An Efficient Method for the Isolation of Toxins from *Pteridium aquilinum* and Evaluation of Ptaquiloside Against Cancer and Non-cancer Cells

Courtney Williams[1], Simon J. Allison[1], Roger M. Phillips[1], Peter A. Linley[2] and Colin W. Wright[2]

[1]School of Applied Sciences, University of Huddersfield, West Yorkshire, HD1 3DH, UK.

[2]School of Pharmacy and Medical Sciences, University of Bradford, West Yorkshire, BD7 1DP, UK;

Correspondence

Prof. Colin W. Wright

School of Pharmacy and Medical Sciences

University of Bradford

West Yorkshire, BD7 1DP, UK

Phone +44(0)1274 234739; FAX +44(0)1274 235600

c.w.wright@bradford.ac.uk;

Dedicated to Professor Arnold Vlietinck on the occasion of his 80th birthday

ABSTRACT

The common fern, bracken (*Pteridium aquilinum*) is well known for its toxic effects on livestock due principally to the carcinogenic constituent ptaquiloside (**1**), although other toxins are present including the cyanogenic glycoside, prunasin (**2**). Here, we report an improved and relatively 'green' process for the isolation of **1** and **2** from fresh bracken fronds and the evaluation of **1** for cytotoxicity against several cancer cell lines. The results indicate that **1** displays selective toxicity against cancer cells relative to non-cancer retinal epithelial cells and the improved method for the isolation of **1** is expected to facilitate further exploration of its pharmacological properties.

Introduction

The common fern, bracken, also known as brake, *Pteridium aquilinum* (L.) Khun. (Dennstaedtiaceae), is one of the most abundant plants globally and is widespread in northern upland areas of the UK [1]. Traditionally, bracken was important in the UK as a packing material and winter bedding for cattle while the ash was used as a substitute for soap and was employed in soap and glass manufacture [2]. Bracken was also used as a fuel or tinder, for bivouac thatching and for making plaited sandals.

However, bracken is notable for its toxic effects especially in animals which may result in several acute or chronic poisoning syndromes depending on the species affected [1]. Acute poisoning due to the presence of the enzyme thiaminase in the plant occurs in horses and pigs as a result of thiamine deficiency while an acute haemorrhagic syndrome may occur suddenly in cattle after the animals have grazed on bracken for several weeks. The latter syndrome may also occur in sheep although it is slower to develop and occurs less commonly than in cattle. Chronic bracken toxicity resulting from the consumption of small amounts of bracken over a prolonged time gives rise to enzootic haematuria in cattle and, in addition to the loss of blood in the urine, this condition is associated with the development of benign and malignant tumours in the bladder [1]. The mechanism of carcinogenesis involves immunosuppressive effects of bracken that activate latent bovine papillomavirus type 2, resulting in the formation of pre-malignant changes in the bladder that may then become malignant through the action of the bracken carcinogen, ptaquiloside (**1**) (**Fig. 1**) [3]. Bracken consumption is also associated with tumours of the digestive tract [1]. In sheep, bracken consumption over a period of time may cause Bright blindness (progressive degeneration of the retina), resulting in blindness and has also been associated with tumours of the intestine and jaw [1]. In Japan, human consumption of the young, uncurled fronds of bracken known as fiddleheads is associated with an increased incidence of tumours of the oesophagus and stomach and as **1** has been detected in milk there have been concerns that milk consumption could be hazardous to humans [1].

Ptaquiloside (**1)**, a norsesquiterpene glucoside of the iludane type, was isolated by bioassay guided fractionation as the principal toxic constituent of bracken by Yamada's group in 1983, [4] and subsequently a number of other terpene glycosides related to **1** have been isolated [5]. When the plant is consumed by animals, loss of glucose from **1** leads to the formation of a dienone (**3**), a highly reactive molecule that alkylates biomolecules (**4**), (**Fig. 1**). DNA alkylating agents such as temozolomide and cyclophosphamide as well as the platinates which are 'alkylating-like' in their mechanism of action are currently used in the clinic against particular cancer indications.

