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Jonquailine, a new pretazettine-type alkaloid isolated from *Narcissus jonquilla quail*, with activity against drug-resistant cancer

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Abstract

A new alkaloid, belonging to the pretazettine group of Amaryllidaceae alkaloids, was isolated from dried bulbs of *Narcissus jonquilla quail* and named jonquailine. Its structure, including the absolute configuration, was elucidated using various NMR, ECD and ESI MS techniques. Initial biological evaluation revealed significant antiproliferative effects against glioblastoma, melanoma, uterine sarcoma and non-small-cell lung cancer cells displaying various forms of drug resistance, including resistance to apoptosis and multi-drug resistance. Jonquailine was also found to synergize with paclitaxel in its antiproliferative action against drug-resistant lung cancer cells. The results obtained compared with literature data also showed that the hydroxylation at C-8 is an important feature for the anticancer activity but this seems unaffected by the stereochemistry or the acetalization of the lactol.

Keywords

Amaryllidaceae; *Narcissus jonquilla quail*; Alkaloids; Jonquailine; Cancer cells; Antiproliferative effects

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Conflict of interest

The authors declare no conflict of interest.

1. Introduction

Drug resistance is one of the main causes for the failure of chemotherapy in the clinic. Many cancers harbor intrinsic resistance to chemotherapeutic agents, normally stemming from their incompetence in initiating apoptosis, which is a common mode of action of the majority of cancer drugs [1–4]. Such cancers include the tumors of the lung, liver, stomach, esophagus and pancreas, as well as melanomas and gliomas, all of which are associated with dismal prognoses [5]. Resistance to apoptosis, and thus to conventional chemotherapy, is also an intrinsic property of tumor metastases, which remains an important clinical challenge as 90% of cancer patients die from metastastic cancer [6,7]. An important solution to chemotherapy resistance entails the complementation of cytotoxic therapeutic regimens with cytostatic agents [8–11], which can often sensitize cancer cells to apoptosis and thus display synergistic effects, making the search for such agents an important pursuit [12–15].

Often, tumors innately respond to chemotherapy but eventually become refractory to the continuing treatment. Such resistance is referred to as “acquired” and it usually involves the development of a multi-drug resistant phenotype (MDR) affecting a broad spectrum of structurally and mechanistically diverse antitumor agents [16,17]. The phenomenon of MDR has plagued conventional chemotherapies, for example the widely applied vinca alkaloids [18] and taxanes [19]. Thus, the search for agents capable of circumventing MDR mechanisms is another important area of research aiming to combat drug-resistant cancers [20].

Isolation and evaluation of new natural products for anticancer activity has been a major method for the discovery of anticancer drugs. According to the analysis of Newman and Cragg, 85 of the 175 small molecule anticancer drugs discovered since the 1940s are actually natural products or their direct derivatives [21]. Among various natural sources that have been investigated in the search of small-molecule constituents with anticancer activity, plants of the Amaryllidaceae family have been particularly useful [22,23]. These ornamental plants with high commercial value because of their beautiful flowers have been used in folk medicine since ancient times. Indeed, the therapeutic effects of these plants were already known to the Greek physician Hippocrates in the fourth century B.C., who used the oil from the daffodil, *Narcissus poeticus* L., for the treatment of uterine tumors [23]. In more recent times, hundreds of structurally diverse alkaloids, possessing a wide spectrum of biological activities have been isolated from the Amaryllidaceae species, including many with promising anti-cancer activities [22–27]. Some of the most promising Amaryllidaceae constituents include the alkaloids such as lycorine (**1**), haemanthamine (**2**) and the non-basic metabolite narciclasine (**3**) (Fig. 1). These have attracted considerable attention, most prominently because of their activity against drug-resistant cancers with dismal prognoses, which is most likely due to their cytostatic non-apoptotic antiproliferative mechanisms [8–11,28]. Among other possible effects contributing to cytostaticity, the inhibition of eukaryotic protein synthesis by these natural products apparently plays a major role in their anticancer action [23,29,30].

