VOL. 53 NO. 7, JULY 2000 THE JOURNAL OF ANTIBIOTICS pp. 657 — 663

Discovery of Novel Ansamycins Possessing Potent Inhibitory Activity in a Cell-based Oncostatin M Signalling Assay

PAUL STEAD, SHAZIA LATIF, ANDREW P. BLACKABY,
PHILIP J. SIDEBOTTOM, ANGELA DEAKIN, NICHOLAS L. TAYLOR,
PAUL LIFE, JOHN SPAULL, FIONA BURRELL,
ROSIE JONES, JANE LEWIS, IAN DAVIDSON and THOMAS MANDER

GlaxoWellcome Medicines Research Centre,
Gunnels Wood Road, Stevenage, Herts SG1 2NY, UK

(Received for publication February 25, 2000)

We describe the isolation and characterisation of novel non-benzoquinone ansamycin metabolites related to geldanamycin from a culture of *Streptomyces* sp. S6699. The compounds possess potent inhibitory activity in a cell-based assay measuring inhibition of oncostatin M signalling in a reporter cell line utilising a secreted placental alkaline phosphatase (sPAP) readout. In this paper we report the isolation and structure elucidation of the compounds and describe some of their biological properties.

Ansmycins are described as antibiotics containing an aliphatic ansa bridge connecting two nonadjacent positions of an aromatic nucleus, and comprise a broad family of microbial secondary metabolites including the rifamycins, streptovaracins, tolypomycins and geldanamycins, which possess numerous diverse biological activities[1]. Geldanamycin, a benzoquinone ansamycin, was discovered in 1970 by DE BOER *et al.*[2] possessing activity *in vitro* against bacteria, protozoa, fungi and mammalian tumour cells. Geldanamycin and related compounds have since been the subject of intensive investigation as potential antitumour agents[3~5]; geldanamycin itself appears to exert its anti-tumour activity principally through binding to the *N*-terminal ATP/ADP binding domain of heat shock protein 90 (Hsp90), a molecular chaperone, causing inhibition of its inherent ATPase activity, which ultimately results in disruption of the cell cycle[6].

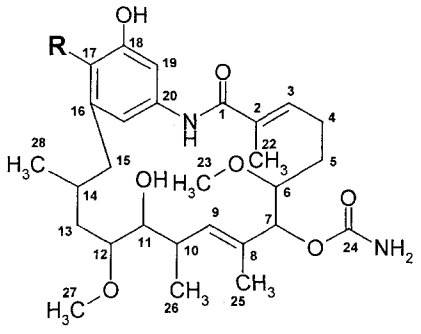
Oncostatin M (OSM), a member of the IL-6 cytokine family, is a pleiotropic cytokine with pro-inflammatory activity. OSM upregulates adhesion molecule expression in endothelial cells[7] synergises with IL-1 to promote cartilage degradation[8] whilst administration of anti-OSM antibodies ameliorates both collagen- and pristane- induced arthritis in mice[9]. Furthermore, OSM is released from *ex-vivo* cultures of inflamed synovial tissue[10] and is present both in the synovium[8] and syovial fluid of patients with rheumatoid arthritis[8]. Inhibition of OSM therefore represents a novel therapeutic opportunity for the treatment of rheumatoid arthritis. In the course of screening in a cellular assay for inhibitors of OSM signalling we have isolated and characterised novel non-benzoquinone ansamycins related to geldanamycin, together with the known compound reblastatin (see Figure 1). The compounds exhibit potent and apparently selective activity, inhibiting OSM effects with low micromolar or submicromolar potency, whilst showing no activity in a cellular assay measuring TNFα signalling.