The aim of this study was to isolate **1** in order to explore its potential as a lead to new anticancer agents. Although dried bracken samples were shown to contain **1** by mass spectrometry, initial attempts that employed extraction and separation methods based on previously published methods, [5], [11], were unsuccessful. This result was not surprising considering that **1** is unstable, likely to be destroyed by glucosidase enzymes present in the bracken and also that yields from previous studies have been low (0.02-0.1% with respect to dried bracken) [5]. Here we report a simplified and relatively 'green' method for the extraction and isolation of **1** and its preliminary

evaluation for cytotoxicity in several human cancer cell lines and one non-cancer cell line.

Results and Discussion

Fresh, uncurled fronds of bracken, (fiddleheads) were collected at the beginning of the growing season (mid-May), as this part of the fern has previously been reported to contain the highest concentration of **1** [5]. A cold extraction process was employed in order to prevent thermal and enzymatic decomposition of **1**. Fresh fiddleheads were placed in a mortar, frozen with liquid N_2 within 2-3 h of harvesting, powdered, and macerated with Me_2CO. The extract was filtered, concentrated under a stream of N_2 gas, diluted with water and extracted with hexane to remove pigment and then partitioned several times with EtOAc. The EtOAc extract was concentrated under N_2 and chromatographed over TLC-grade silica gel 60 by means of low pressure column chromatography eluting with EtOAc:EtOH. Two compounds were eluted; the more polar was confirmed to be **1** as NMR and MS data were consistent with that previously published, [4], (Table 1S, Figs. 1S-3S, Supporting Information), and the less polar isolate was identified as the cyanogenic glycoside, prunasin (**2**) (**Fig. 1**), a known constituent of bracken [6] by comparison of spectral data with published values [7], (Table 2S, Figs. 4S-6S, Supporting Information). The yield of **1** was 0.12% with respect to fresh bracken, equivalent to approximately 0.6% with respect to dried bracken, comparable to previously published data although the content of **1** in dried bracken fern is reported to be very variable, ranging from <0.003 to 0.4% [8]. The content of **2** was found to be 0.08% with respect to fresh plant material, consistent with a study in which young, curled bracken fronds were reported to contain up to 0.05% [6]. However, the yields in this study are underestimates of the contents of **1** and **2** since some fractions that contained a mixture of **1** and **2** were discarded.

In vitro chemosensitivity studies were performed using the isolated ptaquiloside, (**1**) against four human cancer cell lines and one human non-cancer cell line (**Table 1;** Fig. 7S, Supporting Information). Ptaquiloside, (**1**) displayed micromolar activity against all four cancer cell lines with IC_{50} values ranging from 22 to 70μM. These data are consistent with a previous study in which **1** was found to have dose-dependent cytotoxicity, (10 – 100 μM) against L1210 leukaemia cells as well as antitumour activity against L1210 cells in mice [5]. The mechanistic basis for the differential cytotoxicity of **1** towards different cancer cell lines requires further investigation but it raises the possibility that certain cancer cells may be particularly susceptible following exposure to **1**. Ptaquiloside, (**1**) was less active against the non-cancer ARPE-19 cell line, (IC_{50}>100μM) compared to all four cancer cell lines tested, with **1** being over 4-fold more active towards the HCT116 colorectal cancer cell line. The selectivity index (SI), defined as the IC_{50} for ARPE-19 cells divided by the IC_{50} for HCT116 cells was >4.5, and this compares to a previously reported cancer cell selectivity index of ~1.9-2 for the clinically approved platinate, cisplatin against the same cancer/non-cancer cell line pair [9, 10], (Fig. 8S, Supporting Information).

