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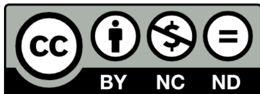
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Modulation of tumour angiogenesis by targeting p38 MAPK signalling in tumour-associated macrophages

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Introduction

Recruitment of tumour-associated macrophages (TAMs) to the tumour site is known to be negatively correlated with patient survival (Balkwill F *et al.* 2001) and indicative of high tumour vascularization and motility (Welm AL *et al.* 2007).

TAM-derived signalling mediators such as IL-1, IL-10, TNF-α, EGFR, VEGF and MMP-9 are able to trigger key signalling pathways and elicit anti-apoptotic stimuli, tumour angiogenesis and dissemination (Condeelis J *et al.* 2005). As a result, targeting TAMs presents itself as a therapeutic strategy and we have shown this to be partially due to inhibition of angiogenesis (Zeisberger S *et al.* 2006). However, the exact implication of TAM-derived signalling on cancer cells is still largely unknown. Consequently, regulating TAM-derived cytokine release is a therapeutic strategy to reduce inflammatory-mediated tumourigenesis.

The p38 family of mitogen-activated protein kinases (MAPK) comprises four isoforms which are involved in the regulation of pro-inflammatory genes including TNF-α, IL-1, IL-6, IL-8 and COX-2 (Herlaar E *et al.* 1999).

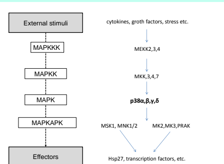

Fig. 1 Schematic overview of MAPK signalling pathway. External stimuli trigger phosphorylation of MAPK kinase kinases (MAPKKK), MAPK kinases (MAPKK) and MAPKs which in turn specifically activate further downstream components.

Aims

Study the underlying cellular and molecular mechanisms of TAM-derived p38-mediated signalling patterns in tumourigenesis. As such, the effects of p38 signalling and inhibition in TAMs on modulation of chemosensitivity, tumour growth and angiogenesis is further investigated with the aid of a small molecule p38 MAPK inhibitor (BIRB796) in free and liposomal formulations.

Methods

p38 MAPK signalling, inhibition and cytotoxicity assays are performed in murine RAW 264.7 macrophages (RAW), Lewis lung cancer carcinoma (LLC) and BL6/B16 melanoma (B16) cells . Conditioned medium (CM) obtained from LLC or B16 cells are used to induce a TAM-phenotype in RAW cells.

Signalling and inhibition of p38 MAPK are assessed by Western blotting using total cell lysates. Dose-response curves and cytotoxicity assays are established with the resazurin reduction assay by measuring fluorescence of resorufin generated by non-apoptotic cells.

Liposomal BIRB796 formulations are prepared along previously described protocols (Seiler P *et al.* 1997) and analyzed for homogeneity and vesicle size.

In vivo angiogenesis assays are conducted in the chick chorio-allontois membrane (CAM) after inoculation with 1.0×10^6 cells (LLC/RAW-CC ratio 3:1) on incubation day (ID) 8.5 with and without B-Lip at 75µg/kg. Angiogenic response is assessed by stereomicroscopic imaging with intralipid and histological haematoxylin-eosin (H&E) staining.

In vivo tumourigenesis assays are performed in 6 week old female C57BL6 MacGreen mice injected under isoflurane subcutaneously (s.c.) with 0.25×10^6 LLC cells in the left flank. Treatment is commenced as of day 3 post-inoculation with 3mg/kg free and liposomal BIRB796. Tumour growth is measured by caliper and volume calculated using the formula $V = 4/3 \times (d/2)^2 \times (D/2)$, where d is the perpendicular tumour diameter and D is the major diameter.