Materials and Methods

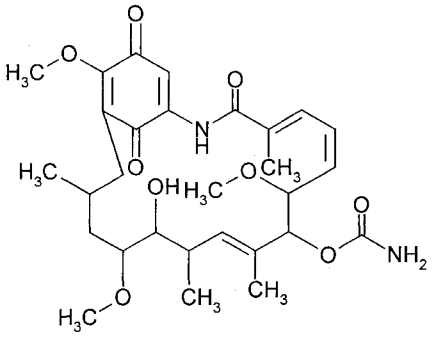
Fermentation of S6699

Streptomyces sp. S6699 (isolated from the roots of a Hazel tree, Black Park, Southern England) was inoculated from agar plugs into seed stage medium (in g/liter: glucose (15), glycerol (15), Soy peptone (LabM; 15), NaCl (3), $CaCO_3$ (1), pH 7) which was incubated at 28°C for 6 days using an orbital shaker. A 1% inoculum was transferred to production medium (in g/liter: soya flour (Arkasoy; 10), glucose (50), peptone (4), Lab Lemco (Oxoid Ltd; 4), yeast

Fig. 1. Structures of compounds **1** to **3** and geldanamycin.



R	Compound
H	1
OH	2
OCH3	3 (reblastatin)



Geldanamycin

extract (1), NaCl (2.5), $CaCO_3$ (1), pH 7.6; 4×250 ml in 1 liter Erlenmeyer flasks) and incubated for 8 days at 28°C using an orbital shaker.

Prefractionation of S6699 Fermentation

Whole fermentation broth was mixed with an equal volume of methanol. After standing for 1 hour at room temperature, cells were removed by centrifugation (2500 rpm×30 minutes). The supernatant was taken, methanol was removed by rotary evaporation, and the volume of aqueous concentrate was adjusted to ~800 ml (0.1% aqueous phosphoric acid). This was pumped through a column containing copolymer resin (Amberchrom CG161, Rohm and Haas), the resin was washed with 0.1% aqueous phosphoric acid and adsorbed components were eluted with methanol (1 liter). The eluate was concentrated and further processed to provide a sample suitable for preparative HPLC. A gradient elution method was used (solvent A: 100 mM ammonium dihydrogen phosphate in water with 3 ml/liter orthophosphoric acid added. Solvent B: 50% v/v acetonitrile, containing 100 mM ammonium dihydrogen phosphate and 3 ml/liter orthophosphoric acid. Linear gradient A to B in 60 minutes at ~75 ml/minute; 40 seconds/fraction). Following interrogation of the UV (210 nm) chromatogram, fractions corresponding to sample components were pooled then de-salted and evaporated to dryness. Pooled HPLC fractions 46~47 (which generated a screen sample) were subsequently deconvoluted to yield **1** (see below).

Isolation of **1** from Bio-active Prefractionated Sample

The prefractionated sample (1.5 ml of a 20 mg/ml solution in DMSO) was diluted to 4.5 ml (10 mM ammonium acetate, 0.1% v/v TFA in water) then processed by preparative HPLC. A Hypersil 5 μm BDS C18 column (15×2 cm) was used. Mobile phase A was 10 mM ammonium acetate, 0.1% v/v TFA in water; mobile phase B was 90% v/v acetonitrile, 10 mM ammonium acetate, 0.1% TFA in water. A linear gradient of 20% B to 100% B in 40 minutes was employed with a flow rate of 20 ml/minute. **1** eluted after 8 minutes. Acetonitrile was removed by rotary evaporation, then the compound was adsorbed onto reverse phase silica (Bond-elut C18 cartridge - Varian Ltd; 200 mg size). After washing with water, the adsorbed component was eluted with methanol (2 ml) and the eluate was evaporated to dryness using a Speedivac concentrator. The yield of **1** was 4.4 mg.

Isolation of **2** and **3** from Scale-up Fermentation of S6699

Whole fermented broth (5 liters) was mixed with methanol (5 liters) and stood at room temperature for 2 hours. Cells were pelleted by centrifugation (2500 rpm for 1 hour) and the supernatant was taken. Methanol was removed by rotary evaporation, and the resulting dark brown aqueous solution was pumped through a column

