NEW APOPTOSOME INHIBITORS FROM IRISH DEEP-SEA ZOANTHID.

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Abstract

Cell death is a normal and a tightly regulated part of many normal physiological processes but when the proper regulation fails, the result can be disease. Consequently, there is great interest in the discovery of pharmacological tools to induce or inhibit cell death that might be of therapeutic use. Apoptosis is an extraordinarily important form of regulated cell death that is implicated in a wide range of human diseases, from degenerative diseases where too much death occurs, to cancer where apoptosis typically fails. Using a split-luciferase to detect Apaf-1 homotypic interactions identified that a methanolic extract of a deep-sea zoanthid blocked apoptosome formation. Purification of this extract and spectroscopic analysis of fractions yielded a series of adenine substituted bromotyrosine derived metabolites. In this presentation we showcase our efforts to find drug-like metabolites from deep-sea collections of Irish invertebrates, to screening, to pure compounds.

Introduction

Comprehensive surveys of some deep-sea environments have compared their biodiversity favourably to that rainforests and tropical coral reefs.[1] Deep-sea organisms live under extreme conditions of high pressures, low oxygen and complete darkness. Survival has necessitated diverse adaptions in an array of biosynthetic and physiological pathways in deep-sea organisms. These adaptions are often coupled with modification in both the gene regulation as well as secondary metabolite pathways.[2,3] These modified pathways increase the likelihood of discovering novel natural products. Metabolites from these organisms account for approximately 2% of all the reported marine metabolites, primarily due to the difficulty in accessing these environments.[4] With the development of new technologies such as ROVs, collection in this unique ecosystem is now possible. Herein we describe our strategy for the discovery of bioactive marine metabolites sourced from Irish deep-water canyons (Fig 1A) and screened in a novel split luciferase assay to identify activators/inhibitors of the apoptosome.

Programmed cell death (PCD) occurs in many pathological situations from inflammatory conditions to ischaemia. PCD is also the mechanism through which many anti-cancer therapies act. The apoptosome is a protein complex formed when seven Apaf-1 molecules oligomerize and recruit the protease caspase-9 (Fig 1B). The apoptosome is part of the mitochondrial or intrinsic death pathway and forms when cell stressors, cellular damage or other signals trigger the release of cytochrome *c* from mitochondria. Cytochrome *c* binds to Apaf-1 and induces a conformational change that allows Apaf-1 to self-associate into a wheel like complex, with caspase-9 binding at the hub of the structure. When bound in the apoptosome caspase-9 is activated, an event that triggers a cascade. of protease activation that can ultimately kill a cell by apoptosis. Activators of Apaf-1 are predicted to be potential anti-cancer agents and while this type of molecule has not yet been identified, small molecules that sensitize Apaf-1 to cytochrome c mediated activation have been reported and these agents show activity against cancer cell lines.[5-7] Recent developments in oncology have shown, unexpectedly, that blocking apoptotic cell death sensitizes cancer cells to radio- and chemotherapy by favouring a different mode of cell death.[8-11] This suggests that Apaf-1 inhibitors, rather than activators, could also be useful anti-cancer agents.

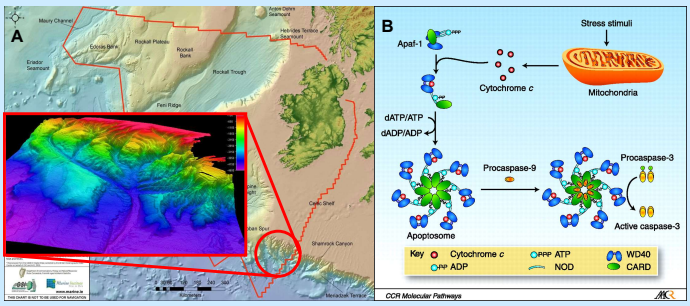


Figure 1: A: The real map of Ireland, area outlined in red incorporate the territorial waters of Ireland with a bathymetry map of the Whittard Canyon inset. B: Graphical representation of a stress induced Cytochrome C storm resulting in the formation of the apoptosome and caspase-3 activation.[12]

Screening:

The chemical extracts of the deep-sea organisms collected in the Whittard Canyon were screened in an initial bioassay designed to identify inhibitors of Caspase-3 activation. Extracts deemed active were additionally screened in a novel split-luciferase apoptosome assay (Fig 2). This assay was designed to identify activators/inhibitors of the Apaf-1/Apaf-1 protein-protein interaction within the apoptosome. The extracts displaying activity were counter-screened against the intact luciferase to ensure the activity noted was due to inhibition of the reporter enzyme. Ultimately, three deep-sea marine extracts were identified as inhibitors of the apoptosome and pursued further, the best of which originated from a zoanthid which grows on *Aphrocallistes beatrix*.