Results

MAPK signalling in resting and stimulated RAW macrophages

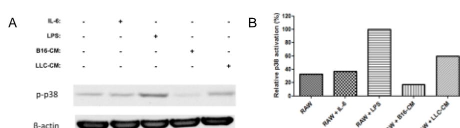

Fig. 2 Phosphorylation of p38 in resting and stimulated RAW macrophages. A: Western blot of total cell lysates in culture for 24h before being stimulated with IL-6 (50ng/ml), LPS (100ng/ml), B16- and LLC-CM, respectively for 60min. **B**: Bar graph showing activation of p38 as percent of control; LLC-CM induces a 2-fold increase in p38 baseline activation in RAW cells.

BIRB796 inhibits LLC-CM-triggered p38 MAPK activation in RAW cells

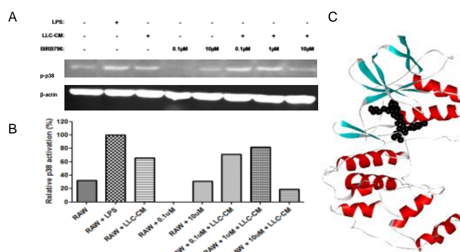

Fig. 3 BIRB796 inhibits p38 baseline activation at low dose and rescues RAW cells from LLC-CM-induced stimulation of p38 activation at high dose. A: Western blot of total cell lysates pre-treated with BIRB796 for 48h before stimulation with LPS (100ng/ml), B16- and LLC-CM, respectively for 60min. **B**: Bar graph showing activation of p38 as percent of control; BIRB796 at 0.1µM reduces baseline activation of p38 to zero and at 10µM rescues RAW from LLC-CM-induced increase in p38 activation. **C**: inactivated p38α (β-stranded N-terminal lobe: cyan, α-helical C-terminal lobe: red) bound to BIRB796 (black); PDB: 1KV2.

Synergistic effects of BIRB796 on chemosensitivity of cancer cell lines

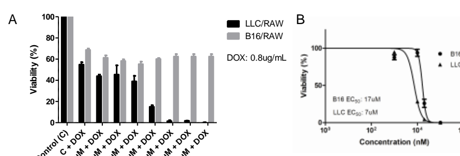

Fig. 4 BIRB796 increases chemosensitivity to doxorubicin (DOX) in sensitive tumour cell lines. A: Bar graph showing increased sensitivity of LLC to DOX by 60% when pre-treating with 10µM BIRB796 for 48h. **B:** Dose-response curve showing cytotoxic effects of increasing concentrations of BIRB796 on LLC- and B16-MC (72h incubation).

Liposomal BIRB796 inhibits angiogenic capillary sprouting *in vivo*

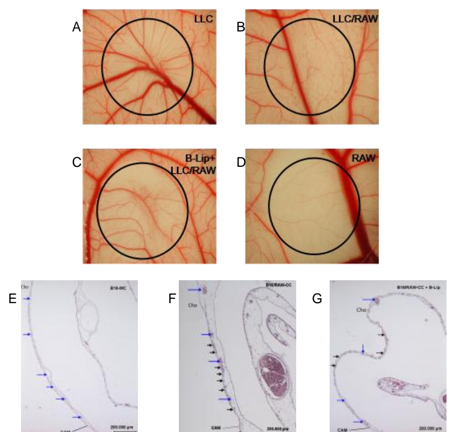

Fig. 5 Angiogenesis response in the CAM at ID 12.5. A: LLC monocultures (MC) elicit an angiogenic response marked by characteristic spokes' wheel formation of vessels towards centre of stimulus **B:** LCC/RAW co-cultures show a significant increase in capillary sprouting. **C:** LLC/RAW co-cultures treated with B-Lip show a marked decrease in vessel steering and capillary sprouting. **D:** RAW-MC show no effect. All images taken at x6.3 magnification. H&E-stained histological sections of CAMs showing capillary network beneath chorion (Cho) with larger vessels marked by blue and capillary sprouting by black arrows for **E:** B16-MC **F:** B16/RAW-CC and **G:** B16/RAW-CC + B-Lip inoculation.