containing polymeric adsorbent (Amberchrom CG161, Rohm and Haas Ltd). The column resin was washed with water (800 ml) then eluted sequentially with 10% v/v acetonitrile/water (1 liter) and 60% v/v acetonitrile/water (1.5 liters). The 60% v/v acetonitrile/water fraction was taken and evaporated to ~600 ml. 100 ml of this was pumped through a loading column containing reverse phase silica (Whatman Partisil 40) which was then connected in-line to a preparative HPLC instrument; the preparative HPLC column (25×5 cm) was packed with C8 reverse phase silica (Kromasil, 7 μm particle diameter). The mobile phase was 20% v/v acetonitrile, 10 mM ammonium acetate, 0.1% TFA in water and the flow rate was 100 ml/minute. Compound **2** and **3** eluted after 28 and 62 minutes respectively. Product-containing fractions were taken and acetonitrile was removed by rotary evaporation. Components were then adsorbed onto reverse phase silica cartridges (C18 Bond-elut; 500 mg size). Adsorbed material was eluted with methanol (5 ml) and the eluates were evaporated to dryness using a Speedivac concentrator. The yields of compounds **2** and **3** were 22 mg each.

Acquisition of NMR, Mass Spectral and UV Data

NMR data was recorded in DMSO-d_6 at 323k to resolve spectral broadening seen at 298K. A Bruker AMX500 with a Nalorac 3 mm Broadband Inverse (Z) Gradient probe and standard pulse sequences were used. Mass spectra were acquired from LC/MS experiments performed using a Micromass Platform LCZ instrument fitted with an electrospray interface, operating in both positive and negative ionisation modes. A linear gradient HPLC method was employed for analysis of compounds as follows: Mobile phase A–10 mM ammonium acetate, 0.1% v/v formic acid in water. Mobile phase B–90% v/v acetonitrile, 10 mM ammonium acetate, 0.1% v/v formic acid in water. A linear gradient from A to B in 30 minutes at 1 ml/minute was used, and the column (15×0.46 cm i.d.) was packed with Hypersil Elite BDS C18 silica (3 μm diamater). Accurate mass measurements were made using a Micromass Autospec Q Sector Mass Spectrometer of E-B-E-Q configuration. Analyses were performed by flow injection using internal calibration (PEG 200, 600 and 1,000) in the mobile phase (60 : 40 acetonitrile : water with 10 mM ammonium acetate, 0.1% v/v formic acid). The PEG-ammonium series (PEG-NH_4) reference table was used. UV spectra were recorded during HPLC analyses in acetonitrile/water containing 10 mM ammonium acetate and 0.1% v/v TFA (pH 2.5 approx.) using a Hewlett Packard HP1050 UV-diode array detector.

OSM-induced sPAP Release from HepG2 B6 Cells

A HepG2 cell line stably transfected with six functional STAT3 response elements (REs) upstream of sPAP cDNA was kindly provided by M. SAUNDERS (Glaxo Wellcome, France). STAT3 is an intermediate in the IL-6 cytokine family intercellular signalling cascade[13]. Following dimerisation of cell surface receptors STAT3 is phosphorylated and will then bind to DNA REs in the nucleus and activate DNA downstream, in this construct that DNA is sPAP. Thus this line (designated HepG2 B6) can be driven to produce sPAP by incubation in Oncostatin M or IL-6.

HepG2 B6 cells were plated into 96 well plates to a final concentration of 3×10^4 cells per well in 100 μl of media (DMEM (Sigma), 10% heat inactivated foetal calf serum (FCS), 1% non-essential amino acids (NEAA), 2 mM glutamine, 500 μg/ml G418, (all from Life Technologies)). Cells were allowed to equilibrate for 48 hours. Prior to assay, the media was replaced with assay media (DMEM, 1% low alkaline phosphatase FCS (Life Technologies), G418, glutamine, NEAA). Compounds (in DMSO) were added to give a final concentration of 0.0001~10 μg/ml, and 0.5% DMSO. Oncostatin M (2 or 4 ng/ml; R&D Systems) or IL-6 (4 or 8 ng/ml; R&D Systems) was added to HepG2 B6 cells 1 hour after addition of the compounds. Following incubation for approximately 18 hours at 37°C, 5% CO_2, 20 μl of media was removed from each well and assayed for sPAP activity using pNPP (*p*-nitrophenyl phosphate; Sigma), as a substrate. Endogenous alkaline phosphatase is blocked with L-homoarginine (Sigma). Optical density of substrate is read at 405 nm using 650 nm as a reference.

