School of Dentistry / Yr Ysgol Ddeintyddiaeth

Identification of Gene Expression Profiles Underlying Preferentially Stimulated Keratinocyte Wound Healing Responses and Re-epithelialisation by Novel Epoxy-Tigliane Pharmaceuticals

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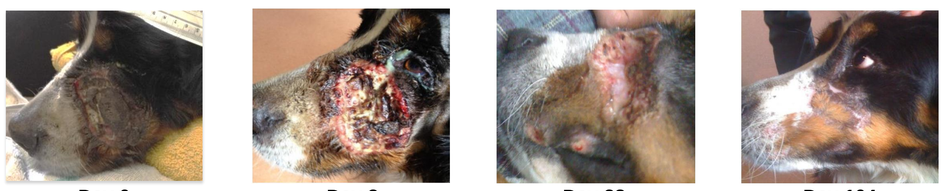
Introduction

- **The novel epoxy-tiglianes, 12-tigloyl-13-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliane-3-one (EBC-46) & a less active related compound, 12-tigloyl-13-(2-methylbutanoyl)-5,6-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliane-3-one (EBC-211), occur within seeds of the Fontain's Blushwood Tree, indigenous to Queensland's tropical rainforest[1].**
- **EBC-46 is currently being developed as an anti-cancer agent by Australian biotechnology company, QBiotics (*www.qbiotics.com/*), for the intra-lesional treatment of cutaneous & sub-cutaneous tumours in humans & animals[2].**
- **In veterinary clinical trials, exceptional dermal wound healing responses, characterised by accelerated re-epithelialisation, closure & reduced scarring, have been consistently observed following tumour ablation by EBC-46[2].**
- **Such observations are reminiscent of the rapid re-epithelialisation rates evident during early gestational, foetal skin or oral mucosal healing[3-4], leading to the potential of EBC-46 & EBC-211 being used to promote dermal re-epithelialisation during impaired healing chronic wounds or burn injuries.**

Fontainea picrosperma

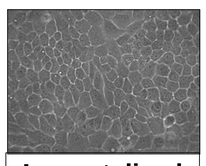


Deep necrosing facial wound that had been unresponsive to 3 months treatment with current standards of veterinary wound care, resolved following treatment with EBC-46.

Aims & Objectives

As little is known how epoxy-tiglianes promote wound re-epithelialisation in treated skin, we examined the effects of EBC-46 & the lesser active analogue (EBC-211), on keratinocyte proliferation & migration *in vitro* & the underlying mechanisms of action.

Materials & Methods



Immortalized human epidermal keratinocytes (HACATs)

EBC-46 or EBC-211 (0 or 0.001-100µg/ml)

Serum-starved (24h), DMEM, antibiotics, L-glutamine (2mM), 1% FCS

168h → MTT assay @ 24h, 72h, 120h & 168h. — **Cell Proliferation & Viability**

In vitro Scratch Wound Re-population

Automated time-lapse confocal microscopy.

ImageJ analysis of wound closure rates. — **Cell Migration & Proliferation**

Serum-starved (24h), DMEM, antibiotics, L-glutamine (2mM), 1% FCS

48h

EBC-46 or EBC-211 (0 or 0.001-10µg/ml)

Cell Cycle Analysis using Draq5 & Flow Cytometry @ 0h, 9h, 17h, 26h, 32h, 40h, 48h. — **Cell Cycle Analysis**

24h & 48h

Global Gene Expression Analyses by Microarrays

(Human HT-12 v4 Expression BeadChips) & GeneSpring. — **Microarrays**

Conclusions

- **Both EBC-46 & EBC-211 significantly increase HACAT proliferative & wound re-population responses *in vitro*.**
- **This study has also identified the genes principally involved in mediating enhanced keratinocyte proliferative & migratory responses following epoxy-tigliane treatment, which potentially occur by similar mechanisms to the favourable rapid healing events observed with epithelial wounds in the oral mucosa[3-4].**
- **Such findings help explain the enhanced re-epithelialisation responses in epoxy-tigliane-treated skin & highlight their potential as novel therapeutics for impaired dermal wound healing situations.**
- **Further studies are elucidating the mechanisms by which epoxy-tiglianes induce these stimulatory effects.**

