate experiments.

Table 4. Cell viability of non-stimulated and PMA-stimulated HIG-82 cells after 72 h treatment

Compound	Concentration (µM)	Cell Viability (%) Non-induced	Cell Viability (%) PMA-induced
Control (Normal cells)	0	100.90 ± 5.10	-
PMA-Induced (Negative Control)	0	-	44.94 ± 8.40*
2-Benzoylbenzoic Acid	3.125	100.17 ± 6.02**	83.29 ± 14.35
	6.25	102.99 ± 3.86**	78.91 ± 16.25
	12.5	87.78 ± 8.21**	76.52 ± 17.96
	25	92.55 ± 8.74**	79.81 ± 17.09
	50	89.29 ± 7.65**	77.32 ± 14.58
	100	88.76 ± 6.81**	75.15 ± 16.99
	200	92.75 ± 8.99**	71.42 ± 9.32
	300	89.43 ± 8.19**	61.87 ± 20.11
Ibuprofen	100	89.91 ± 6.59**	68.10 ± 11.76
Diclofenac	100	89.91 ± 8.14**	73.21 ± 14.92

All values are presented as mean ± SEM of 3 separate experiments.
*; significant difference compared with normal control.
**; significant difference compared with negative control.

function without affecting viability directly.[21] To clarify these effects, complementary assays such as live/dead staining, DNA quantification, or automated cell counting should be employed, as suggested by other investigations into flavonoid and phenolic acid effects on synovial cells.[22]

PMA is well-established as an activator of inflammatory signaling, including NF-êB pathways,[16] which are central to synovial inflammation and rheumatoid arthritis (RA) progression. NF-êB activation leads to increased production of proinflammatory cytokines, matrix metalloproteinases, and other mediators that contribute to joint destruction.[16,17] Therefore, agents that modulate this pathway hold therapeutic potential.

In terms of inflammatory mediator production, we observed that nitrite levels were low and highly variable, with no consistent suppression by 2-benzoylbenzoic acid. Although NO is implicated in RA pathogenesis, contributing to cartilage matrix degradation under IL-1 stimulation[10], synovial fibroblasts may not produce a robust nitrite response upon PMA stimulation alone, as reported elsewhere.[23] This suggests that nitrite as a sole endpoint may lack sensitivity in this context, especially when evaluating modest anti-inflammatory effects. Similar limitations have been noted in studies where NO levels do not reliably reflect anti-inflammatory activity.[24] To improve assay sensitivity, future experiments could employ cytokine stimulation regimens involving TNF-á or IL-1â, which more directly activate iNOS pathways.[18, 19] Including positive controls for NO inhibition, such as aminoguanidine, would also strengthen data interpretation.

The observed lack of reproducible nitrite suppression aligns with the known mechanisms of 2-benzoylbenzoic acid, which primarily inhibits AKR1C3, an enzyme involved more closely in prostaglandin and steroid metabolism than NO biology.[3] This suggests that its anti-inflammatory effects may be mediated through pathways other than NO suppression. Supporting this, previous studies have shown that phenolic acids can modulate prostaglandin synthesis and steroidogenic pathways, which are relevant in inflammatory diseases.[25] Therefore, future research should broaden outcome measures to include COX-2 expression, prostaglandin E, production, and cytokines such as IL-6, providing a more comprehensive assessment of anti-inflammatory potential.[20]

In comparison to other studies, phenolic acids and related compounds have demonstrated varying degrees of anti-inflammatory activity, often

linked to their antioxidant capacity and modulation of enzymatic pathways involved in prostaglandin synthesis.[26] Our findings contribute to this body of evidence, suggesting that 2-benzoylbenzoic acid may exert its effects primarily through modulation of prostaglandin metabolism rather than direct NO inhibition.

CONCLUSION

Overall, within the limits of the current HIG-82/PMA conditions and the variability of the nitrite assay, the data do not provide convincing evidence for robust NO-linked anti-inflammatory activity of 2-benzoylbenzoic acid. More informative stimulation and additional endpoints are needed before firm conclusions can be drawn.[21, 22] The nitrite readout was close to baseline and variable, suggesting that PMA alone may not have induced a sufficiently robust iNOS/NO response to detect modest inhibition. Because MTT reflects cellular metabolic activity rather than direct cell number, orthogonal viability measures such as live/dead assays or direct counting are recommended. Follow-up experiments should consider cytokine-based stimulation (e.g., TNF-á and/or IL-1â), include a positive control for NO/iNOS inhibition, and expand inflammatory endpoints to COX-2/PGE2 and cytokines especially IL-6.

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Conflicts of interest

The author(s) declare no conflicts of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

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Not Applicable.

Author contributions

Wan Nooremira Wan Rashidi conducted experiments and drafted the initial manuscript; Nurul Syuhada Nordin, Che Ku Dahlan Daud, Mohd Sofian Omar Fauzee, and Zuraini Ahmad contributed to study design, supervision, and manuscript review; Muhammad Nazrul Hakim supervised the project and approved the final version.

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