TNFα Induced sPAP Release from A549 Cells

This assay used A549 cells that had been stably transfected with a reporter gene, comprising the cytokine responsive region of the E-selectin gene coupled to alkaline phosphatase[11]. This transfected cell line can be driven to produce sPAP by incubation with TNFα. A549 cells were plated into 96 well plates to a final concentration of 5×10^4 cells per well in 100 μl of media. Cells were allowed to equilibrate for 24 hours. Prior to assay, the media was replaced with assay media (DMEM, 1% low alkaline phosphatase FCS (Life Technologies), G418, glutamine, NEAA). Compounds (in DMSO) were added to give a final concentration of 0.0001~10 μg/ml, and 0.5% DMSO. TNFα (3 ng/ml; R&D Systems) was added to the cells 1 hour after the compounds. Following a further 18 hours incubation (37°C, 5%CO_2), 20 μl of the supernatant was removed and assayed for sPAP as described above.

MTS Assay for Cell Viability

(MTS is converted to a soluble formazan by dehydrogenase enzymes found in metabolically active cells). To cell cultures remaining after incubation with **1** as described above, 20 μl of MTS solution (1 vol. phenazine methosulphate (0.92 μg/ml; Sigma) in 9 vol. (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2*H*-tetrazolium, inner salt; MTS; Promega) (2 mg/ml) was added. Following an incubation of 30 minutes~1 hour, viability was measured (as $OD_{490\,nm}$ minus $OD_{630\,nm}$).

^{14}C-Leucine Incorporation as a Measure of Total Protein Synthesis

Cell cultures were set up as described above, with the exception that in place of cytokine, ^{14}C-leucine was added (0.2 μCi/well; Amersham). Cells were harvested using Skatron cell harvester following 18 hours incubation, and incorporated ^{14}C-leucine measured using a scintillation counter.

PPD Proliferation Assay

Blood from healthy volunteers was diluted 1 : 3 with phosphate buffered saline (PBS), layered onto Lymphoprep (Nycomed UK) and centrifuged at 600×g for 30 minutes at

Table 1. NMR data and assignments for compounds **1** to **3**.

	^{1}H-NMR data			^{13}C-NMR data		
Atom	**3**	**2**	**1**	**3**	**2**	**1**
1	-	-	-	169.5	169.8	170.0
2	-	-	-	132.1	131.3	131.1
3	5.93	5.80	5.78	134.2	133.6	133.5
4	2.24;2.15	2.21;2.09	2.22;2.08	23.4	23.3	22.8
5	1.41	1.34;1.28	1.31;1.23	29.5	29.6	29.0
6	3.32	3.25	3.26	79.5	79.5	79.0
7	4.91	4.88	4.89	80.1	80.6	80.1
8	-	-	-	129.3	129.1	129.1
9	5.36	5.29	5.29	132.9	133.1	132.4
10	2.45	2.42	2.38	33.3	33.6	33.4
11	3.36	3.38	3.41	73.6	73.5	72.8
12	3.09	3.05	3.04	80.8	80.9	80.1
13	1.57;1.25	1.61;1.16	1.53;1.17	34.4	33.9	33.0
14	1.79	1.83	1.81	30.8	30.5	30.0
15	2.59;2.40	2.66;2.34	2.56;2.30	35.6	35.7	42.1
16	-	-	-	133.1	126.7	140.6
17	-	-	6.29	142.2	140.1	111.9
18	-	-	-	*	*	156.8
19	6.94	6.77	6.69	106.8	106.7	105.1
20	-	-	-	134.1	129.8	131.0
21	6.37	6.25	6.28	114.0	116.0	114.1
22	1.72	1.70	1.74	12.5	13.0	12.7
23	3.36	3.35	3.34	57.7	57.9	57.6
24	-	-	-	155.8	155.8	155.4
25	1.50	1.46	1.44	11.4	11.3	11.2
26	0.93	0.93	0.94	15.4	16.2	15.9
27	3.26	3.25	3.25	56.0	56.2	55.7
28	0.80	0.81	0.79	19.7	19.2	18.3
29	3.67			59.4		
Ar-OH	-	7.66				
Ar-OH	9.04	9.01	9.11			
11-OH	4.10	4.10	*			
NH2	6.33	6.31	6.32			
NH	9.08	8.94	9.18		* Signal not observed	