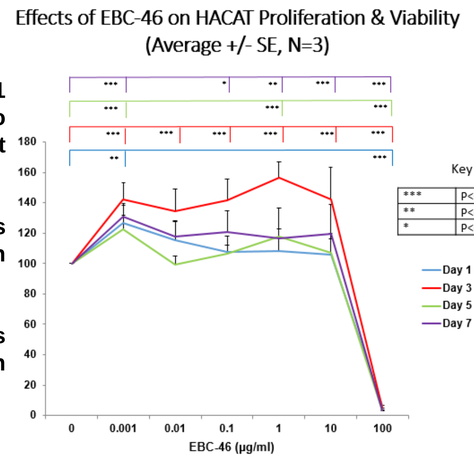
References

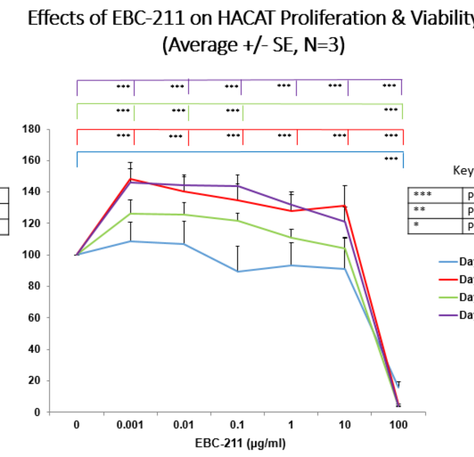
1) Dong L *et al.* Anticancer agents from the Australian tropical rainforest: Spiroacetals EBC-23, 24, 25, 72, 73, 75 and 76. *Chem Eur J* (2009); **15:**11307-18.
2) Boyle GM *et al.* Intra-lesional injection of the novel PKC activator EBC-46 rapidly ablates tumours in mouse models. *PLoS One* (2014); **9:**e108887.
3) Yun-Shain L *et al.* Wound healing in development. *Birth Defect Res, C Embryo Today* (2012); **96:**213-22.
4) Glim JE *et al.* Detrimental dermal wound healing: What can we learn from the oral mucosa? *Wound Rep Regen* (2013); **21:**648-60.

Results

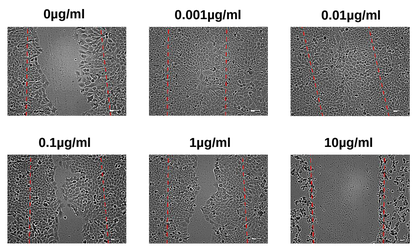
HACAT Proliferation

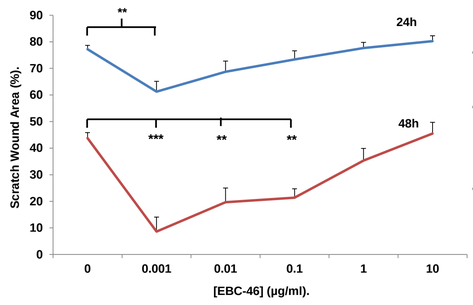
- **EBC-46 & EBC-211 are cytotoxic to HACATs at 100µg/ml.**
- **EBC-46 stimulates HACAT proliferation at 0.001-10µg/ml.**
- **EBC-211 induces HACAT proliferation at 0.001-10µg/ml.**



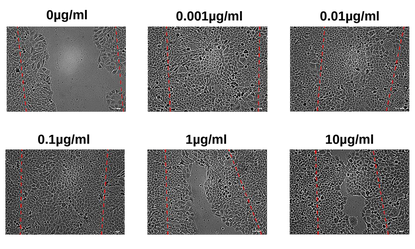


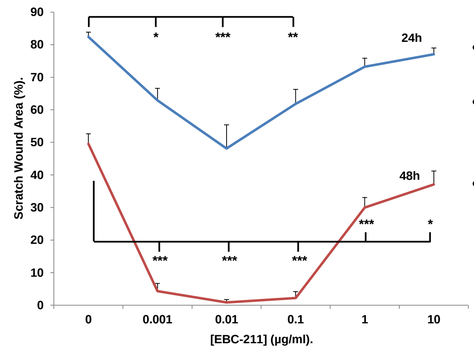
In vitro scratch wound re-population, EBC-46 @ 48h (N=3)