In vivo effects of BIRB796 on tumour growth and angiogenesis

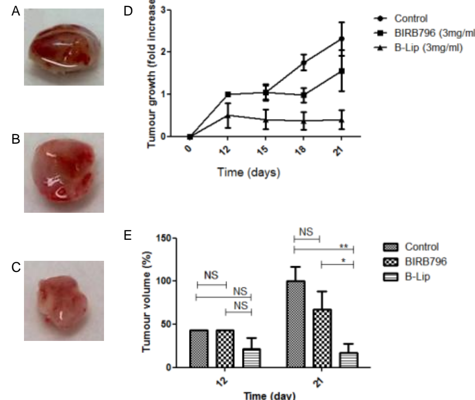

Fig. 6 Effects of BIRB796 treatment on s.c. LLC growth and angiogenesis in MacGreen mice. A: Macroscopic appearance of excised tumours on day 23 post-inoculation. Control tumour from untreated animals; large, solid and highly vascularised. **B:** LLC tumour from group treated with free inhibitor (BIRB796); decreased vascularisation. **C:** B-Lip treated tumour; significantly smaller and paler tumour showing decreased vascularisation. **D:** The relative increase in tumour volume is presented as fold increase ±SEM as a function of time. Tumour volume for vehicle- (control), free BIRB796- and B-Lip-treated mice (n=3) was calculated **E:** Bar graph showing tumour volume at days 12 and 21 as calculated means ±SEM. Statistical analysis: *P<0.05, **P<0.005, NS=not significant.

Specific targetting of phagocytic macrophages by liposomal drug

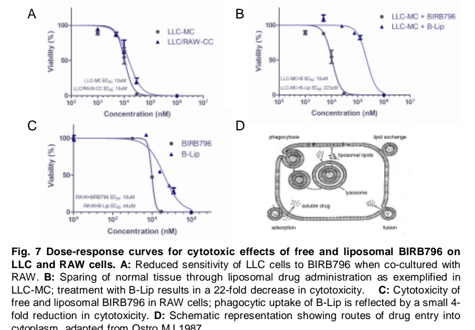

Fig. 7 Dose-response curves for cytotoxic effects of free and liposomal BIRB796 on LLC and RAW cells. A: Reduced sensitivity of LLC cells to BIRB796 when co-cultured with RAW. **B:** Sparing of normal tissue through liposomal drug administration as exemplified in LLC-MC; treatment with B-Lip results in a 22-fold decrease in cytotoxicity. **C:** Cytotoxicity of free and liposomal BIRB796 in RAW cells; phagocytic uptake of B-Lip is reflected by a small 4-fold reduction in cytotoxicity. **D:** Schematic representation showing routes of drug entry into cytoplasm, adapted from Ostro MJ 1987.

Discussion

The preliminary *in vitro* and *in vivo* data undermine the role of p38 MAPK signalling in the cellular response of sensitive tumour cell lines to TAM-derived signalling mediators.

Inhibition of p38 can down-regulate baseline activation of p38 and rescue LLC-CM-induced activation of p38 MAPK signalling in RAW cells. As adjuvant p38 MAPK inhibition can increase chemosensitivity of LLC cells to doxorubicin treatment by nearly 60%.

In vivo tumourigenesis assays demonstrate an increased angiogenic effect of LLC/RAW-CC on capillary sprouting in comparison to LLC-MC and a potent anti-angiogenic effect of B-Lip on capillary vessel formation induced by LLC/RAW-CC on the CAM. Furthermore, free and liposomal BIRB796 can reduce tumour growth and vascularisation of tumours in an induced murine LLC tumour model.

Liposomal formulations of BIRB796 show reduced cytotoxicity in normal tissue and allow passive targetting of macrophage-derived cell lines.

Conclusion

Inhibition of p38 MAPK signalling in TAMs leads to:

- increased tumour chemosensitivity to conventional chemotherapeutics;
- decreased angiogenesis;
- decreased tumour growth.

Liposomal formulations of BIRB796 show reduced cytotoxicity in normal tissue and allow passive targetting of macrophage-derived cell lines. TAM-targetted p38 MAPK inhibition shows potential for adjuvant chemotherapy in sensitive tumours.

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