Isolation and Characterization of a Cyclic Hydroxamic Acid from a Pollen Extract, Which Inhibits Cancerous Cell Growth in Vitro

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Introduction

Cernilton is a multipotent extract from rye pollen which is commercialized by Cernitin SA, Lugano, Switzerland; it is one of the most popular phytoherapeutic drugs in Europe. The extract contains two main components, namely the hydrophobic Cernitin GBX1 fraction, both of which are devoid of allergens and other high molecular weight substances.

Clinical trials reported the efficacy of this drug in the treatment of benign prostatic hyperplasia (BPH) and chronic prostatitis with no reported side effects. Subsequently, it was demonstrated in vitro that the water soluble Cernitin T-60 fraction was the active component in the “Cernilton” mixture as this inhibited the growth of prostate cancer cells and primaty culture from BPH specimen. In addition, in vivo studies on rats showed significant reductions in the weight of the rat prostate following an intake of Cernilton over a period of 21 days. Significantly there was no change in any of the other organs of the animals tested.

More recently we have identified a number of constituent components in Cernitin T-60 of which only one fraction, designated V-7 (FV-7), was found to be biologically active with respect to its strong inhibitory effect on the growth of prostate cell lines; this prompted us to identify and characterize this active ingredient. We now report on the isolation, characterization, and biological evaluation of one natural component present in the pollen extract and believed to be responsible for the biological activity of this drug.

Results

Purification of Biologically Active Fractions from a Pollen Extract (Cernitin T-60). The aim of the present investigation was to single out one or more components present in a pollen extract (referred to as T-60 water soluble fraction) which could be responsible for the growth inhibition of the human prostate cell line DU-145. The first step of purification consisted of the dialysis of the spissum through a spectrapor dialysis membrane with a cutoff limit of 1 kDa. Using the in vitro DU-145 cell growth inhibition assay, the material inside the bag proved to be inactive (as tested in the range of 0.1-0.5 mg/mL) and was discarded. All of the inhibitory activity was recovered in the diffusate.

Diffusate (2g) was then loaded onto a Sephadex G-25 superfine column. Up to eight fractions could be resolved. They were separately lyophilized, and the total recovery was ≈ 90% of the starting material. Each fraction was assayed for the inhibitory activity: only one fraction (FV) was found to exhibit significant inhibitory activity toward DU-145 prostatic cancerous cell line at a concentration range of about 100μg/mL. Fraction V, which eluted at the same position as N-2-4-DNP-L-alanine (MW=255 Da) on the same column (i.e., 3.7- fold void volume), accounts for ≈10% of the components present in the diffusate, i.e., ≈ 3.6% (w/w) of the Cernitin T-60 fraction extract. Fractions IV and VI also exhibited some inhibitory activity, which

was likely to be due to the cross-contamination by fraction V.

Purification of the Active Fraction V. Pooled fraction V (≈50mg from five to six preparative G-25 runs) was then loaded onto a Sephadex G-10 column, equilibrated, and developed in distilled water. Fraction V yielded eight subfractions. The inhibitory activity was found to be associated with the seventh subfraction only (elution volume = 9.2-fold void volume), at a concentration as low as 15-20μg/mL. FV-7 accounts for ≈ 13% of the whole FV, which in turn corresponds to .3% (w/w) of the whole Cernitin T-60.

G-10 pooled fraction v-7 (500μg) was further loaded on a Lchro CART C18 reverse phase column (4.6 mm x 100 mm) on a Varian 5000 liquid chromatograph to isolate the active compound to homogeneity, i.e., in a form suitable for chemical characterization. The eluting system was .1% TFA, pH 2.0, with an acetonitrile, was separated from several minor contaminants which were estimated to account for no more than 10% of the total fraction V-7, as monitored at 254 nm. The in vitro biological test confirmed that the major HPLC peak had dramatic inhibitory effect on DU-145 cells at 10μg/mL.

Chemical characterization of the Natural Product Contained in Fraction V-7. Ultraviolet Spectrum (UV). The UV spectra of the HPLC-purified component V-7 at pH 7.0 exhibited a shoulder at 279 nm, an absorption maximum at 253 nm, and a minimum at 232 nm.

