Effects of 4 Thiopurine Compounds on Nitric Oxide Production and Cell Viability of HIG-82 Synoviocytes and RAW 264.7 Macrophages

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The 6-Mercaptopurine (6-MP) is a purine sulphur derivative used in the treatment of children with leukaemia as an antitumor medication.Nonetheless, the anti-proliferation and anti-rheumatoid arthritic activities of the drug have not been previously described. Therefore, the current study objective was to determine the cytotoxicity effects of 6-MP, 6-mercaptopurine riboside (6-MPR), 6-thioguanine (6-TG) and 6-thioxanthine (6-TX)on murine macrophages (RAW 264.7) and rabbit synoviocytes (HIG-82) cell lines. Methods:An inflammatory stage of RAW 264.7 and HIG-82 cell lines stimulated with *Escherichia coli* lipopolysaccharide (LPS) and phorbol-12-myristate acetate (PMA), respectively were treated with the drugs at a serials concentration $3.125 - 100\,\mu M$. Subsequently, the MTT assay was used to determine the viability of cells. Theinhibitory effectswere measured based on nitric accumulation in the conditioned media. Results: The results showed that all drugs tested did not show any cytotoxic effect on both cell lines at low and medium concentrations, which the HIG-82 cell viability was more than 80%.However, the drugs display anti-proliferation property on RAW 264.7 cells compared to control. Reduction on cell proliferation wasfound on all tested drugs. Among its, the diclofenac significantly reduced the proliferation on HIG-82 cellcompared to other drug compounds. Inhibitory effects of compound on nitric oxide production PMA-stimulated HIG-82 had only a small inductive effect, but excellent inhibition wasobserved on RAW 264.7 cell. Meanwhile, 100ìM 6-thioguanine and diclofenac had cytotoxic effect to RAW 264.7 andHIG-82 cell respectively. Conclusion:All thiopurinesenhancedthe proliferation of HIG-82 and RAW 264.7 cells with no cytotoxic effects. This finding opens new avenues for treating RA and the anti-rheumatic activities during synovial inflammation stage of RA. The inhibitory effects of 6-MP and 6-MPR to inflammatory cells marker such as synovial fibroblast and macrophages by proliferating healthy synoviocytes more potently than 6-TG and 6-TX. The current study has demonstrated the potential use of 6-MP and its associated thio compounds in the in-vitro approach of HIG-82 cell culture in curing rheumatoid arthritis disease.

Keywords: Thiopurines, HIG-82, RAW 264.7, cytotoxicity, nitric oxide.

Drugsfor the treatment of inflammatory arthritis particularly related to rheumatoid arthritis (RA) have been notcomprehensivelyexplored.

Worldwide, RA is a serious health problem that affectsup to 1.0% of adults in the developed countries (Parada-Turska*et. al.*, 2006; Wang *et. al.*,

2006).In the USA alone, the total annual cost of arthritis to society is compensated for by more than 20 million RA patients with severe developmental disabilities on a daily basis and estimated at $100 billion(Scott *et. al.,* 2010). In Malaysia, 5 in 1000 adults are reported to be the RA patient by a year (Shahrir*et. al.,* 2008). As consequent, RA has a significant impact on physical, emotional, psychological and social activities of patients in the daily basis; they restricted in most functioning on works as well reducing life expectancy, premature mortality further causing the massive economic burden to the society (Too *et. al.,* 2012).

To date, patients suffering from arthritis such as Crohn's, ulcerative colitis, and chronic arthritis have been prescribed with a long-single potent drug that has experienced serious side effects.As example, the potent drugs like disease-modifying anti-rheumatic drugs (DMARD's) (e.g: azathioprine, methotrexate and tofacitinib), non-steroidal anti-inflammatory drugs (NSAID's) (e.g; diclofenac) and glucocorticoids (Nielsen *et. al.,* 2001). DMARD drugs are often used with a combination with NSAIDs or glucocorticoids greatly improved the quality of life for many patients after 4-6 months to be fully effective. Unfortunately, DMARDs target the immune system of the patients, which weaken the immune system's ability to fight infections. Subsequently, the chemotherapy to the cells effected disease become resistance toward the drugs. This is a major factor in the failure of many forms of chemotherapy especially to cancer patients. These problems of course reduce the drug's efficacy (Shanker*et. al., 2010).*

Hyper-proliferation of synovial fluid (SF) and synoviocytes in the synovial membrane lead to the development of pannus which subsequently cause the invasion to the joint and bone that cause damage to the affected area (Smolen *et. al*., 2014; 2007; Firestein, 1996) and SF play a vital role in facilitating inflammatory action, cartilage degradation and joint destruction in RA (Mor*et. al*., 2005). A study using SCID mouse revealed that intrinsic factor with the immune system support contributes to join damage and degradation. Thus, it indicates that the invading products, if synoviocytes may be isolated from inflammatory cells and serve as a key target for progression of arthritis (Muller-Ladner *et. al*.,

2007). In this research, the HIG-82 synoviocyte cell line, which retains both the morphology and differentiation markers of synovial fibroblast, was used as a RA cell model to evaluate the therapeutic action of thiopurine compounds.Phorbol myristate acetate (PMA) is a well-known stimulator to activate necrosis factor-kappa B (NF-êB) in the cell was applied to resemble the inflammatory and arthritic condition in activated synoviocytes (Kim and Ro, 2005; Smith *et. al.,* 1998; Baeuerle and Henkel, 1994; Hasan *et. al.,* 2012). NF-êB regulates the expression of target genes that encode inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12, tumor necrosis factor-alpha (TNF-á), interferons (IFNs), chemokines and enzymes that cause inflammatory response such as COX-2 and inducible nitric oxide synthase (iNOS)(Piccinini and Midwood, 2010).