NMR spectra were recorded in DMSO-d_6 at 323K. ^{1}H- and ^{13}C-NMR data were recorded at 500 MHz and 125 MHz respectively. ^{13}C NMR data were recorded by inverse methods and referenced to DMSO-d_5 at 39.7 ppm.

room temperature. Harvested peripheral blood monoculear cells (PBMC) were washed 3 times in PBS and resuspended in media (RPMI 1640 (Sigma), 10% heat-inactivated FCS, glutamine) and plated at 1×10^5 cells/well. Compounds were added at 0.001~10 μg/ml with or without PPD (1 μg/ml). Plates were incubated for 4 days (37°C, 5% CO_2) with ^{3}H-thymidine (0.15 μCi/well; Amersham) present for the last 18 hours. Plates were harvested with a Skatron Harvester and counted on a scintillation counter.

Results and Discussion

The structure of **3** was identified by using 2D Correlation NMR experiments (HMQC, HMBC, Gradient assisted PSDQFCOSY). NMR data was in accordance with that previously reported for reblastatin[12], an inhibitor of Rb phosphorylation. Compound **2** gave data similar to **3**, but with the absence of OCH_3 at 3.67/59.4 ppm (^{1}H/^{13}C respectively) and the presence of a new signal (17-OH) at 7.66 ppm in the ^{1}H spectrum which gave no HMQC correlation. For **1**, 2D-NMR data experiments were employed. **1** gave data similar to both **2** and **3**, however differences in the chemical shifts about the aromatic ring combined with the loss of OCH_3 or OH signal and the appearance of an HMQC correlation for CH-17 gave rise to the reported structure. An HMBC correlation from H-15 to C-17 supported this conclusion. MS analysis showed that **1** gave a protonated molecular ion at *m/z* 519 ($M+H^+$) and a deprotonated molecular ion at *m/z* 517 ($M-H^-$) indicating that the molecular weight was 518. Similarly, **2** gave a protonated molecular ion at *m/z* 535 ($M+H^+$) and a deprotonated molecular ion at *m/z* 533 ($M-H^-$) indicating its molecular weight was 534, whilst **3** gave a protonated molecular ion at *m/z* 549 ($M+H^+$) and a deprotonated molecular ion at *m/z* 547 ($M-H^-$) indicating that its molecular weight was 548. Accurate mass values were also consistent with the molecular formulae proposed. The accurate mass values were: **1** (536.333576 (fits for $C_{28}H_{46}N_3O_7$ (MNH_4^+) error 1.1 ppm) **2** (552.328491 (fits for $C_{28}H_{46}N_3O_8$ (MNH_4^+) error 2.3 ppm and **3** (566.344141 (fits for $C_{29}H_{43}N_3O_8$ (MNH_4^+) error 2.4 ppm). Compounds **1**~**3** possessed identical ultraviolet absorption spectra, exhibiting absorption maxima at 220 nm and 290 nm.

The high throughput screen assay used for this work was based upon inhibition of OSM driven sPAP production in sPAP transfected human HepG2B6 cells. In order to discriminate genuine oncostatin M signalling-inhibition from non-specific effects *e.g.* cellular cytotoxicity, a secondary selectivity assay was employed. This assay utilised a human lung epithelial cell line—A549—also transfected with sPAP downstream of NF kappa B. By measuring inhibition of TNFα driven sPAP production in the A549 cells, it was anticipated that both inhibitors of the sPAP readout as well as non-selective cytotoxic agents would be readily identified.