Another promising Amaryllidaceae alkaloid that has received significant attention, particularly from the synthetic community due to its interesting structure [31], is pretazettine

(**5**) [32]. Compared with its congener tazettine (**4**) that is devoid of anticancer activity [8], pretazettine has shown promising anticancer effects and has been studied in numerous models of murine cancer [33–36]. Particularly noteworthy is a dated report of its activity against Lewis lung carcinoma in mice, known to be resistant to standard chemotherapy [36]. Specifically, this alkaloid was found to be effective against this tumor by synergizing with adriamycin, 5-fluorouracil, methotrexate and vincristine; this effect most likely stems from its ability to inhibit eukaryotic protein synthesis [29]. An important impediment that has apparently halted its further studies in models of human cancer is the lack of stability associated with the rearrangement of 8-OH in pretazettine to the 6a position in tazettine upon standing in free form and in an aqueous solution [32].

As part of a research program aimed at the anticancer development of the Amaryllidaceae metabolites (**1**, **2** and **3**) and identification of novel alkaloids with potential activities against drug-resistant cancers, our team reinvestigated the crude organic extract of bulbs of *Narcissus jonquilla quail* collected in Middlesex county of southeast England. This narcissus cultivar with fragrant flowers native to Spain and Portugal has now become naturalized in many regions of Europe and the United States. Although a GC analysis carried out by Gotti and coworkers [37] identified galanthamine and haemanthamine as the main alkaloid constituents of *N. jonquilla quail*, our analysis showed abundant amounts of haemanthamine, lycorine and narciclasine. When these main anticancer metabolites were separated, the residual fractions still preserved strong anticancer activity. The purification of these fractions yielded a new pretazettine-type alkaloid that was named jonquailine (**6**) (Fig. 1). The current paper reports the isolation and structural determination of jonquailine as well as its activity against drug-resistant cancer cells.

2. Experimental

2.1. General

ECD spectra were recorded on a JASCO J-815 spectrometer in MeOH; IR spectra were recorded as deposit glass film on a Thermo Electron Corporation Nicolet 5700 FT-IR spectrometer and UV spectra were measured in MeOH on a Jasco V-530 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 600/125 MHz in $CDCl_3$ on Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra [38]. DEPT, COSY-45, HSQC, HMBC, and NOESY experiments [38] were performed using Bruker microprograms. HRESI and ESI, and APCIMS spectra were recorded on Agilent Technologies 1100 LC/MSD TOF and Agilent Technologies 6120 Quadrupole LC/MS instruments, respectively. Analytical and preparative TLC were performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively, Merck, Darmstadt, Germany) plates. The spots were visualized by exposure to UV radiation (253), or iodine vapor. Column chromatography (CC) was performed using silica gel (Kieselgel 60, 0.063–0.200 mm, Merck, Darmstadt, Germany).

2.2. Plant material

Bulbs of *N. jonquilla quail* were purchased from the Jacques Amand International, Ltd nurseries in Middlesex, England.

2.3. Extraction and purification of jonquailine

Fresh bulbs (8.5 kg) of *N. jonquilla quail* were dried at 40 °C for 48 h and then finely minced. The resultant powder (3 kg) was extracted with a Soxhlet using first petroleum ether (1 × 2 L, 24 h) and then MeOH (1 × 2 L, 72 h). The MeOH extract (80.2 g), obtained as a brown-red oil, was further fractionated by column chromatography on silica gel eluted with EtOAc/MeOH/H_2O (85:10:5 v/v/v). The fractions were monitored by TLC using the same solvent and nine homogeneous fraction groups were collected. The residue (3.5 g) of the third fraction group was further purified by column chromatography on silica gel eluted with CH_2Cl_2/MeOH (9:1 v/v) yielding eight groups of homogeneous fractions. The residue of the third fraction (103 mg) was further purified by two TLC steps on silica gel eluted with EtOAc/MeOH/H_2O (97:2:1 v/v/v) followed by $CHCl_3$/*i*-PrOH (95:5 v/v) yielding jonquailine (**6**) (5.1 mg) as a homogeneous amorphous solid.