Nitric oxide (NO) is a short-lived, reactive species molecule synthesized by NO synthases (NOS) catalyzed by L-arginine (Nathan, 1991). Many biological responses involved by NO molecule such as inflammation, cell-mediated immune response, relaxation of the blood vessels and neurotransmission (Miyasaka, 1997). There are at least two NOS isoforms; $Ca2 +$ Calmodulin-dependent constitutive NOS (cNOS), present primarily in endothelial cells and brain, and $Ca2 +$ Calmodulin-independent inducible NOS (iNOS), residing predominantly in macrophages and smooth muscle cells (Nagy *et. al.,* 2007). Nitric oxide (NO) is generally produced in large quantities by activated macrophages as one of the host defence during inflammation. NO can catalyse by distinct three isoforms of nitric oxide synthase. However, the enzyme which plays the most critical role in inflammation is iNOS. After activated by endotoxin or pro-inflammatory cytokines such as IL-1, IFN-gamma (IFN-ã), TNF-á (Miyasaka, 1997), murine macrophage produces large amount of NO (Laroux*et. al*., 2000). Purinethiol or 6-mercaptopurine (6-MP) (Figure 1) is a derivative of purine sulphur approved by the Food and Drug Administration (FDA) as an antitumor drug in 1953 (Pilar *et. al.,* 1996). 6-MP was among the first effective drugs for childhood leukaemia and subsequently refined as an effective anti-cancer and immunosuppressant medication in the previously poor prognosis of the disease (Zaza *et. al*., 2005; Bell *et. al.*, 2004; Elion, 1989).

Thiopurine compounds are purine anti-metabolic, which interferes with biochemical process involving endogenous purines, which are vital components of DNA, RNA and certain co-enzymes (Coulthard, 2012; Cara *et. al.,* 2004;Coulthard*et. al.,* 2002; Lennard, 1992). The thiopurines were initially tested for leukaemia treatment and as immunosuppressant for organ transplantation. The compounds of thiopurine have a fairly small therapeutic index and are capable of life-threatening toxicity (Sahasranaman*et. al.,* 2008). Thiopurines can have a cytotoxic effect in a myriad of areas.Previous reported usesthe thiopurines for inflammatory bowel disease and ulcerative colitis, eventually by controlled clinical trials (Konidari and El-Matary, 2014; Frei*et. al.,* 2013; Mahadevan*et. al.,* 2000; Ricketts, 1998). 6-MP and its derivatives, blocks the bio-activation of NF-êB and its related cytokines (Chang *et. al.*, 2012). They prevent bio-activation by inhibiting de novo purine synthesis and integrate specific cells, including neutrophils, macrophage, lymphocyte, and endothelial cells, into nucleic acids (Ordentlich*et. al.,* 2003; Hussein-Al-Ali et. al., 2012). Possessions are dose-related, low doses of drugs are anti-inflammatory, but higher doses are immunosuppressive and cytotoxic (Polifka and Friedman, 2002).Therefore, effects of thiopurine compounds namely, 6-mercaptopurine (6-MP), 6-MP riboside (6-MPR), 6-thioguanine (6-TG) and 6-thioxanthine (6-TX) on cell viability and inhibitory effects on nitric oxide production were examined on PMA-activated HIG-82 synoviocytes fibroblast and LPS-induced murine macrophage, RAW 264.7 cell lines. The therapeutic effects of thiopurines compounds, particularly on the cell viability with less cytotoxicity is crucial. Therefore, the objectives of the current study: firstly, to examine the effects of immunosuppressive thiopurine anti-metabolites compounds on cell viability of phorbol myristate acetate (PMA)-activated HIG-82 synoviocytes fibroblast and *Escherichia coli* lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophage cell lines. Secondly, to examine inhibitory effects of immunosuppressive thiopurine anti-metabolites on inducible nitric oxide production of phorbol myristate acetate (PMA)-activated HIG-82 synoviocytes fibroblast and *Escherichia coli* lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophage cell lines

and thirdly, to investigate the cytotoxicity effects of immunosuppressive thiopurine anti-metabolic compounds due to nitric oxide production in responding to compound dosage. We hypothesized that these thiopurine compounds will be potentially useful for the treatment of RA.

MATERIALS AND METHODS

Thiopurine compounds

All reagents and chemical used were purchased from commercial manufactures. Antibiotics (glutamine-penicilin-streptomycin 100x), fetal bovine serum (FBS) and trypsin (0.25% - EDTA in HBSS w/o calcium w/o magnesium w/ phenol red) from Biowest (France), nutrient mixture Ham's F-12 from Sigma Chemicals (St. Louis, USA), dimethylsulfoxide (DMSO) and phosphate buffer saline (PBS) from Amresco (Solon, USA), phorbol-12-myristate 13-acetate (PMA) and thiopurine compounds (6-mercaptopurine and 6-mercaptopurine riboside) from Acros Organic (New Jersey, USA), 6-thioguanine and 6-Hydroxy-1,6-mercaptopurine (6-thioxanthine) from Sigma-Aldrich(St. Louis, USA), diclofenac sodium from Sigma-Aldrich(St. Louis, USA), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] from PhytoTechnology Laboratories (KS, USA); Griess reagent from Merck-Chemical (Germany); sodium nitrate (NaNo$_2$) from Sigma Chemicals (St. Louis, USA).

The 6-MP,6-MPR, 6-TG and 6-TX and diclofenac (Figure 1) were dissolved in 100% dimethyl sulfoxide (DMSO) at stock 0.05 M stock solution. Further, from the stock, a serial dilution with Dulbecco's Modified Eagle' medium (DMEM)(Sigma-Aldrich, St Louis, USA) to give a final concentration 100 μM with 0.2% DMSO is used for Murine macrophage RAW 264.7 cell line. Meanwhile, a serial dilution with nutrient mixture Ham's F12 with L-glutamine (Sigma-Aldrich, St Louis, USA) is used for Rabbit synoviocytes HIG-82 cell line. Further diluted with diluents to 6 different serial concentrations between 100 μM and 3.125 μM. The final concentration of DMSO as a vehicle remained constant at 0.15%.

