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Crispene E, a *cis*-clerodane diterpene inhibits Stat3 dimerization in breast cancer cells

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Crispene E, a new clerodane-type diterpene, inhibited STAT3 dimerization in a cell-free fluorescent polarisation assay and was found to have significant toxicity against STAT3-dependent MDA-MB 231 breast cancer cell line and selectively inhibited the expression of STAT3 and STAT3 target genes *cyclin D1*, *Fascin* and *Bcl-2*. Molecular docking studies suggest the molecule inhibits STAT3 by interacting with its SH2 domain. The compound has been isolated from *Tinospora crispa* and characterized using standard spectroscopic techniques.

STAT (signal transducer and activator of transcription) proteins — especially STAT3 and, to a large extent, also STAT5 — have emerged as promising molecular targets for cancer therapy[1]. It is an attractive molecular target for novel cancer therapies, as a number of *in vivo* studies have shown that STAT3 is constitutively active in a variety of malignancies ranging from breast, prostate, and head and neck tumours to multiple myelomas and haematological cancers[2-7]. Although cancer cells are often dependent upon activation of STAT3, non-cancerous cells are fairly tolerant of loss of its function, likely reflecting redundancies in normal signal transduction. Thus, STAT3 inhibitors have a high therapeutic potential[1, 8]. Furthermore, resistance to targeted therapies often arises from activation of an alternative signalling pathway, many of which also converge on STATs. This suggests that inhibition of these proteins may forestall resistance[9, 10]. The STAT3 signalling pathway is stimulated by growth factors or cytokines which leads to receptor dimerization and activation[3, 4, 7, 11-13]. Phosphorylation of the tail of the receptor creates a docking site for the recruitment of un-phosphorylated STAT3 (uSTAT3) which becomes phosphorylated at the Tyr705 position (near the C-terminus) by JAK kinases. The phosphorylated STAT3 (pSTAT3) protein is then released, forming a homodimer through reciprocal binding of the SH2 domain of one monomer to the pTyr-containing PYLKTK sequence of another[14, 15]. This dimeric STAT3:STAT3 complex then translocates to the nucleus where it binds to its DNA consensus sequence, thus regulating transcription of numerous genes critical for the survival and proliferation of cancer cells[3-5, 7, 12, 13, 16]. Each step of the STAT3 activation process offers a potential molecular target for selective inhibition of aberrant STAT3 signalling. In this context, there is a greater focus upon developing small molecules that selectively target the STAT3 proteins themselves[17-21]. This is based on increasing experimental evidence suggesting that inhibition of the formation of the active dimeric phosphorylated STAT3 complex is an effective way to down-regulate STAT3 transcriptional activity[7, 22-28]. A number of different approaches have been taken to identify novel small molecules as STAT3:STAT3 dimerization inhibitors. Early prototypes were based on peptidic and peptidomimetic molecules that bind to the STAT3 SH2 domain[7], but in the last five years progress has been made with small molecules derived from STAT3 structural information and *in silico* design[11, 16, 22].

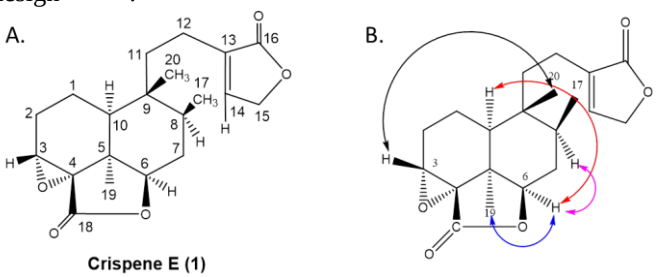

Figure 1:A) Structure of the novel STAT3 dimerisation inhibitor Crispene E (**1**); B) NOE relationships in Crispene E.