Jonquailine (**6**): amorphous solid; R_f 0.44, $CHCl_3$/*i*-PrOH (95:5 v/v); UVλ_{max} (log ε) 287 (4.00), 240 (4.09) nm; CD ([θ] $_\lambda$): [θ] $_{242}$ + 18,588, [θ] $_{291}$ + 5163 (6-*O*-methylpretazettine [39] CD [θ] $_{244}$ − 4398, [θ] $_{299}$ + 4524; tazettine [40] CD MeOH ([θ] $_\lambda$):[θ] $_{207.2}$ + 194,500, [θ] $_{240.4}$ + 32,600, [θ] $_{289.2}$ − 3090; pretazettine [40] CD MeOH ([θ] $_\lambda$): [θ] $_{217.6}$ + 51,600, [θ] $_{233.7}$ 0, [θ] $_{249.6}$ − 9830, [θ] $_{263.9}$ 0, [θ] $_{291.0}$ + 13,130); IR ν_{max} 1616, 1500, 1493, 1497 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; (+)-APCI MS *m*/*z* 346 $[M + H]^+$, 314 $[M - CH_3OH]^+$; (+)-HRESIMS *m*/*z* 713 $[2 M + Na]^+$, 363 $[M + NH_4]^+$ and 346.1664 $[M + H]^+$ (calcd for $C_{19}H_{24}NO_5$ 346.1654).

2.4. Ethanol extraction of N. jonquilla quail bulbs and alkaloid GC/MS analysis

The powder (1.0 g) of the same specimen of *N. jonquilla quail* was extracted with a Soxhlet using first petroleum ether then with EtOH in the same conditions reported above. The EtOH extract obtained as a brown-red oil was dissolved in 2% H_2SO_4 (20 mL) and extracted with Et_2O (3 × 20 mL). The acidic aqueous phase was basified with 25% of NH_4OH to pH 9–10 and extracted with $CHCl_3$ (3 × 20 mL). The combined chloroform extracts were dried (Na_2SO_4), filtered and evaporated under reduced pressure. For GC/MS analysis, the alkaloid extracts were dissolved in MeOH (5 mg of extract in 250 ml MeOH). The GC/MS analyses were performed on Agilent Technology instrument 5975B operating in electron impact mode (EI, 70 eV). The temperature condition worked with the following program: 100–180 °C at 15 °C × min^{-1}, 180–300 °C at 5 °C × min^{-1} and 10 min hold at 300 °C. The injector was 250 °C. The flow rate of the carrier gas (helium) was 0.8 mL × min^{-1}. An HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The split ratio was 1:20. The alkaloids and narciclasine were identified by applying co-chromatography with standards previously isolated from *N. jonquilla quail* bulbs and other Amaryllidaceae plants, by comparing the mass spectral fragmentation with standard reference spectral data from NIST (NIST Mass Spectral Database, PC-Version 5.0 (2005), National Institute of Standardization and Technology, Gaithersburg, MD, USA), and by comparing mass spectral data with those of literature [41,42].

2.5. Cell lines

Human cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), the European Collection of Cell Culture (ECACC, Salisbury, UK) and

the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The human glioblastoma U87 cells (ATCC HTB-14) were cultured in DMEM culture medium. Human melanoma SKMEL-28 cells (ATCC HTB72), U373 glioblastoma cells (ECACC 08061901) and A549 NSCLC cells (DSMZ ACC107) were cultured in RPMI culture medium (Lonza; Braine-l'Alleud; Belgium) supplemented with 10% heat-inactivated FBS (Lonza). Anaplastic oligodendroglioma Hs683 cells (ATCC HTB-138) were cultivated in DMEM culture medium supplemented with 10% FBS (Lonza). NSCLC cell lines H1993 and H2073 were obtained from the Hamon Center for Therapeutic Oncology Research at UT Southwestern Medical Center and cultured in RPMI-1640 medium (Mediatech, VA, USA) supplemented with 5% heat-inactivated FBS (Mediatech, VA, USA), 100 units/mL penicillin and 0.1 mg/mL streptomycin (Sigma, MO, USA). Human uterine sarcoma MES-SA (ATCC CRL-1976) and MES-SA/Dx5 (ATCC CRL-1977) cells were cultured in RPMI-1640 medium supplemented with 10% FBS with MES SA/Dx5 maintained in the presence of 500 nM doxorubicin (Sigma). All cell lines were cultured in T25 flasks, maintained and grown at 37 °C, 95% humidity and 5% CO_2.