Prefractionated natural product samples derived from *Streptomyces* sp. S6699 appeared to have potent inhibitory activity in the OSM-induced sPAP assay, whilst having no discernible activity in the TNF-induced sPAP assay. For this reason, one of the most potent and selective samples was deconvoluted to yield compound **1** (19-demethoxylebstatin). The fact that this compound does not possess the fully oxidised benzoquinone ring system of geldanamycin and related compounds, offered hope that its biological effects might be somewhat dissimilar to that of geldanamycin, and that it would prove to be a selective modulator of oncostatin M signalling. Fermentation of the producing organism was therefore scaled up to provide more of **1** and possibly related fermentation metabolites. Indeed, the scale-up fermentation yielded two additional compounds **2** (19-demethylreblastatin) and **3** (reblastatin). The IC_{50} values of compounds **1**, **2** and **3** in the OSM sPAP assay were 0.7 μM, 4.9 μM and 0.16 μM respectively.

Compound **1** produced a concentration dependent inhibition of OSM-driven sPAP production in sPAP transfected HepG2B6 cells, with an IC_{50} of *ca.* 0.7 μM (see

Fig. 2. Inhibition of OSM and IL-6 driven sPAP production in HepG2B6 cells by compound **1**.

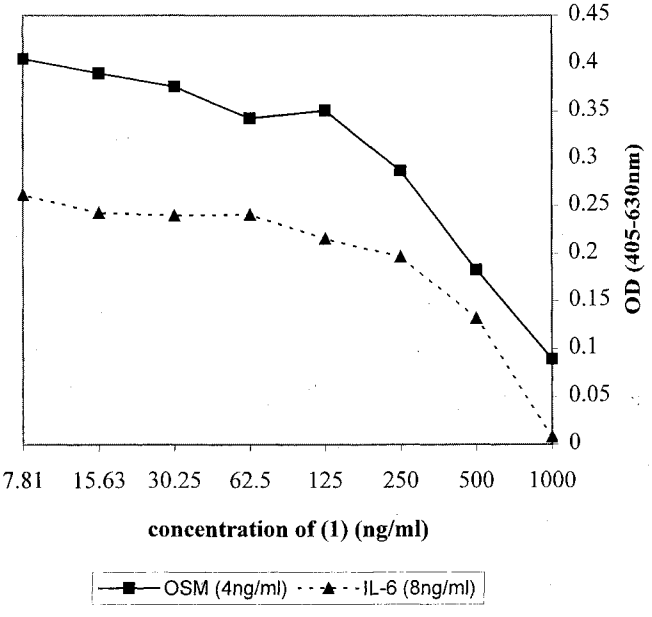


Fig. 3.1. The effect of (**1**) on sPAP production, cell viability and protein synthesis in HepG2 cells.

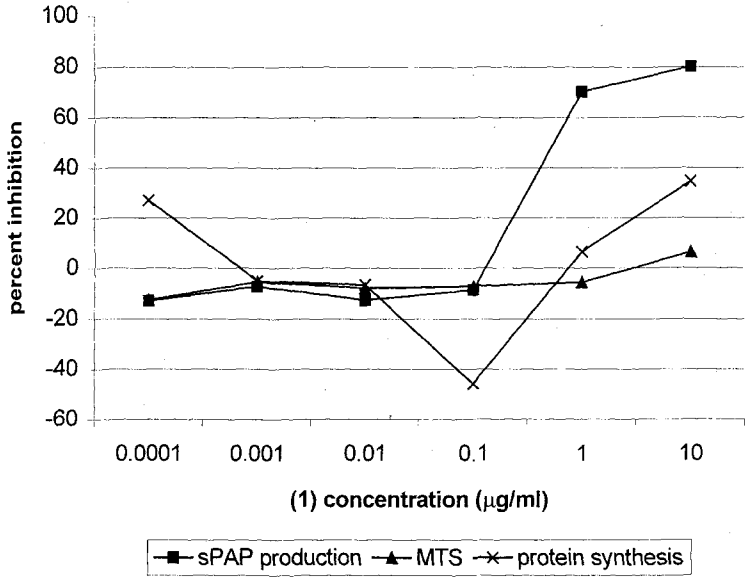


Fig. 3.2. The effect of (**1**) on sPAP production, cell viability and protein synthesis in A549 cells.