Overall, these initial chemosensitivity studies suggest that ptaquiloside, (**1**) has selective *in vitro* cytotoxicity towards certain cancer cells and this warrants further investigation. Future studies will explore whether differential cytotoxic effects are linked to differences in the activity or expression of glycosidases in these cancer cells that can metabolise ptaquiloside (**1**) to **3** (**Fig. 1**) and which might serve as a biomarker of likely cytotoxicity of **1**. In contrast to previously published methods, the extraction and isolation method described above ensures that the decomposition of **1** during the extraction process is minimised, does not require the use of resins and is completed with fewer steps [5], [11]. This method may also form a basis for the isolation of minor iludane glycosides from bracken, thus facilitating the exploration of the pharmacology and therapeutic potential of these interesting compounds.

Materials and Methods

General Experimental Procedures

Solvents and reagents were purchased from commercial suppliers and used without further purification. ^{1}H-NMR spectra at 400 MHz and ^{13}C-NMR spectra at 100.6 MHz were recorded in CD_3OD.

Plant Material

Bracken fern (*P. aquilinum)* was identified by CWW and young, uncurled bracken fronds were collected from Baildon Moor, Bradford, West Yorkshire, BD17 5PH, UK soon after they appeared in mid-May and processed within 2-3 h. A voucher specimen, SPMS 190521, has been deposited at the School of Pharmacy and Medical Sciences, University of Bradford, West Yorkshire, BD7 1DP, UK.

Extraction and Isolation

Bracken fiddleheads (50 g), were placed in a mortar, frozen with liquid N_2, powdered and extracted by macerating with Me_2CO for 10 minutes. The extract was decanted and extraction repeated twice. The pooled extracts were concentrated to a small volume under a stream of N_2, diluted with water and then extracted with hexane (3 x 10 mL). The aqueous layer was then repetitively partitioned with EtOAc, (20 mL) and the pooled extracts concentrated to a small volume under a stream of N_2. The concentrated extract was chromatographed over TLC-grade silica gel 60 (Merck Kieselgel 60, 20 g), in a glass column under low pressure and eluted with ETOAc:EtOH 98.5:1.5 until **2** eluted followed by ETOAc:EtOH 98:2 to remove **1** from the column. This was repeated if necessary for further purification of **1**. Fractions were monitored by TLC on Merck Kieselgel 60 F_{254} analytical plates, using Me_2CO:benzene 7:3 visualised by spraying with vanillin 1% and H_2SO_4 5% in EtOH followed by heating. Ptaquiloside (**1**), 60 mg, (R*f* = 0.25), colourless glassy solid and prunasin (**2**)**,** 24 mg (R*f* = 0.34), white powder, were obtained and their identities confirmed by comparison of spectroscopic data with published values [4, 7].

Cell Lines and Cell Culture

All cell lines were cultured in antibiotic-free media supplemented with 10% foetal calf serum, 1mM sodium pyruvate and 2mM L-glutamine and were maintained at 37°C in a humidified atmosphere of 5% CO_2. Human cancer cell lines used were the colorectal carcinoma cell line HCT116, [12] the pancreatic carcinoma cell line MIA PaCa-2, [13] and the paediatric neuroblastoma cell lines SHEP, [14] and SK-N-AS, [15]. Human non-cancer cells that were used as a non-cancer cell model for a preliminary evaluation of ptaquiloside *in vitro* selectivity towards cancer cells were human retinal epithelial ARPE-19 non-cancer cells, [16] as has previously been described [17-19]. All cell lines were purchased from ATCC except for SHEP and SK-N-AS which were a gift from Dr Christopher Cooper, University of Huddersfield.