Macrophage abelsen murine leukaemia virus transformed (RAW 264.7 cell line)

Mouse macrophage RAW 264.7 cell line,

obtained from ATCC (American Type Culture Collection, Menassas, VA, USA) was stored at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2.The culture medium constituted of Nutrient Mixture DMEM (Sigma Chemicals, St. Louis, MO, USA) supplemented by 10% foetal bovine serum (FBS;Biowest, South America), 100 U/ ml penicillin (Biowest) and 100 ìg/ml streptomycin (Biowest). The cells were sub-cultured every 2–3 days at 1:5 split ratios after 85 -100% confluency.

Rabbit synoviocytes fibroblast (HIG-82 cell line)

HIG-82 synoviocyte cell line (ATCC CRL-1832) was obtained from ATCC was kept at 37 °C in a humidified 95% O_2 and 5% CO_2. The culture medium consisted of Nutrient Mixture F-12 Ham (Sigma Chemicals, St. Louis, MO, USA) 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.

Induction and stimulation of inflammation phase

Methods previously described by Parada-Turska*et. al.* (2008) and Jeoung*et. al.* (2013) were employed with slight modifications. The cells growing at exponential phase with 80–90% confluence were detached from the plastic surface by gently trypsinized with Trypsin-EDTA(0.25%) solution.After the cells had completely detached from the surface of the flask, then the completed media was added to stop the trypsin activity.The cell suspension was relocated into a centrifuge tube of 15 mL and centrifuged at 4 ° C at 190 x g for 10 minutes.After removal of supernatant, the cell pellets were re-suspended with 1 mL of completed growth medium; RAW 264.7 cell in DMEM with 10% fetal bovine serum and HIG-82 cell line in Ham's F12 containing 10% fetal bovine serum and the concentration of cells were adjusted to 1 x 10^6 cells/mL and 1 x 10^4 cells/mL for both cells respectively by adding calculated volume of completed growth medium above. Basically, 10 uL of suspension cells was mixed at ratio 1:1 with 0.4% trypan blue solution and the number of cells was calculated using a haemocytometer. Dead cells

absorbed the blue colour of trypan blue due to the membrane permeability, while the viable cells remained unstained. The viability of cells for the assay had to be at least 95%. The cell viability and the volume of completed growth medium needed were calculated.

For cell stimulation and treatment,50 µL of the diluted cell suspension (1 x 10^6 cells/ mL) were dispensed into a tissue culture grade 96–well flat bottom plate (Becton Dickinson, NJ, USA) except for blank. The plate was incubated for 3 hours for RAW 264.7 cell line and 24 hours for HIG-82 cell line at 37 °C, 5% CO_2 to allow the cells to attach to the surface. While waiting the cell attachment, the tested compound stock was serial diluted to a decreasing concentration with completed growth medium. For preliminary screening, six concentration of tested compound were prepared (100, 50, 25, 12.5, 6.25 and 3.125 µM). After incubation hours, the media was gently removed to discard unattached cells and the attached cells were then induced with 50 mL of 10 µg/mL (final concentration) of *Escherichia coli* lipopolysaccharide (LPS) from serotype 055:B5 (Sigma, USA) and 10 nM (final concentration) phorbol myristate acetate (PMA) for the cells respectively. Then, 50 µL from each of serially diluted samples were transferred into each well of the prepared tissue culture plate except the control groups. The five controls were set up and the control group were prepared in the last row of the tissue culture plate. The plate was then incubated for another 24 hours overnight at 37 °C, 5% CO_2. After incubation, the culture cells were prepared for MTT cytotoxicity assay.

3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assay

The cell viability was determined by assaying the reduction of MTT reagents to formazan salts. MTT is a water soluble tetrazolium salt which yellow in colour. Metabolically active cells are able to convert MTT molecule into a water-insoluble dark blue formazan salt by reductive cleavage of the tetrazolium ring (Pozzolini*et. al.,* 2003). After treatment, the supernatant of 96-wells plate containing cells were replaced with the fresh media and 20 µL of MTT reagents (5 g/mL) were added into each well. After 4 hours, the spent media was removed completely and the formazan salts

were dissolved with 100 µL 100% DMSO. The absorbance was then measured at 570 nm using an ELISA microplate reader (Infinite M200 Tecan Microplate Reader; Tecan Inc. Durham, North Carolina, USA). The percentage of cell viability was calculated.

Percentage cell viability (%) = [OD sample / OD Control] x 100

Griess assay

The production of nitric oxide (NO) was determined by measuring the increase of nitrite in the supernatant of the samples. The inhibitory activity of compounds on NO production assayed using Griess reaction (Ahmad *et. al.*, 2006). Briefly, 50 µL of supernatant from each well transferred into corresponding well of clean non-sterile 96-well plate. The exact volume 50 µL (1:1) of Griess reagent [1% sulpanilamide, 0.1% N-(1-naphtyl)-ethylene diamine dihydrochloride in 2.5% phosphoric acid] and mix well with the cell culture supernatant. The absorbance was read at 550 nm with microplate reader (Tecan Infinite 200 PRO, Switzerland). The amount of nitrite in a sample was calculated from sodium nitrite standard curve

Table 1. The effects of Thiopurine compounds on cell viability of HIG-82 synoviocytes and RAW 264.7 cell lines

Compound	Concentration (µM)	Cell Viability (%)	
		HIG-82	RAW 264.7
None	Normal	127.87±29.34	154.65±52.72
6-Mercaptopurine	3.125	92.79±7.02*	159.85±65.82
	6.25	99.20±6.77	158.25±61.40
	12.5	94.41±9.24*	149.42±55.27
	25	90.62±10.00*	127.39±46.38
	50	90.45±10.57*	105.73±37.15
	100	88.53±7.13*	93.90±34.16
6-Thioguanine	3.125	103.25 ± 6.75	83.66±3.02*
	6.25	103.84 ± 5.08	85.77±4.11*
	12.5	108.07 ± 0.75	82.93±4.06*
	25	107.10 ± 6.70	79.23±3.59*
	50	102.99 ± 5.07	73.03±1.31*
	100	96.60 ± 5.09	69.22±5.21*
6-Mercaptopurine riboside	3.125	100.99±17.29	177.49±69.86
	6.25	97.59±15.28*	169.07±63.34
	12.5	98.76±9.52*	167.52±58.32
	25	94.12±6.14*	146.61±65.93
	50	91.19±3.73*	145.70±67.37
	100	92.31±10.60*	140.96±63.67
6-Thioxanthine	3.125	108.57 ± 19.89	98.99±7.08
	6.25	104.01 ± 3.50	98.63±9.68
	12.5	106.98 ± 4.76	98.61±14.07
	25	103.03 ± 2.88	99.37±13.60
	50	103.89 ± 9.54	95.28±21.42
	100	103.70 ± 7.25	93.78±17.37
Diclofenac	3.125	92.91±1.51	167.21±61.01
	6.25	90.31±5.84	151.54±55.58
	12.5	86.58±2.03	147.98±72.35
	25	83.58±1.52*	136.74±67.60
	50	89.42±1.47	101.38±26.44
	100	69.02±19.59*	91.84±35.22