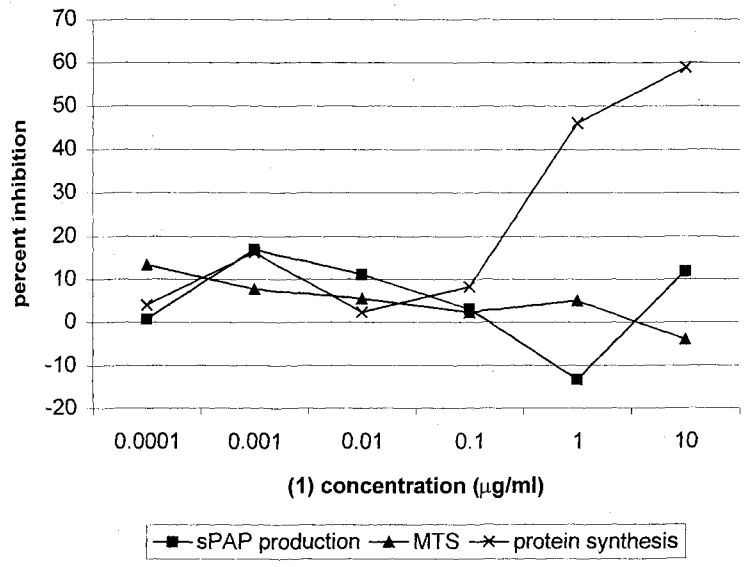
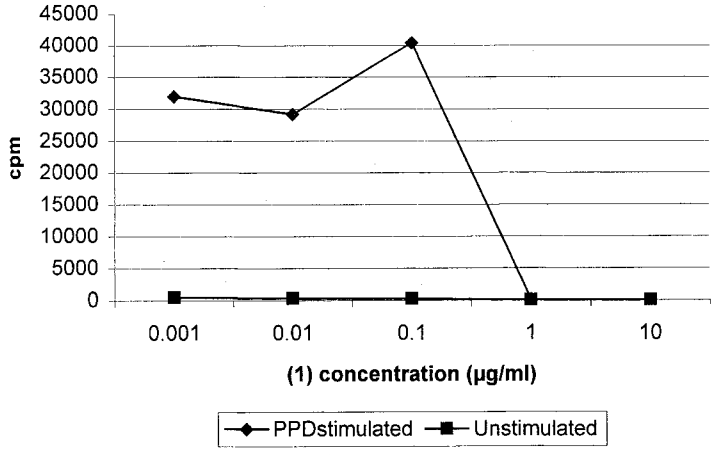


Figure 2). The compound also exhibited roughly equipotent activity as an inhibitor of IL-6 driven sPAP production (Figure 2). It did not inhibit TNFα-induced sPAP production in A549 cells. In order to investigate the biological activity of **1** in more detail, its effects on cell viability and protein synthesis were also evaluated. It can be seen (Figure 3.1) that in HepG2B6 cells, the compound has no significant effect on either protein synthesis or cell viability up to a concentration of 10 μg/ml.

However, the compound did inhibit protein synthesis in A549 cells in a concentration dependent manner (Figure 3.2). **1** inhibited protein synthesis by approx. 46% at a concentration of 1 μg/ml. In this cell line, this degree of protein synthesis inhibition was not accompanied by an inhibition of either sPAP production or cell viability. This implies that in A549 cells, sPAP production is largely unaffected by partial block of protein synthesis caused by **1**. In a further experiment, **1** produced near maximal

Fig. 4. The effect of (**1**) on PPD-induced lymphocyte proliferation.



inhibition of lymphocyte proliferation (induced by PPD) as measured by inhibition of ^{3}H-thymidine incorporation (Figure 4).