Mass Spectrometry (MS). The strongest signals were obtained at 200° C during evaporation of the bulk of the sample. The spectrum showed a strong signal at *m/e* 181, which was thought to be the most reasonable candidate molecular ion (data not shown). High-resolution, narrow scan data provided an assigned mass of 181.0375. The only composition found within the maximum estimated error (0.1 mmu) was C8 H7NO4, 181.0375 calcd (error 0.1 mmu).

Nuclear Magnetic resonance (NMR). The HNMR spectrum of the natural product V-7 recorded in DMSO-d 6 showed seven H resonances. Four of these, appearing as a multiplet at 7.0-7.3 ppm, immediately suggested the presence at 10.9 ppm was indicative of an acidic hydrogen atom. Two further resonances at 8.1 and 5.7 ppm, respectively, were found to couple with 5.5 Hz (vicinal coupling). Two hydrogen atoms of the molecule (at 10.9 and 8.1 ppm) underwent isotopic exchange with D2O.

The C-NMR spectrum showed eight resonances at 92.0, 112.9, 117.1, 122.5, 123.8, 128.7, 140.6, and 157.6 ppm. Together with the results from high-resolution MS, these spectra definitely confirm the molecular formula C8H7NO4 for the compound V-7. Two-dimensional heteronuclear shift correlation spectroscopy (H/C-COSY) revealed that only five of the eight C atoms are bound to hydrogen, i.e., three of the C atoms of the compound bear no H (those at 157.6, 140.6, and 128.7 ppm). The compound has neither a CH2 nor a CH3 group. Consequently, and in agreement with the isotope exchange experiment mentioned above, the two remaining H atoms must be bound to a heteroatom. The most reasonable fit between the structure and the NMR spectra was found for 2, 4-hydroxy-2H-1, 4-benzoxazin-3 (4H)-one (DIBOA).

DIBOA

DIBOA is a cyclic hydroxamic acid, FV (G-25) and FV-7 (G-10) were the only two fractions of all the Sephadex fractions which exhibited a positive blue complex formation, characteristic for hydroxamic aids, with the ferric chloride spray method. The ultimate proof that fraction V-7 was indeed identical to the known compound DIBOA resides in the independent chemical synthesis. This was carried out and then naturally occurring product V-7 was found indistinguishable by NMR, MS, UV spectra, and HPLC profile from an authentic synthetic sample of DIBOA.

Figure 1. Growth of the androgen insensitive DU-145 human prostate cancer cell line following treatment with different concentrations of either fraction V-7 or DIBOA. Cells at a density of 1.5x 103 cells/ well were incubated for periods up to 6 days, and the results ate expressed as percentage of thymidine incorporated relative to the untreated control. Each point is the mean of three separate experiments each run eight times. Bars represent coefficient of variation.

Comparison of the Biological activity of V-7 and synthetic DIBOA. At an initial cell density of 1.5x 103 DU-145 cells/ well, the inhibitory patterns for both the naturally occurring fraction V-7 and an authentic synthetic sample of DIBOA were tested at 1, 10, and 100μg/mL. The results outlined in Figure 1 demonstrate an identical inhibitory pattern for the two compounds, thus confirming tht there is no difference between DIBOA and V-7 in term s of their biological activities. At a concentration of 1μg/mL of either V-7 of DIBOA, no growth inhibition can be observed from day 1-6. However at a concentration of 10μg/mL of, the inhibitory effect at day 1 wa found to be in the region of 50%, but this increased to 80% at day 5. When concentrations of 100μg/mL inhibitory material were used, a complete shutdown of the proliferative effects was achieved from day 1 and this remained as such up to day 6. Similar patterns of inhibition were recorded when the initial cell density was increased to 2.5 x 103 cells/well (results not shown), suggesting

that the inhibitory effect is independent of cell concentration.

Discussion

We described here the isolation of a cell growth inhibitor, designated fraction. V-7 from a commerical pollen extract, Cernitin T-60. V-7 was shown to be inhibitory at a concentration as low as 5-10μg/mL when added to human prostatic cell line DU-145 in culture, and its content was evaluated to averafe 1% of the pollen extract. High-resolution mass spectrometry and nuclear magnetic resonance allowed us to characterize V-7 as the cyclic hydroxamic acid, 2-4-dihydroxy2H- 1, 4-benzoxazin-3(4H)-one (DIBOA). Furthermore an authentic synthetic sample of DIBOA was found structurally indistinguishable fronV-7. The in vitro comparative inhibition curves obtained with V-7 and DIBOOA were virtually superimposable (Figure 1).