2.6. Antiproliferative properties

Antiproliferative properties were evaluated by MTT assay. Jonqualine was dissolved in DMSO at a concentration of either 100 mM or 50 mM prior to cell treatment. The cells were trypsinized and seeded at 4×10^3 cells per well into 96-well plates. The cells were grown for 24 h, treated with compounds at concentrations ranging from 0.001 to 100 μM and incubated for 48 h in 200 μL media. 20 μL of MTT reagent in serum free medium (5 mg/mL) was added to each well and incubated further for 2 h. Media was removed and the resulting formazan crystals were resolubilized in 200 μL of DMSO. A_{490} was measured using a Molecular Devices Thermomax plate reader. The experiments were performed in quadruplicate and repeated at least twice per cell line. Cells treated with 0.1% DMSO were used as a negative control; 1 μM phenyl arsine oxide (PAO) was used as a positive control.

2.7. Evaluation of jonquailine's synergism with paclitaxel

Paclitaxel (Teva, PA, USA) was dissolved in polyethoxylated castor oil. Jonquailine was dissolved in DMSO. Both paclitaxel and jonquailine were diluted to different concentrations in medium. Appropriate numbers of cells (4×10^3 cells per well for H1993, 7×10^3 cells per well for H2073) were seeded into 96-well plates. The cells were grown for 24 h and treated with paclitaxel and jonquailine, either alone or in combination. After 96 h, the viability of cells was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, WI, USA), according to the manufacturer's instructions. Predicted additivity was calculated based on Bliss Independence, as defined by $E_{xy} = E_x + E_y - E_xE_y$, where E_{xy} is the additive effect of drugs x and y as predicted by their individual effects E_x and E_y [43].

2.8. Selection of doxorubicin resistant cells

Selection of the MES-SA/Dx5 cell line was done according to Harker et al. [44]. The cells were split and allowed to adhere overnight. The next day cells were initially exposed to a DOX concentration of 100 nM, which represented the GI_{50} concentration. The cells were maintained at this DOX concentration until their growth rate reached that of the untreated cells. The DOX concentration was then increased in two-fold increments following the same

growth criteria at each concentration to a final DOX concentration of 500 nM. Each new DOX concentration required approximately 2 passages to reach the growth rate of the untreated cells.