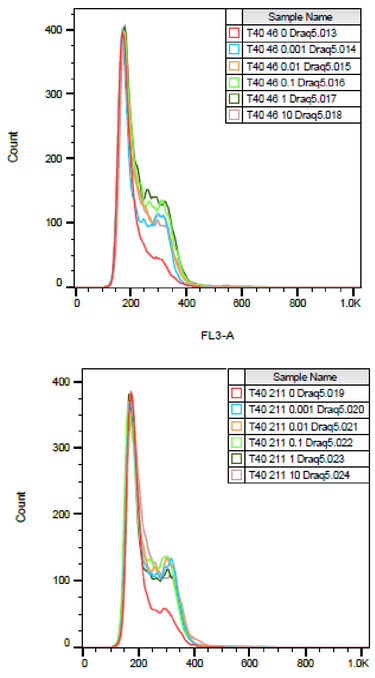
In vitro scratch wound re-population, EBC-211 @ 48h (N=3)





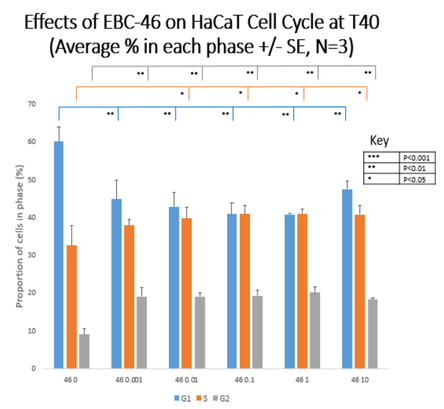
HACAT Wound Re-population

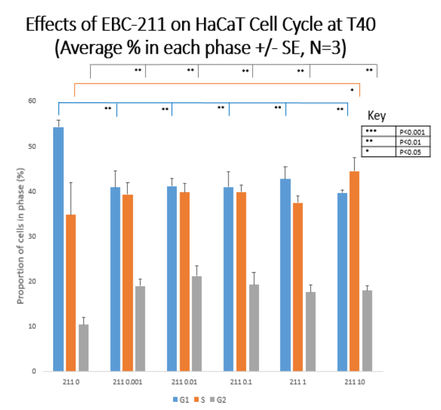
- **EBC-46 accelerates migration @ 0.001-0.1µg/ml.**
- **Full wound closure @ 0.001-0.01µg/ml EBC-46, vs untreated controls.**
- **Significant wound re-population evident at 0.001µg/ml (24 & 48h) & 0.01-0.1µg/ml (48h), vs. controls (*N=3*, **p<0.01, ***p<0.001).**
- **EBC-211 accelerates migration @ 0.001-10µg/ml.**
- **Full wound closure @ 0.001-0.1µg/ml EBC-211, vs untreated controls.**
- **Significant wound re-population evident at 0.001-0.1µg/ml (24 & 48h) & 1-10µg/ml (48h), vs. controls (*N=3*, *p<0.05, **p<0.01, ***p<0.001).**



Cell Cycle Analysis

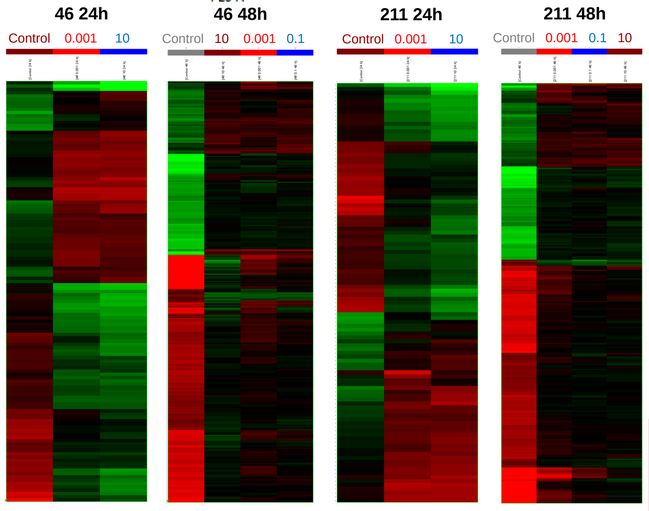
- **EBC-46 accelerates HACAT cell cycle progression @ 0.001-10µg/ml, compared to untreated control.**
- **EBC-211 accelerates HACAT cell cycle progression @ 0.001-10µg/ml, compared to untreated control.**
- **EBC-46 & EBC-211 treated cells progress out of G1 phase through S phase & into G2 phase ahead of the untreated control.**





Microarrays

- **Heatmap visualization through hierarchical clustering of genes differentially expressed ≥2 fold by HACATs (*n=4* biological repeats).**
- **Cultured in the presence of 0.001µg/ml, 0.1µg/ml or 10µg/ml EBC-46 (A) or EBC-211 (B); & compared to untreated controls at 24h & 48h.**
- **Data from the 4 biological repeats were grouped for combined analysis between treatments & vs the untreated controls.**



Red = up-regulated genes,
Green = down-regulated genes.