DIBOA is the enzyme hydrolysis product of the glycoside derivative. It was recently shown that he parent glycosides of DIBOA and its methoxy derivatives occur in significant amount in some members of the Gramineae family of plants. Cernitin T-60 extract, which contains up to 95% pollen (w/w), appears to comprise at least one member of the DIBOA family. The cyclic hydroxamic acids have attracted much attention in agronomic research because of their role as phytotoxic agents, even though the glycoside derivatives appeared to be less potent.

Until now, there were no reports in the literature concerning the role of DIBOA and its dihydroxy, methoxy, and glucoside derivatives as potential agents in the treatment of human neoplastic diseases. To the best of our knowledge, this is the first report on the use of DIBOA to inhibit human prostate cancer cell growth in culture. In this context, evaluation and antitumoral activities of hydroxamic acids have been reported. It was suggested that a series of polyhydroxy-substituted benzohydroxamates act as inhibitors of ribonucleotide reductase activity. It remains to be seen whether the mechanism of action of DIBOA in the human prostate is similar. It is tempting to speculate that cell growth inhibition may result from the chelating or radical scavenger properties of DIBOA, which in turn may be the rate-limiting step of nucleotide biosynthesis; this is a possibility, which we are at present investigating.

Experimental Section

Cernitin T-60. Cernitin T-60 is a pollen extract preparation from AB Cernelle, Helsingborg, Sweden. The water-soluble T-60 fraction accounted for more than 90% of the pollen extract.

Purification of Active Natural Product(s) from T-60 Fraction. This was carried out by a combination of dialysis, gel filtration, and reverse phase HPLC steps, as outlined in ref.6. Dialysis of T_60 fraction was carried out against distilled waster using spectra/Por (cutoff, 1 kDa) porous membranes. Contents inside and outside the bag were lyophilized. Twenty milliliters of the concentrated diffusate at a concentration of 200mg/mL was loaded onto a Sephadex G-25 superfine column (2.6x140cm) developed with distilled water at a flow rate of about 15mL/min, and the effluent was monitored at 280nm. The pooled biologically active material (20-30mg) was further fractionated through a Sephadex G-10 column (2.0X100cm) using distilled water as eluent and monitoring at 280 nm. Biologically active subfraction from Sephadex G-10 chromatography was further purified to homogeneity by reverse phase HPLC on a semi-preparative Nucleosil C18RP column (1x25cm); the eluting system was .01% aqueous TFA at pH 2.0 and an acetonitrile gradient was from 0 to 40% at a flow rate of 0.7mL/min. Monitoring was done at 254nm; 500-700μg of the active sub-fraction from the G-10 step was repeatedly loaded, and the practical recovery was about 30-40%.

Physicochemical Characterization. Mass Spectrometry. The mass spectrum was acquired by direct insertion on a MS 50S instrument (Kratos Ltd., Manchester, England) under conditions of electron impact ionization (70 eV) with a source temperature of 200° C.

Nuclear Magnetic Resonance. The H-and C-NMR spectra of the active compound were recorded in deuterated dimethyl sulfoxide (DMSO-d6) on a Bruker AMX-400 spectrometer (9.4T) operating at 400 and 100 MHz, respectively. Assignments were ascertained by two-dimensional homonuclear and heteronuclear shift correlation spectroscopy.

Chemical Synthesis of the Active Compound. An authentic sample of the active compound was obtained by an independent synthesis in four steps following procedures properties of the synthetic and the natural compound isolated from the T-60 fraction was made by HPLC, UV, MS, and NMR.

Biological Assay. Cell Culture. To monitor and evaluate the inhibitory activity of each of the purified fractions from dialysis, gel filtration, and RP-HPLC steps, an assay employing the human prostate cancer cell line DU-145 was under-taken. Conditions for growth of these cells have been described previously as well as thymidine incorporation and statistical analysis of the data.

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