There is increasing interest in searching for natural products with drug-like properties as potential pre-clinical candidates[29]. To search for natural products with these properties we used a fluorescent polarisation assay using a uSTAT3 monomer and a surrogate peptide to study plant

isolates, and identified the clerodane-type diterpene Crispene E (**1**, Figure 1) from *Tinospora crispa*, a woody climber native to Malaysia, Indochina, Indian subcontinent and China[30]. In traditional medicine, it is used for the treatment of hypertension, diabetes mellitus, malaria, diarrhoea and as a vermifuge[31, 32]. As part of our on-going work to identify bio-active pant metabolites[33-37], we report here a novel chemical scaffold with STAT3-dimeerization inhibitoty activity. This is the first report of a clerodane diterpene of this type possessing selective STAT3 inhibitory activity, and provides a new chemical scaffold to design novel analogues with improved activity. It should be noted that Crispene E is structurally different compared to other reported STAT3 dimerization inhibitors and therefore, provides a new chemical scaffold for a drug-discovery program. An important feature is that **1** is drug-like, according to Lipinski's rule of five[38], making it amenable for further med-chem optimization to enhance its STAT3 inhibitory activity and cytotoxicity in STAT3-dependent tumours.

Crispene E was isolated from the methanolic extracts of *T. Crispa* (†ESI). The ^{1}H NMR spectrum of 1 is summarized in Table S1 and confirmed that it contains five sp3-hybridized methylenes (C-1, C-2, C-7, C-11 and C-12), and one oxygenated methine group (C-6). The molecular formula $C_{20}H_{26}O_5$ of **1** was determined by ESI mass spectrometry measured in the positive ion mode (*m/z* 369.17 [M+Na]+) and this was further confirmed by high resolution mass spectrometry (Exact Mass: 369.1678 [M+ Na], observed mass 369.1670 [M+ Na)+].. The ^{13}C NMR decoupled spectrum (**Table S1**) exhibited 20 signals which are in agreement with the molecular formula. The ^{1}H and ^{13}C NMR spectra (δH 7.11 bs and 4.77 d, J = 1.6Hz; δC 134.23, 143.91, 70.25 and 174.15) indicated the presence of a butenolide function[39a], lactone ring (δC 173.46) and an epoxide unit (δH 3.64 s, δC 58.24 and 60.13). In addition, three methyl groups are present and were identified from their spectral data as doublet for C-17 (δH 0.88 d, J = 6.0Hz; δC 15.03) and two singlets for C-19 (δH 1.27 s; δC 27.30) and C-20 (δH 0.80 s; δC 19.63). Considering the quaternary carbons (δC 34.94 and 38.94) and the methine groups (δC 32.16 and 41.05) and their heteronuclear correlations displayed in the HMBC spectrum, these signals could be assigned to C-5, C-9, C-8 and C-10, respectively, of the decalin moiety. The one proton broad signal at δH 7.11 and no cross connection between C-14 proton and any olefinic protons in COSY experiment, confirmed the absence of proton at C-13 and exclude the possibility of the double bond at C-14 and C-15. The position of the epoxide group (C-3 and C-4) was deduced from the HMBC spectrum correlations between C-3 (δC 58.24) with H-1, H-2 and C-4 (δC 60.13) with H-2, H-6, H-19. The downfield double doublet showed by H-6 at δH 4.53 (1H, J = 10.0, 8.0Hz) revealed the presence of another lactone ring between C-6 and C-18 and the axial orientation of H-6 was indicated by the large coupling of 10.0 Hz. The ^{13}C NMR signal of the shielded Me-19 (δC 27.30) suggested that the A/B ring junction is *cis*-fused[40]. The α-orientation of the epoxy group (C-3 and C-4) was proposed on the basis of the ^{1}H NMR signal of the shielded Me-19 protons at δH 1.27 s[41]. The signals were assigned with the aid of the ^{13}C and ^{1}H NMR spectral analysis and the assignments were verified by ^{1}H-^{1}H COSY, HSQC and HMBC experiments (†ESI). The relative configuration of **1** was determined on the basis of NOESY interactions. The correlations between H-6 with H-8, H-10 and Me-19 indicated that all these are in the same side of ring, i.e α-orientation. Hence Me-17 and Me-20 are obviously β-oriented. Furthermore, H-3 showed connectivity with Me-20, confirming it to be in β conformation. Thus **1** was identified as a new *cis*-clerodane type diterpene, and given a trivial name Crispene E.