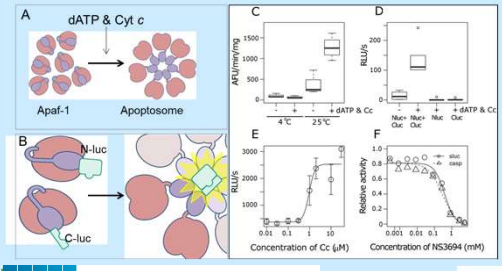


Figure 2: A: Cartoon diagram of the assembly of the apoptosome from Apaf-1 subunits in the presence dATP and Cytochrome *c* B: Cartoon diagram showing the assembly of the split-luciferase on the formation the apotosome. C: Temperature dependence of the split-luciferase assay (sluc) D: Activity of the components of the sluc assay. E: Dose response of the sluc-apoptosome to increasing concentration of Cytochrome *c*. F: Dose response of the known inhibitor NS3694 in the sluc and caspase-3 assays.

Purification and Chemical Analysis:

The greatest inhibition was observed in a methanoic extract of a yet-to-be-described zoanthid growing on *Aphrocallistes beatrix* (Fig 3A). Bioguided fractionation identified the major metabolite as well as some minor metabolites as active against the apoptosome. The major metabolite was identified as aphrocallistin A (**1**), previously reported by Wright et al.[13] from a sample collected off the Florida coast. Further purification of the fractions yielded four new aphrocallistins. High resolution mass spectrometry showed all these metabolites were dibrominated and molecular networking showed these metabolites were all structurally related to **1**.

All the metabolites showed a dibrominated isotope pattern in the HRESIMS. The ^{1}H NMR spectrum showed resonances which could be attributed to various *N*-methyl protons, an acetyl moiety (**1, 3** & **5**) and an A_2X_2 spin system observed in all compounds. Correlations observed in the HMBC from the *N*-methyl showed the presence of an amide in the metabolite as well as linking this amide to the brominated benzene ring through an ethyl linker (Fig 3B). The spectra of the right-hand side fragment showed two methine protons (δ_H 8.66 (s), δ_C 149.4; δ_H 8.44 (s), δ_C 145.5), three sp^2 hybridized quaternary carbons (δ_C 153.4, 149.7, 112.5) and the remaining *N*-methyl (δ_H 4.08 (3H, s), δ_C 36.5), ultimately resulting in the unusual 3-methyladenine moiety. Further analysis of the HMBC and COSY data connected the adenine functionality to the dibromophenyl ring through a propyl ether linker resulting in the final structure

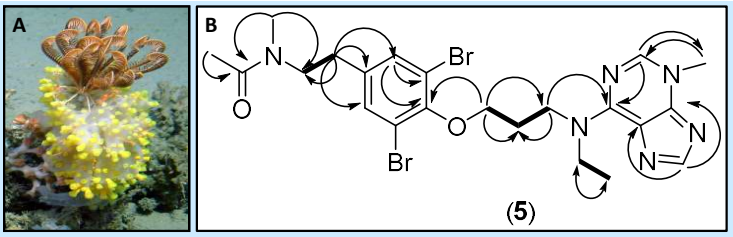
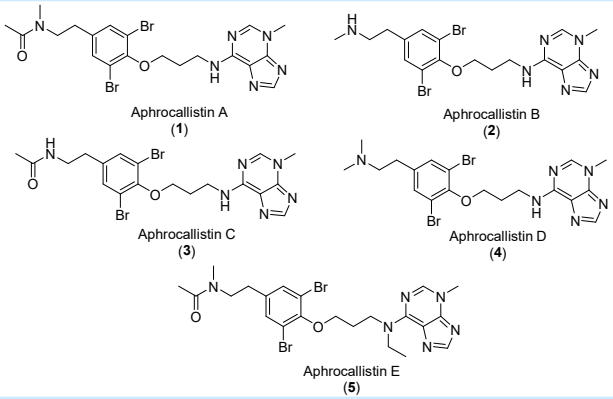


Figure 3: A: Underwater photograph of the zoanthids responsible for the bioactive extract. B: Chemical structure of a representative zoanthid metabolite **(5)**. Bold lines signify key ^{1}H-^{1}H COSY correlation used to determine the final structure. Arrows denote key HMBC correlations used to elucidate the structure.



Acknowledgments

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