Fold change for untreated control against the five conditions for EBC-46 (A) & EBC-211 (B); 0.001µg/ml at 24h & 48h, 0.1µg/ml at 48h, & 10µg/ml at 24h & 48h.

A Gene	Control vs EBC-46, 0.001 24h	Control vs EBC-46, 0.001 48h	Control vs EBC-46, 0.1 48h	Control vs EBC-46, 10 24h	Control vs EBC-46, 10 48h
KRT13		5.3	5.3		6.3
KRT15		4.6	4.6	-2.8	5.7
KRT9		2.1	2.1		2.2
KRT81	2.3				
KRT16		-4.3	-4.3	-3.1	-4.1
KRT17		-4.1	-4.1	-3.1	-4.0
KRT6B		-3.2	-3.2		-3.5
KRT17P3		-4.3	-4.3	-2.5	-4.2
Cyclin B2		4.1	4.1		4.2
Cyclin A2		3.0	3.0		3.0
Cyclin B1		2.7	2.7		2.7
CDKN3		3.3	3.3		3.2
CDKN1A		-2.0	-2.0	-3.2	-2.2
MMP-1		5.8	5.8	2.5	5.1
GINS2	2.5	2.8	2.8	2.4	2.9
POLE2	2.4			2.41	
UBE2C		3.8	3.8		4.3
PTHLH	2.7	2.5	2.5	2.8	2.4
IL-6		-5.3	-5.3	-2.0	-7.2
IL-8		-5.6	-5.6	-4.4	-8.1
IL-32		-6.7	-6.7		-8.6
IFNB1	-3.8	-4.3	-4.3	-4.2	-5.0

B Gene	Control vs EBC-211, 0.001 24h	Control vs EBC-211, 0.001 48h	Control vs EBC-211, 0.1 48h	Control vs EBC-211, 10 24h	Control vs EBC-211, 10 48h
KRT13		6.9	5.6		4.7
KRT15		5.4	3.8		5.6
KRT9		2.8	2.4		2.2
KRT81	2.5				
KRT16			-3.2	-3.0	-4.0
KRT17		-2.3	-3.1	-3.0	-3.6
KRT6B			-2.89		-3.7
KRT17P3		-2.6	-3.3		-3.9
Cyclin B2		3.8	4.2		3.8
Cyclin A2		2.9	3.0		2.7
Cyclin B1		2.7	2.7		2.7
CDKN3		3.2	3.4		3.1
CDKN1A				-2.8	-2.1
MMP-1	4.1	6.0	4.7		6.2
GINS2	2.5	3.1	3.1	2.3	
POLE2	2.19		2.0	2.3	
UBE2C		3.6	3.6		3.7
PTHLH	2.7		2.6	2.9	2.3
IL-6		-4.3	-5.4		-6.2
IL-8		-2.8	-3.8	-3.2	-6.3
IL-32		-4.6	-6.9		-7.3
IFNB1	-3.3	-4.3	-5.1	-4.1	-4.4

- **Up-regulated genes included certain keratins (KRT9, KRT13, KRT15, KRT81), positive cell cycle & proliferation regulatory genes (CCNB2, CDKN3, CDCA7, GINS2, KIAA0101); & proteinases (MMP-1, MMP-7, MMP-10).**
- **Down-regulated genes included other keratin (KRT6B, KRT16, KRT17) & cytokines (e.g. IL-6, IL-8, IL-32).**

Acknowledgments: This study is funded by QBiotics Ltd., Queensland, Australia & the Cardiff Institute of Tissue Engineering & Repair (CITER), Cardiff University, UK.
This work is covered by filed patent, **WO2014169356.**
We wish to acknowledge & thank Professor Rachel Errington, Dr Rachel Howard-Jones & Marie Wiltshire, School of Medicine, Cardiff University, for their help & advice in performing the FACS analysis.