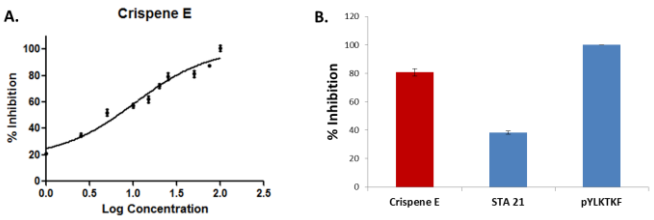

Figure 2: FP assay inhibition graphs for **1**. a) PPI IC_{50} graph for **1**. b) Inhibition relative to natural hexapeptide pYLKTKF,

We used unphosphorylated STAT3βtc protein to develop a fluorescent polarization (FP) based primary protein-protein binding assay to assess the STAT3 dimerisation inhibitory activity of **1**. The FP assay was carried out using the fluorescein-labelled FAM-pYLPQTV peptide as the surrogate peptide and pYLKTKF peptide as a control inhibitor peptide to measure the activity of **1**. Protein expression and purification protocols for U-STAT3βtc were adapted from the method previously reported by us[42, 43]. **1** disrupted binding of STAT3 binding to phosphorylated high-affinity peptide pYLPQTV-NH, with an IC_{50} of 10.27μM (**Figure 2a**). It showed 80% inhibition relative to pYLKTKF and 210% inhibition relative to the SH2 domain interacting molecule STA-21 at 100 μM (**Figure 2b**).

To evaluate the effect of STAT3 dimerisation inhibition on the viability of STAT3 dependent tumour cell lines, **1** was tested using

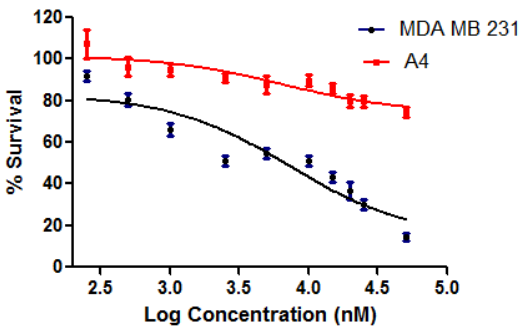

Figure 3.MTT cell-viability assay profile in MDA-MB-231 (STAT3-dependent) and A4 (STAT3-null) cells treated with **1** for 24 hours.

an MTT cell viability assay against both MDA-MB-231 breast (STAT3-dependent) and A4 (STAT3 null) cancer cell lines. The compound showed an IC_{50} of 5.35µM against MDA-MB-231 and >100 µM against A4 cell lines suggesting a STAT3-specific inhibition. This was consistent with the activity observed in the FP assay in which the compound showed greater potency compared to the SH2 domain targeting molecule STA 21. A Trypan Blue exclusion assay in MDA-MB-231 demonstrated 85% of dead cells after exposure to **1** for 24 hour period suggesting a cytotoxic rather than cytostatic effect.