Evaluation of *In Vitro* Cytotoxicity

Cell lines were seeded in 96-well flat-bottomed cell culture plates at an optimal cell seeding density for logarithmic phase growth over 4-5 days. This was 1000 cells/well for all cell lines except HCT116 (1500 cells/well) and MIA PaCa-2 (2000 cells/well). 24h following cell seeding the cells were treated with a 2-fold dilution series of ptaquiloside, (**1**) ranging from 100 µM to 0.391 µM. Isolated **1**, stored at -20^{0}C was freshly dissolved in DMSO just before use and diluted in cell culture media to a final DMSO concentration of 0.4% for all wells. Following 96 h cell exposure to **1**, MTT was added to all wells at a final concentration of 0.5 mg/mL for 4 h at 37 ^{0}C to allow for the formation of formazan crystals. Crystals were then dissolved in 150 µl DMSO and absorbance at 540 nm was measured to enable determination of IC_{50} values as described [18-20]. For each independent biological repeat (n=3), mean absorbance values at 540nm from eight technical replicates were determined for each tested drug concentration. Mean raw absorbance values were blank corrected using the 'no cells, no drug' control lane to enable determination of the true absorbance. Percentage cell survival for each tested drug concentration was calculated relative to that of DMSO vehicle control-treated cells. The formula used to calculate the IC_{50} values was, IC_{50} = ((a/b) multiplied

by the concentration at a) + concentration at a. a = % survival at the tested drug concentration that was nearest to and just above 50% survival - 50; b = % survival at the tested drug concentration that was nearest to and just above 50% survival minus the % survival at the tested drug concentration that was nearest to and just below 50% survival. Cancer cell selectivity of ptaquiloside was compared to that of clinically utilised cisplatin as a reference compound (Fig. 8S, Supporting Information).

To check that **1** did not react with MTT, following 96 h cell exposure to **1** and prior to the addition of MTT, the effects of the different concentrations of **1** on cellular phenotype was examined under the microscope. The decrease in absorbance values with the different cell lines correlated well with phenotypes observed under the microscope. Additionally, we tested whether 100 µM **1** in a cell-free system (in cell culture media) results in formation of formazan crystals over a 4-hour period. This was compared with vehicle control with no formation of crystals or change in absorbance observed. We also tested the cell system with and without a wash step before development with MTT and saw no change is absorbance levels or IC_{50} values.

Supporting Information

Tables 1S and 2S present NMR data for **1** and **2** respectively. ^{1}H- and ^{13}C-NMR and HRMS spectra for **1** are shown in Figs. 1S, 2S and 3S respectively; ^{1}H- and ^{13}C-NMR and HRMS spectra for **2** are shown in Figs. 4S, 5S and 6S respectively. *In vitro* chemosensitivity data of **1** are shown in Fig. 7S and *in vitro* selectivity of **1** compared to cisplatin is shown in Fig. 8S.

Acknowledgements

We thank Andrew Healy, Haseeb Ur-Rehman and Damian Yeadon for technical assistance.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Cooper MR, Johnson AW. Poisonous Plants and Fungi in Britain. London: The Stationary Office; 1998: 85-92

[2] Mabey R. Flora Britannica. London: Reed International Books; 1996: 15-17

[3] Campo MS, Jarrett WFH, Barron R, O'Neill BW, Smith KT. Association of bovine papilloma virus type 2 and bracken fern with bladder cancer in cattle. Cancer Res 1992; 52: 6898-6904

[4] Niwa H, Ojika M, Wakamatsu K, Yamada K, Hirono I, Matsushita K. Ptaquiloside, a novel sesquiterpene glucoside from bracken *Pteridium aquilinum* var. *latiusculum*. Tet Lett 1983; 24: 4117-4120

[5] Yamada K, Ojika M, Kigoshi H. Ptaquiloside, the major toxin of bracken, and related terpene glycosides: chemistry, biology and ecology. Nat Prod Rep 2007; 24: 798-813

[6] Bennett WD. Isolation of the cyanogenetic glucoside prunasin from bracken fern. Phytochem 1968; 7: 151-152

[7] Nahrstedt A, Rockenbach J. Occurence of cyanoglucoside prunasin and II corresponding mandelic acid amide glucoside in *Olinia* species (Olinaceae). Phytochem 1993; 34: 433-436

[8] Agnew MP, Lauren DRJ. Determination of ptaquiloside in bracken fern (*Pteridium esculentum*). Chromatog 1991; 538: 462-468