Value were mean ± SD (n=3 independent test)
(*) Significant different (P < 0.05) when compared to normal

freshly prepared in deionised water (0-100 μM). Then, the percentage on nitrite inhibition of each sample was calculated using formula below:

NO inhibitory activity (%) = [$[NO_2^-]$ control - $[NO_2^-]$ sample / $[NO_2^-]$ control] x 100

Statistical analysis

Data is expressed as mean ± SEM of duplicates using GraphPad Prism 5 software, using different experiments of triplicate sets of each template. Using variance analysis, statistical significance was established at p Â0.05. Significant treatment means were further subjected to Bonferroni post-tests.

RESULTS

Results in Table 1 and 2 showed a dose-dependent suppression effects to dose increment compounds on HIG-82 synovicytes and murine macrophage RAW 264.7 cell lines due to

Table 2. The effect of thiopurine compounds on NO inhibition of HIG-82 cell lines and RAW 264.7 cell lines

Compound	Concentration (μM)	NO inhibitory Activity (%)	
		HIG-82	RAW 264.7
6-Mercaptopurine	3.125	1.24 ± 0.42	59.67±24.66
	6.25	0.81 ± 0.44	55.90±25.37
	12.5	1.04 ± 0.78	48.10±23.76
	25	1.01 ± 0.88	34.41±20.79*
	50	0.94 ± 0.71	33.41±20,29
	100	1.24 ± 0.66	11.61±10.85**
6-Thioguanine	3.125	1.41 ± 0.68	75.68±17.71#
	6.25	1.02 ± 0.66	60.65±13.90
	12.5	0.83 ± 0.43	34.84±27.02*
	25	0.80 ± 0.57	37.35±15.09*
	50	1.35 ± 0.23	30.92±22.21*
	100	0.93 ± 0.53	34.70±13.10*
6-Mercaptopurine riboside	3.125	1.01 ± 0.53	70.66±18.08
	6.25	0.43 ± 0.43	80.66±9.59#
	12.5	0.59 ± 0.44	72.07±17.05
	25	1.07 ± 0.54	56.58±15.57
	50	0.86 ± 0.44	49.26±28.30
	100	1.37 ± 0.65	22.61±10.12**
6-Thioxanthine	3.125	1.44 ± 0.78	69.45±15.28
	6.25	0.66 ± 0.55	55.46±11.29
	12.5	1.02 ± 0.91	59.40±35.03
	25	0.90 ± 0.59	39.78±25.09
	50	1.14 ± 0.41	43.33±29.63
	100	2.10 ± 0.64	29.71±18.76*
Diclofenac	3.125	0.99 ± 0.58	NA
	6.25	0.75 ± 0.40	NA
	12.5	0.74 ± 0.41	NA
	25	1.05 ± 0.46	NA
	50	0.95 ± 0.42	NA
	100	1.44 ± 0.47	NA
L-NAME	250	1.37 ± 0.38	33.99±12.82*
	Normal-Induced	1.21 ± 0.48	79.69± 6.88#

Value were mean ± SD (n=3 independent test).
(*) Significant different (P < 0.05) when compared to normal-induced.
(#) Significant different (P < 0.05) when compared to L-NAME.
NA, not available

cytotoxicity effects. Interestingly, the inhibitory effect of four thiopurine compounds on NO production were only demonstrated in LPS-induced RAW 264.7 cell line but not on PMA-induced HIG-82 cell line based on NO production. This is due to very less or untraceable concentration of NO was produced by the PMA-activated HIG-82 cell line. Thus, it cannot be compared with the control group due to there are no any significant different between treated group and control. In general, all compounds were not showed the high degree of cytotoxicity effects (cell viability <70%) at all concentration tested for both type of cells. L-NAME, a standard NO inhibitor was used as a positive drug control; which significantly inhibited NO production at the concentration of 250 µM. The efficacy of the active compounds on cell viability and nitrite oxide production of HIG-82 synoviocytes and murine macrophage RAW 264.7 cell lines were shown in the Tables respectively below.

The effect of thiopurine compounds on cell viability

Table 1 indicates the percentage viability of HIG-82 synoviocyte and RAW 264.7 cells used in this analysis undergoing viability MTT assay for

24-hour incubation with various 6-MP doses and derivatives.HIG-82 synociocyte cell displayed a dose-dependent reduction in cell viability in all thiopurine compounds examined after 24 hours incubation.Eventually, 6-MP and 6-MP riboside compounds were demonstrated a good and moderate potentially candidate of rejuvenalization cell respectively compared to others. Furthermore, 6-MP compound was more potent compared with others on HIG-82 synoviocyte cell where cell viability calculated at 90.62, 90.45 and 88.53% at the same concentration (25 µM, 50 µM and 100 µM doses) doses respectively. In the other hand, diclofenac, a control drug, demonstrated highly reduction in cell viability compared to thiopurine compounds. The result shown there are significant different in cell viability of diclofenac treatment on PMA stimulated HIG-82. The reduction effect of diclofenac in cell viability at 25 µM (cell viability = 83.58±1.52), 50 µM (cell viability = 89.42±1.47), 100 µM (cell viability = 69.02±19.59) compared to control group; PMA stimulation HIG-82 without treatment.