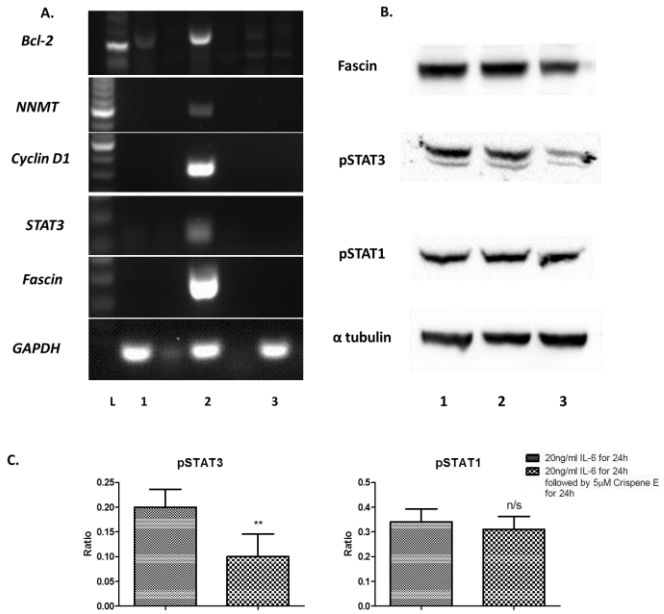

Figure 4.A) RT-PCR gel showing effect of **1** on the expression of STAT3 and STAT3-dependent genes in STAT3 dependent MDA-MB-231 cell line (lane 1, untreated cells; lane 2, 500µg/ml LPS for 24h; lane 3, 500µg/ml LPS for 24h, 100µM **1** for 24h); B) Western blot showing selective downregulation of pSTAT3 and Fascin with little or no effect on STAT1 and α-tubulin (lane 1, untreated cells; lane 2, 20ng/ml IL-6 for 24h; lane 3, 20ng/ml IL-and 5µM **1** for 24h) in MDA-MB-231 cells. C) Statistical analysis on western blots of pSTAT3, and pSTAT1 protein expression in MDA-MB-231 showing the significant pSTAT3 (**P<0.05) down-regulation after treatment with 5µM Crispene E for 24 h. In contrast, pSTAT1 expression was insignificantly affected by the STAT3 inhibitor under the same conditions (P>0.05). Error bars represent S.D, N/S = not significant.

To probe the apparent STAT3-specific cytotoxicity of **1**, the mRNA expression profile of STAT3 and the STAT3 target genes *bcl-2*, *cyclin D1*, *NNMT* and *fascin* were compared to the reference gene *GAPDH* by RT-PCR after 24 hour exposure to 100 µM ligand concentration. **1** produced notable downregulation of *STAT3* and *STAT3* dependent genes as outlined in **Figure 4a**. The down-regulation was particularly significant for *Bcl-2* which is a major anti-apoptotic protein (**Figure 4a**). This suggests a relation between the decreased viability of various tumour cell lines (see supporting information), including MDA-MB-231, with a change in STAT3 expression. Further confirmation of the specificity of **1** for STAT3 inhibition was obtained by investigating its effect on pSTAT3 compared to pSTAT1 by Western blot analysis in serum-starved MDA-MB-231 (**Figure 4b**). **1** significantly down-regulated pSTAT3 with no effect on pSTAT1 at 5 µM concentration. This finding confirmed that although **1** inhibited STAT3 dimerisation by interacting with SH2 domain, this nteraction is specific to STAT3 and does not affect STAT1, which has tumour suppressive properties[44].

To further confirm that the observed effect on phosphorylation of STAT3 and STAT1 (Figure 4B) is due to the ability of the molecule to disrupt STAT3 dimerization, and not mediated by its potential interaction with IL6, we carried out a detailed molecular modelling study on the IL-6 alpha receptor-gp30 complex (PDB ID 1P9M) in an effort to ascertain the molecule's ability to disrupt IL-6. Docking studies were undertaken on the full protein structure, and the study failed to dock the molecule in the protein as a suitable binding cavity for the ligand could not be located.. This suggests that Crispene E does not have the appropriate conformation for interaction with the gp130 protein and is unlikely to interact with IL-6 directly.