[9] Song H, Rogers N, Allison SJ, Brabec V, Bridgewater H, Kostrhunova H, Markova L, Phillips R, Pinder E, Shepherd S, Young L, Zajac J Scott P. Discovery of selective, antimetastatic and anticancer stem cell metallohelices *via* post-assembly modifications. Chem Sci 2019; 10: 8547-8557

[10] Nichol R, Khalaf A, Sooda K, Hussain O, Griffiths H, Phillips R, Javid F, Suckling C, Allison SJ, Scott F. Selective *in vitro* anticancer activity of non-alkyl

minor groove binders. MedChemComm 2019; 10: 1620-1634

[11] Oelrichs PB, Ng, JC, Bartley, J. Purification of ptaquiloside, a carcinogen from *Pteridium aquilinum*. Phytochem 1995; 40: 53-56

[12] Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. Science 1998; 12:1497-1501

[13] Yunis AA, Arimura GK, Russin DJ. Human pancreatic carcinoma (MIA PaCa-2) in continuous culture: sensitivity to asparaginase. Int J Cancer 1977; 19: 128-135

[14] Ciccarone V, Spengler BA, Meyers MB, Biedler JL, Ross RA. Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages. Cancer Res 1989; 49: 219-225

[15] Sugimoto T, Tatsumi E, Kemshead JT, Helson L, Green AA, Minowada J. Determination of cell surface membrane antigens common to both human neuroblastoma and leukaemia-lymphoma cell lines by a panel of 38 monoclonal antibodies. J Natl Cancer Inst 1984; 73: 51-57

[16] Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Exp Eye Res 1996; 62: 155–169

[17] Allison SJ, Knight J, Granchi C, Rani R, Minutolo F, Milner J, Phillips RM. Identification of LDH-A as a therapeutic target for cancer cell killing via (i) p53/NAD(H)-dependent pathways and (ii) p53 independent pathways. Oncogenesis 2014; 3: e102

[18] Allison SJ, Cooke D, Davidson FS, Elliot PIP, Faulkner RA, Griffiths HBS, Harper OJ, Hussain O, Owen-Lynch P, Phillips RM, Rice CR, Shepherd SL, Wheelhouse RT. Ruthenium-containing linear helicates and mesocates with tunable p53-selective cytotoxicity in colorectal cells. Angew Chem Int Ed Engl 2018; 57: 9799-9804

[19] Lord RM, Allison SJ, Rafferty K, Ghandhi L, Pask CM, McGowan PC. Cytotoxic hydrogen-bridged rhuthenium quinaldamide complexes showing induced cancer cell death by apoptosis. Dalton Trans 2016; 45: 13196-13203

[20] Allison SJ, Milner J. RNA interference by single and double-stranded siRNA with a DNA extension containing a 3’ nuclease resistant mini-hairpin structure. J Mol Ther Nucleic Acids 2014; 2: e141

AUTHOR INFORMATION

Corresponding Author

Colin W. Wright – School of Pharmacy and Medical Sciences, University of Bradford, West Yorkshire, BD7 1DP, UK; Orcid.org/ 0000-0002-9815-5806; Email: c.w.wright@bradford.ac.uk

Authors

Courtney Williams – School of Applied Sciences, University of Huddersfield, West Yorkshire, HD1 3DH, UK. Email: Orcid.org/ 0000-0003-1093-5208; courtney.williams@hud.ac.uk

Simon J. Allison – School of Applied Sciences, University of Huddersfield, West Yorkshire, HD1 3DH, UK. Orcid.org/ 0000-0002-5766-2377; Email: s.allison@hud.ac.uk

Peter A. Linley – School of Pharmacy and Medical Sciences, University of Bradford, West Yorkshire, BD7 1DP, UK; Email: p.a.linley@btinternet.com

Roger M. Phillips – School of Applied Sciences, University of Huddersfield, West Yorkshire, HD1 3DH, UK. Orcid.org/ 0000-0003-3504-177X; Email: r.m.phillips@hud.ac.uk