However, on the right-most column, demonstrated the percentage of viability of murine macrophage RAW 264.7 cells treated with
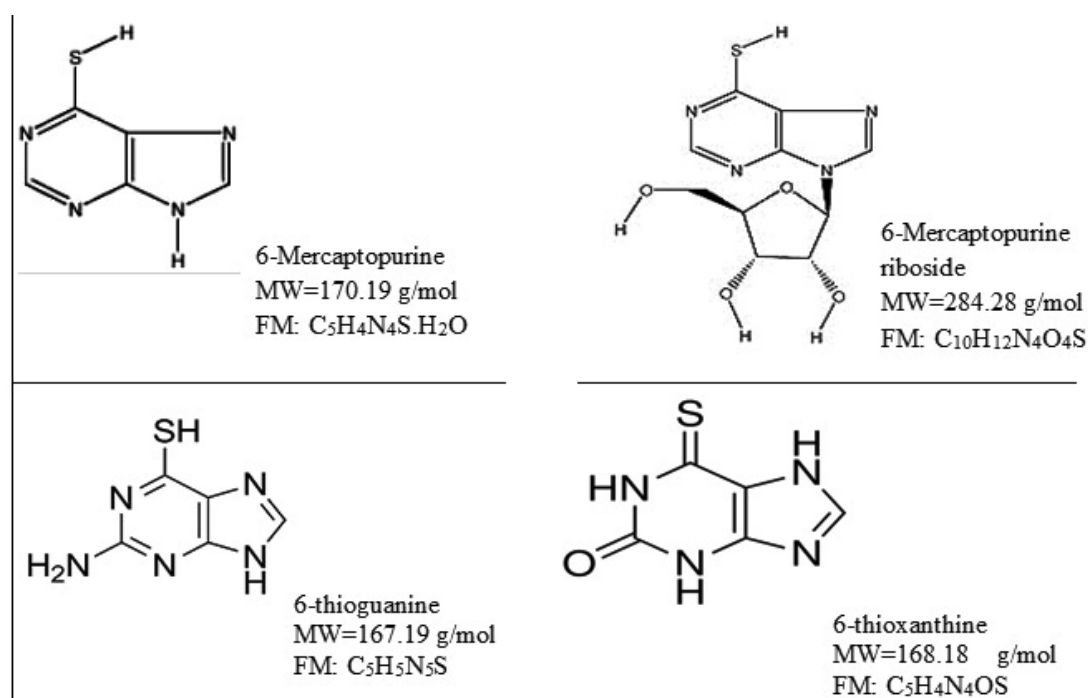

6-Mercaptopurine
MW=170.19 g/mol
FM: $C_5H_4N_4S \cdot H_2O$

6-Mercaptopurine riboside
MW=284.28 g/mol
FM: $C_{10}H_{12}N_4O_4S$

6-thioguanine
MW=167.19 g/mol
FM: $C_5H_5N_5S$

6-thioxanthine
MW=168.18　g/mol
FM: $C_5H_4N_4OS$

Fig. 1. Chemicals structures of Thiopurine compounds

Thiopurine compounds after 24 hrs incubation. The findings showed that for RAW 264.7 cells similar pattern to HIG-82 synoviocyte cells, both compounds have a dose dependent reduction in cell viability.Treatment with thiopurine compounds were caused dose dependent effects where increasing the concentration decreasing the cell viability except 6-Thioxanthine (6-TX) with low dose dependent similar reaction in HIG-82 synoviocytes cell. Similar to the HIG-82synoviocyte cell, the effect of 6-MP in RAW 264.7 murine macrophages was more potent than the 6-MPR or RAW 264.7 macrophages less sensitive than the HIG-82 synoviocyte cell. The viability of RAW 264.7 cell murine macrophage was 145.70 and 140.96% at the two highest 6-MPR concentrations.The most potency Thiopurine compound in this dosages on the RAW 264.7 murine macrophage cell was 6-Thioguanine. The two highest concentrations of 6-Thioguanine were demonstrated a moderate toxicity effect where the cell viability obtained are 73.03and 69.22%, respectively.

Interestingly, 6-MP and diclofenac possess a similar effect on RAW 264.7 macrophages cell same trend effects to HIG-82 synoviocyte cell line. However, from the both findings, HIG-82 synoviocyte cell was seem more sensitive and less viability to thiopurine compounds when compared to RAW 264.7 cell within the dosage range of the compounds but the HIG-82 cell was more consistent pattern effects to thiopurine compounds. The result findings from cell viability MTT assay showed that anti-metabolic thiopurine compounds namely 6-MP, 6-MPR, 6-TG and 6-TX were not cytotoxicity to HIG-82 synoviocyte cell and RAW 264.7 murine macrophage cell line of the selected dosages range. This is because the percentage of cell viability for all four compounds were showed more than 70% viability cell at all concentrations tested.

Effects of thiopurine compounds on nitrite oxide (NO) production

Table 2 demonstrated the inhibitory effects of thiopurine compounds on nitric oxide upon PMA-activated HIG-82 synoviocytes cell line.Among the thiopurine compound tested, there are no inhibitory action of NO showed on PMA-activated HIG-82 cells. This is due to the PMA probably does not cause the production of NO oxide. Thus, the inhibitory assessment of thiopurine compound cannot be determined. This same goes to control drug, diclofenac and the iNOS inhibitor L-NAME action in suppressing NO cannot be determined. The dosages selected were same with dosages range with cytotoxicity effects. The evaluation of NO inhibitory effects of thiopurine compounds and its derivatives is based on the dose response activities effect at six different concentrations (3.125–100µM; 2-folds). Among four thiopurine compounds and diclofenac were tested, the inhibitory effects of NO were not determined. This is probably due to very less or undetectable concentration of NO was produced by the PMA-stimulated HIG-82 cell line. Positive control (L-NAME) also was demonstrated same respond like the compounds due to mechanism of PMA to the type of cell.