In conclusion therefore, we have shown that compound **1** inhibits OSM and IL6-driven sPAP production in HepG2B6 cells whilst having no effect on TNFα-driven sPAP production in A549 cells. The mechanism by which compound **1** causes inhibition of sPAP production in HepG2B6 cells remains unclear, and does not appear to be associated with inhibition of either protein synthesis or cell viability. However, the protein synthesis inhibitory activity of the compound in A549 cells coupled to its ability to inhibit lymphocyte proliferation suggest that its biological activities are somewhat non-specific, and not necessarily attributable to inhibition of either OSM or IL6 signalling.

References

1) RINEHART, K. L.: Antibiotics with ansa rings. Acc. Chem. Res. 5: 57~64, 1972
2) DE BOER, C.; P. A. MEULMAN, R. J. WNUK & D. H. PETERSON: Geldanamycin, a new antibiotic. J. Antibiotics 23(9): 442~447, 1970
3) GALLASCHUN, R. J.; M. P. MOYER & R. C. SCHNUR: Ansamycin derivatives as antioncogene and anticancer agents. WO94-IB160 940616, 1994
4) Anticancer pharmaceuticals containing geldanamycin derivatives (Kaken Chemical Co. Ltd). JP 79-17869 790220, 1979
5) SASAKI, K. & Y. INOUE: Geldanamycin derivative and antitumour agent containing it. JP 79-17218, 790219, 1979
6) ROE S. M.; C. PRODROMOU, R. O'BRIEN, J. E. LADBURY, P. W. PIPER & L. H. PEARL: Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumour antibiotics radicicol and geldanamycin. J. Med. Chem. 42(2): 260~266, 1999
7) MADHUR, V.; M. J. FELDHAUS, A. S. WEYRICH, D. L. JICHA, S. M. PRESCOTT, G. A. ZIMMERMAN & T. M. MCINTYRE: Oncostatin M is a proinflammatory mediator—*In vivo* effects correlate with endothelial cell expression of inflammatory cytokines and adhesion molecules. J. Clin. Invest. 100: 158~168, 1997
8) CAWSTON T. E.; V. A. CURRY, C. A. SUMMERS, I. M. CLARK, G. P. RIPLEY, P. F. LIFE, J. R. SPAULL, M. B. COLDRING, P. J. T. KOSHY, A. D. ROWAN & W. D. SHINGLETON: The role of oncostatin M in animal and human connective tissue collagen turnover and its localization within the rheumatoid joint. Arthiritis and Rheumatism 41(10): 1760~1771, 1998
9) PLATER-ZYBERK. C.; J. BUCKTON, S. THOMPSON, J. SPAULL, E. ZANDERS, J. PAPWORTH & P. LIFE: Amelioration of arthritis in two murine models using abtibodies to oncostatin M (OSM). Arthritis and Rheumatism 42 (9 supplement): 313, 1999
10) BOYCE, S.; R. MALHOTRA, J. WORDEN, J. SPAULL, T. CAWSTON, J. SANCHEZ-BALLESTER & P. LIFE: Spontaneous secretion of oncostatin M (OSM) by rheumatoid arthritis and osteoarthritis (OA) synovial tissue: A putative role in inflammation and cartilatge breakdown. Arthritis and Rheumatism 42 (9 supplement): 735, 1999
11) RAY K. P.; S. FARROW, M. DALY, F. TALABOT & N. SEARLE: Induction of the E-selectin promoter by interleukin 1 and tumour necrosis factor, and inhibition by glucocorticoids. Biochem. J. 328: 707~715, 1997
12) TAKATSU, T.; A. MURAMATSU, M. OTSUKI, S. KURAKATA & R. ENOKIDA: Cell cycle inhibitor reblastatin manufacture with *Streptomyces*. Japanese patent 96-306035, 19961118
13) LAI, C.-F.; J. RIPPERGER, Y. WANG, H. KIM, R. B. HAWLEY & H. BAUMANN: The STAT3-independent signaling pathway by glycoprotein 130 in hepatic cells. J. Biol. Chem. 274(12): 7793~7802, 1999