Molecular docking calculations were conducted on **1** bound to the SH2 domain to investigate its affinity for the STAT3 protein. A second docking study was also undertaken involving the DNA binding domain of STAT3 as this is also known to be a potential binding point for STAT3 inhibitors[45]. **1** was found to be capable of binding in both locations. However, free energy of binding calculations conducted on the most favourable conformations of **1** bound to both the SH2 domain and DNA binding domain suggested that the molecule has a greater affinity for the SH2 domain (-40.67kcal/mol compared to -32.06kcal/mol), thus indicating preference for binding in the SH2 domain.

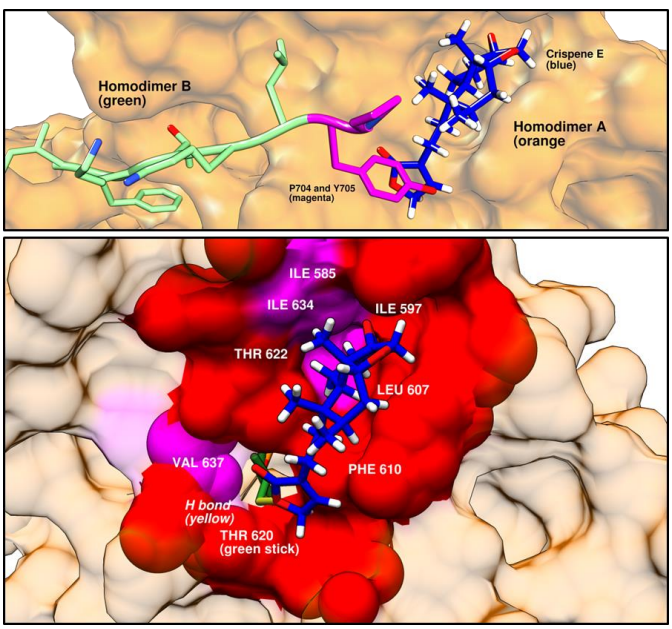

Figure 5 Figure: Top: Model of STAT3 monomer (PDB ID: 1BG1) showing 1 (blue) docked in the SH2 domain of Homodimer A (orange), preventing the interaction of Homodimer B (green), and particularly residues P704 and Y705 (highlighted in magenta) contained in the PYLKTKFI fragment. Bottom: Zoomed in model of 1 docked in a binding pocket in the SH2 domain (red) of Homodimer A. Interactions between hydrophobic residues (magenta) and 1 (blue) are shown, with a stabilising H-bond (yellow) between the oxygen of the furanone ring and THR 620 (green) contributing to the affinity of the molecule for the pocket. Homodimer B has been removed from the lower image for clarity.

A detailed investigation of the **1**/SH2 complex was then undertaken. Analysis of the docking results suggested that binding occurs in a hydrophobic pocket located alongside the pYLKTKF hexapeptide binding domain of the SH2 monomer. In particular, the fused ring structure of **1** occupies a cavity consisting of Ile597, Leu607, Phe610, Thr622 and Ile634, forming hydrophobic interactions in the process. A hydrogen bond is also formed between the oxygen group of the furanone ring and oxygen of Thr620, further stabilising the complex. Finally, hydrophobic interactions between the ligand structure and Ile585 and Val637 assist in restraining the molecule in the cavity. Interestingly, the binding mode of **1** to the pocket allows the furanone moiety of the molecule to directly inhibit the interaction of both Pro704 and Tyr705 of homodimer B. This mechanism of action is in accord with previously-published studies, which illustrate molecules preventing the interaction of Tyr705 in a similar manner[4, 20].

In summary, we have identified a novel clerodane diterpene with selective STAT3 inhibitory activity. Molecular modelling experiments supported experimental observations suggesting this molecules works by interacting with the SH2 domain of STAT3. It provides a novel natural scaffold to develop STAT3-targeting anticancer therapy.

Notes and references

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† Electronic Supplementary Information (ESI) available: [Spectroscopic data, Stat3 expression and purification procedure, details of cell culture, RT-PCR, Western blot and molecular modelling methods]. See DOI: 10.1039/c000000x/

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