The concentration used for screening NO production of RAW 264.7 murine macrophage cell was covered a wide range (3.125–100 µM) to determine the effective working concentration for
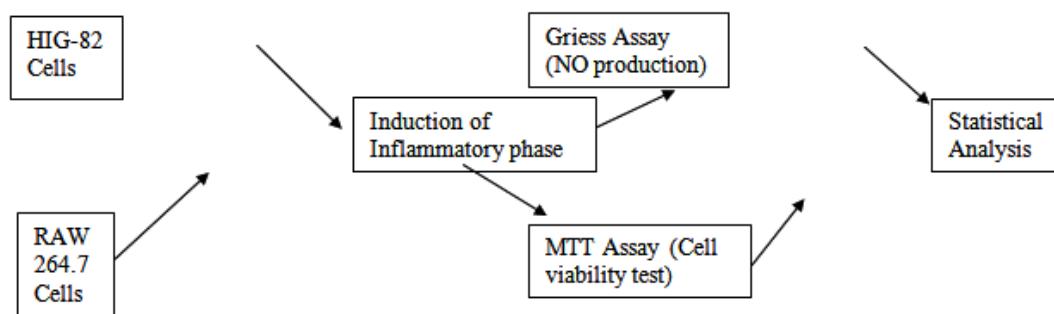

Fig. 2. Summary of methods

each compounds. Table 2 showed the effects of 6-MP, which dose dependent effects decreasing the level of nitrite concentration. The decreasing level of NO production pattern also demonstrated by other thiopurine compounds. Among four thiopurine compounds used, 6-mercaptopurine showed most potent NO inhibitory effect on LPS-induced RAW 264.7 murine macrophage cells with 11.61±10.85% NO inhibition activity at 100 μM compared to the rest compounds. The potency thiopurine compounds as NO inhibitor from strongest to weakest compounds as following in order; 6-mercaptopurine > 6-mercaptopurine riboside > 6-thioxanthine > 6- thioguanine. Interestingly, the inhibitory effects of NO production of all thiopurine compounds were showed at very strong activity of immunosuppressive when compared to L-NAME, a standard iNOS inhibitor as a positive drug control, which significantly inhibited NO production at the concentration of 250 μM. The efficacy of the thiopurine immunosuppressive compounds on nitrite production of RAW 264.7 murine macrophage cell line is shown in Table 2. Table 3 and Table 4 demonstrated the percentage of cell viability reduction and inhibitory effects on NO production at 100 μM respectively compared to Normal after 24 hrs incubation.

Percentage (%) = [Normal – Cell viability at 100 μM]/ [Normal] x 100

DISCUSSIONS

Results of the present study demonstrated the cytotoxicity effects and NO production by HIG-82 synociocyte and RAW 264.7 cell lines after treated with four immunosuppressive thiopurine anti-metabolic compounds, namely the 6-mercaptopurine (6-MP), 6-mercaptopurine riboside (6-MPR), 6-thioguanine (6-TG) and 6-thioxanthine (6-TX). As mentioned these compounds exhibits dose-dependent cytotoxicity effects on HIG-82 synoviocytes and murine macrophage RAW 264.7 cell lines after incubation 24 hours. In general, the cytotoxicity effect on PMA-activated HIG-82 and LPS-induced RAW 264.7 cell lines after treated with thiopurine compounds were demonstrated a dose-dependent manner with higher proliferation activity on HIG-82 synoviocytes cells compared to murine macrophage RAW 264.7 cells approximately 70% (30.77% reduction) and 60% (39.28% reduction) proliferation activity after 24 hrs incubation (Table 3). Furthermore, all thiopurine compounds except 6-MP riboside were showed more toxicity effects on LPS-induced RAW 264.7 murine macrophage cell line especially after treated with 6-thioguanine. In other side, only 6-MP riboside was demonstrated less toxicity to RAW 264.7 murine macrophage cell, thus promoting more proliferation activity (Mawatari*et. al*., 2001). According to our finding in this study, 6-thioguanine was demonstrated a very potent thiopurine as well at 100 μM on RAW 264.7 murine macrophage cell (Table 3) was believed involved in HGPRT up-regulation in inflammation mechanisms. Endless, the toxic accumulation of 6-TG in cells has activated an apoptosis or cell death programme which therefore provokes tumour regenericity in tissues and organs(Yatscoff and Aspeslet, 1998).

NO is an important modulator of the inflammatory cascade (Korhonen *et. al*., 2005).

Table 3. Percentage of cell viability reduction at 100 μM compare to the normal after 24 hours incubation.

Cell line	Percentage (%)	
Compound	HIG-82	RAW 264.7
6-MP	30.77 ± 4.119*	39.28 ± 17.08
6-MPR	27.81 ± 6.120	8.85 ± 31.84
6-Thioguanine	24.45 ± 5.094	55.24 ±3.005
6-Thioxanthine	18.90 ± 7.258	39.36 ±10.03
Diclofenac	46.02 ± 11.31*	40.61 ± 17.61

(*) Significant different (P<0.05) when compared to Normal

Table 4. Percentage of inhibitory effects on NO production at 100 μM compare to the normal-induced after 24 hours incubation

Cell line	Percentage (%)	
Compound	HIG-82	RAW 264.7
6-MP	2.48 ± 0.6562	85.43 ±6.267*
6-MPR	13.22 ± 0.6544	71.63 ±5.842*
6-Thioguanine	23.14 ±0.5288	56.46 ±7.563*
6-Thioxanthine	73.55 ±0.6416	62.72 ±10.83*
L-NAME	13.22 ±0.3769	57.35 ±8.678*

(*) Significant different (P<0.05) when compared to Normal-Induced

However, due to the instable and volatile properties of NO if long air exposure, it is difficult to quantify the level of NO production in biological samples. Griess assay is a commonly used, simple and rapid spectrophotometric method to detect the presence of organic nitrite compounds in biological samples, which is also one of the short stable end product(s) of NO formation (Moshage, 2009). Nitric oxide (NO) is a free radical that commonly involved in biological process (Stadler*et. al.,* 1991). However, overproduction of NO can cause cytotoxic and cytostatic effect (Choudhari*et. al.,* 2013; Connelly *et. al.,* 2001; Palmer *et. al.,* 1988). Few years back, several study in RA patients had revealed with strong evidences that overproduction of NO may be important in the pathogenesis of RA suggested a predominant M1 macrophage phenotype (McInnes and Schett, 2011) and the inflammation joints in RA are the main source of NO (Korhonen *et. al.,* 2005; Miyasaka, 1997). In human, level of nitrite in synovial fluid was highly elevated compared to serum in RA patient thus suggesting that inflamed synovial joint and synoviocytes were contributed to RA (Moshage, 2009; Jovanovic *et. al.,* 2002).

NO producing cells can be varied inflammatory synoviums. Several specialized cells are capable of providing NO in the inflamed synovium, including osteoblasts, osteoclasts, macrophages, fibroblasts, neutrophils, and endothelial cells. (Otero and Goldring, 2007). Articular chondrocytes synthesise NO in response to IL-1 and lipopolysaccharide (LPS) reactions(Otero and Goldring, 2007; Stadler *et. al.,* 1991). Nevertheless, the number of chondrocytes existing in the synovium may not be sufficient to compensate for the level of NO produced in the synovium. Synovial fibroblasts develop NO in response to IL-1 and TNF-á, while its development is inhibited by the transforming growth factor-â (TGF-â) (Stichtenoth*et. al.,* 1995). In comparison, it also notes the localization of iNOS immunoreactivity in synovial macrophages(Miyasaka, 1997). The NOS inhibitor (L-NAME) has been reported to reduce disease activity in experimental RA (McCartney-francis*et. al.,* 1993). However, in this study, the level of nitric oxide produce cannot be determined due to no or too little of NO production being induced by the PMA-activated HIG-82 synoviocyte cell. It seems PMA suppresses its production as same reaction demonstrated by

TGF-â (Stichtenoth*et. al.,* 1995). This is because PMA was not an appropriate activator for the HIG-82 synoviocyte cell to produce NO instead of activating protein kinase C (PKC) signalling pathways for modulating diverse cellular responses such as gene transcription, cell proliferation and differentiation, induction of apoptosis, immune response, and receptor desensitisation (Chang *et. al.,* 2005). PMA has a minimal effect on NO synthesis (Hulkower*et. al.,* 1992; Stadler *et. al.,* 1991; Georgescu*et. al.* 1988; Zakaria *et. al.,* 2006). On the other hand, PMA is a protein kinase C (PKC) activator alone had no effects, whereas PMA with recombinant interferon (rIFN)-gamma synergistically increased NO synthesis (Yoon *et. al.,* 1994). PMA-sensitive PKC isoforms (a and e) function in such a negative regulatory role of NOS and PMA-sensitive isoforms (a, b or e) may play a role in the inhibition of NOS induction (Paul *et. al.,* 1997). These findings revealed that the dosages of thiopurine compounds were not toxic to synoviocytes HIG-82 cells.

In contrary, the thiopurine compounds have pronounced NO-suppressing activity on RAW 264.7 cell line. Off these, 6-MP was most potent in inhibiting the production of nitrite oxide compared to 6-MPR, 6-TG and 6-TX. Previous study suggested that thiopurines did not act as inhibitor on iNOS system, but they were significantly influenced many respective pharmacological proteins functions which responsible expressing the iNOS especially necrosis factor-êappa B (NF-êB) (Chang *et. al.,* 2012). The central transcriptional mediator in the pathogenesis of several diseases, including stress response, cerebral ischemia and various immune reactions, which have been activated in abundance of cells and stimulated by oxidative stress-related cytokines, is considered to be a vital role of NF-êB. (Chang *et. al.,* 2012). A common response to both cytokines and bacterial lipopolysaccharide (LPS) is an increase in apparent nitric oxide synthase (NOS) activity. This corresponds to the induction of the 130 kDa isoform of the enzyme and has been described in macrophages, smooth muscle cells, renal mesangial cells and hepatocytes (Förstermann and Sessa, 2012; Kunz *et. al.,* 1994; Hortelano*et. al.,* 1993; Förstermann*et. al.,* 1991).

Our results revealed that 6-MP and 6-TG were decrease more cell viability compared

to 6- MPR and 6-TX. This might be due to metabolism of 6-MP to S-methyl-thioinosine 5'- monophosphate (6-Me-Thio-IMP), which is strong inhibitor of purine *de novo* synthesis (Sahasranaman*et. al.*, 2008). Purine de novo synthesis inhibition is well known to achieve the impact of immunosuppression and block the proliferation of different forms of lymphocyte lines and contribute to the cytotoxic behaviour (Erb*et. al.*, 1998). Thus, 6-TG metabolism produces 5 "triphosphate (dGS) deoxy-6-thioguanosine. It has been shown that integrating dGS into DNA causes cell-cycle arrest and apoptosis via a mechanism involving the mismatch repair pathway (Swan *et. al.*, 1996). It is commonly assumed that cell damage caused mainly by the incorporation of the thioguanine nucleotides (TGN) into DNA is of decisive importance for the cytotoxic effect of 6-MP as well as 6-TG. At the other hand, the antagonists of purine de novo synthesis (PDNS) in vitro are methylthioinosine monophosphate (MeTIMP) (Erb*et. al.*, 1998) and, to a lesser degree, methylthioguanosine monophosphate (MeTGMP) (Karim*et. al.,* 2013; Coulthard*et. al.,* 2002; Allan and Bennett, 1971).

Previous studies have also shown that DNA has been impaired by the introduction of 6-TG into p388 murine leukemic cells and human cells, which has shown morphological alterations size enlargement and multinucleated nuclei (Almosailleakh and Schwaller, 2019; Daehn*et. al.*, 2011).It's terribly difficult to synthesise, and since its mode of action emerged to be close to that of 6-MP, its metabolic fate and clinical behaviour was addressed a little subsequently (Kowalska*et. al.,* 2015; Mathews, 2012; Elion, 1989). Those were possibly the key reasons behind 6-TG that have never been used in the maintenance treatment of acute lymphoblastic leukaemia, however, unlike 6-MP, it is more active and less harmful than 6-TG that is further converted to cytotoxic TGN and can be degraded only after deamination by xanthine oxidase (Erb*et. al.*, 1998). In other hands, 6-MPR is a derivative of 6-MP, while 6-TX is major products of metabolism of 6-MP by enzyme xanthine oxidase (Polifka and Friedman, 2002). From the results, 6-MPR decrease more cell viability compare to 6-TX. Although 6-MPR is a minor metabolite, it may be a clinical importance compare the known metabolite, 6-TX which is has

less anti-neoplastic activity (Ono *et. al.*, 1997). In contrast, 6-MPR has known has anti-neoplastic activity (Miron*et. al.*, 2009; Solomon *et. al.*, 1984).

Interestingly, our results showed that 6-MP and derivatives exhibits a very good therapeutic ranges in term of less cell dead and high cell lives than diclofenac, a common NSAID used for treating inflammation diseases. Diclofenac is a type NSAID that commonly used to relieve the join swelling but only have minor effect on the disease progression. Diclofenac was expected to inhibit COX synthesis of prostaglandin and help regulate symptoms but fail to prolong even the healing of RA-related joint damage (Wang *et. al.*, 2011; Andreas *et. al.,* 2009). Diclofenac therapy has been shown to have mild impacts on gene expression in SFs (Andreas *et. al.*, 2009).All thiopurine compounds tested in this study were effective in promoting proliferation activity with low toxicity towards HIG-82 synoviocytes and RAW 264.7 murine macrophage cell lines after 24 hrs treatment. However, critical adverse drug reactions (ADR's) in the human body such as fever , rash, diarrhoea, hypertension, hepatitis, pancreatitis, bacterial liver abscesses, cytomegalovirus infections, life-threatening myelosuppression, bone marrow suppression, gastrointestinal symptoms, hypersensitivity reactions, and tumorigenicity may be caused by long-term use of potent drug such as 6-MP in high dose(Gaya *et. al.*, 1995). In addition, the introduction of 6-MP pro-drug; azathioprine may effect the second generations of animal infant in pregnancy's mother (Ramsey-Goldman and Schilling, 1997) and birth defects after first trimester exposure to azathioprine, but the issue is still under debates (Rubinstein and Weinberg, 2012). Intracellular absorption of 6-thioguanine nucleotides in infants is also considered to be responsible for the cytotoxic effects of these drugs by blocking purine synthesis involving DNA synthesis and replication of RNA to new infant tissues and organ (Lennard, 1992).

In regard to these side effects, this present research was performed in observations of very beneficial therapeutic doses as a novel approach to reduce the ADR caused by 6-MP inflammatory arthritis treatment. The usage of glucocorticoids with sufficient thiopurine derivative dosages is another approach to reduce ADRs.Glucocorticoids are often the most successful remission-inducing

medications, however regrettably, for patients that neglect to react, and for others that experience side effects or need long-term glucocorticoid treatment, may still show severe side effects, therefore immunomodulatory or immunosuppressive medications combined with an adequate dosage are necessary treatments or substitutes (Nielsen *et. al.*, 2001).Immunomodulatory medication such as 6-MP pro-drug, azathioprine is able to mitigate and reduce the ADR caused by the usage of 6-MP. However, the replacement of 6-MP by azathioprine still facing with immediate and long-term adverse drug reactions of 6-MP via 6-MP metabolism where involved by three routes with superior enzymes in liver and gut; (a) thiopurine-S-methyltransferase (TPMT), added the methyl group to 6-MP through methylation to form 6-methyl-MP; (b) xanthine oxidase, which catalyses 6-MP to thiourate; and (c) hypoxanthine-guanine-phosphoribosyltransferase (HGPRT), which converts 6-MP to 6-thioguanine nucleotides (Karran and Attard, 2008).

A more approach beyond the usage of glucocorticoids and pro-drug replacement is the use of a nano-encapsulated drug delivery system. Recently study was demonstrated, cell proliferation levels in the presence of anti-cancer drugs provided by gold nanoparticles conjugated have been shown to be significantly smaller than those in cells subjected to cytostatic drugs alone (Cuin*et. al.*, 2011), indicating that the transmission of nano-participants allowed an improved sensitivity of cancer cells to drugs evaluated with ribavirin. (Tomuleasa*et. al.*, 2012). Nano-particles of disease-modifying anti-rheumatic nano (DMARNs)-medicines medication would have increased effectiveness at a lower dosage of the product concentration in the intended tissues, resulting in a decrease of adverse drug reactions of patients (Rubinstein and Weinberg, 2012*)*. Treatment with DMARD azathioprine/purine derivatives, gold sodium thiomalate and methotrexate successfully restored RA-related chondrocyte gene expression to 'healthy' levels(Andreas *et. al.,* 2009). Anti-metabolic thiopurins are thought to be a potential pharmacological agent for the nano-encapsulated drug delivery mechanism for the treatment of multiple pathological disorders with intrinsic inflammatory pathways and components.It often inhibits systemic inflammation by inhibiting several forms of inflammatory cell activation,

including antioxidants of free radical molecules in the body system, such as superoxide anion generation (Ordentlich*et. al.,* 2003), cytokine development (Chang *et. al.,* 2005), and molecular adhesion expression (Chang *et. al.,* 2012).As a consequence, by retarding the trans-nuclear transcription of NF-kâ and associated cytokines, thiopurine effectively reduced the synthesis of nitric oxide synthase (Chang *et. al*., 2011).

CONCLUSION

This research demonstrated the possible use of 6-MP for the in-vitro model of HIG-82 cell culture in treating rheumatoid arthritis disease. Drug candidates are promising usefulness to be identified as a new drug compound used in the treatment of inflammatory arthritis in the future, and further studies need to be conducted with a view to reducing the adverse drug reactions in patients using a nano-encapsulated drug delivery system.

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