3. Results and discussion

Jonquailine (**6**) had a molecular formula of $C_{19}H_{23}NO_5$, as was deduced from its HRESIMS spectrum consistent with a hydrogen deficiency index of nine. A preliminary investigation of its ^{1}H and ^{13}C NMR spectra indicated that this alkaloid belonged to the pretazettine subgroup of the Amaryllidaceae alkaloids. Specifically, ^{1}H NMR spectrum (Table 1) contained two singlets at δ_H 6.78 (H-9) and 6.76 (H-12), typical of a tetrasubstituted benzene ring A present in several Amaryllidaceae alkaloids as well as two doublets (J = 1.3 Hz) at δ 5.91 and 5.90, characteristic of the methylene group in the joined 1,3-dioxole ring [45]. Furthermore, a doublet of triplets (J = 10.9 and 1.6 Hz) and a broad doublet (J = 10.9 Hz) at δ_H 5.54 and 5.87, typical of the protons (H-1 and H-2) of a *cis* double bond, likely located in the C ring, as well as a broad doublet of doublet of doublets (J = 9.5, 6.8 and 1.6 Hz) at δ_H 4.16 corresponding to the H-3 proton of the adjacent secondary hydroxylated carbon, were observed and the couplings confirmed with a COSY spectrum [38]. Three singlets corresponding to two methoxy groups at C-8 and C-3 and an *N*-methyl group resonated at δ_H 3.55, 3.45 and 2.49 [46]. The COSY spectrum also showed that H-3 is the X part of an ABMX system, in which the AB are the two protons of the adjacent methylene group at C-4 resonating as a multiplet and a doublet of doublet of doublets (J = 11.9, 9.4 and 2.0 Hz) at δ_H 2.50 and 1.76, while the M part is the proton (H-4a) at the junction point between the C and D rings appearing as a multiplet at δ 2.93 [46]. The ^{1}H NMR spectrum also contained a singlet of H-8 of the methylated hemiacetal at δ_H 5.59 and signals of another ABX system, in which the X part, resonating as a doublet of doublets (J = 11.1 and 7.6 Hz) at δ_H 4.27, is the proton (H-6a) at the junction between the B and D rings. The AB part appeared as two doublet of doublets (J = 11.1 and 9.3, and 9.3 and 7.6 Hz) at δ_H 3.00 and 2.66, which were attributed to the two protons of the adjacent methylene group at C-6, was also confirmed from the couplings observed in the COSY spectrum [38]. The couplings observed in the HSQC spectrum [38] allowed the assignments of the signals of the methine groups at δ_C 129.8, 129.3, 108.1, 105.3, 100.5, 73.4, 72.8 and 63.9 to C-1, C-2, C-9, C-12, C-8, C-6a, C-3, C-4a, correspondingly. Similarly all methylene groups at δ_C 101.1, 54.0, and 29.7 were assigned to O–CH_2–O, C-6, and C-4, whereas the methyl carbons at δ_C 55.7, 56.2, and 43.1 were attributed to OMe–C-8, OMe–C-3 and N–Me in the ^{13}C NMR spectrum [47]. The four quaternary aromatic and the spiro carbons at the junction between B, C and D rings resonating at δ_C 147.7, 146.4, 135.3, 126.8, and 46.1, were assigned to C-11, C-10, C-8a, C-12a, and C-12b on the basis of the couplings observed in the HMBC spectrum [38] (Table 1) between C-11/H-12, C-10/H-9, C-8a/H-9 and C8a/H-8, C-12a/H-12 and C-12a/H-8, and C-12b/H-2.

On the basis of these data the chemical shifts of all protons and carbons were assigned as shown in Table 1 and jonquailine was determined to be 3,8-dimethoxy-5-methyl-3,4,4a, 5,6,6a-hexahydro-8*H*[1,3]dioxole[4′,5′:6,7]isochromeno[3,4-*c*]indole.

The structure assigned to **6** was further supported by the similarity of the ^{1}H and ^{13}C NMR data with those of pretazettine (**5**) (Fig. 1) obtained under the same conditions and differing only due to the presence of the methyl ether at C-8 in **6** [48]. The final piece of evidence came from the data of its HRESIMS and APCI spectra. The HRESIMS showed the dimeric sodiated form $[2\ M + Na]^+$, the ammonium cluster $[M + NH_4]^+$ and the pseudomolecular ion $[M + H]^+$ at *m*/*z* 713, 363 and 346.1664, respectively. The APCI MS contained a significant ion generated by the loss of methanol $[M–CH_3OH]^+$ at *m*/*z* 314, as well as, the pseudomolecular ion $[M + H]^+$ at *m*/*z* 346.

The relative stereochemistry at the junction between rings B and D, and C and D, as well as those of the chiral carbons of ring C were deduced from the couplings observed in the ^{1}H NMR spectrum (Table 1) and were confirmed by the correlations recorded in the NOESY spectrum [38]. In fact, considering that the C ring assumes a half-chair conformation, the values of 9.5 and 6.8 Hz recorded for the couplings between H-3 with H-4B and H-4A allowed us to assign an α- and β-pseudoaxial orientation of H-3 and H-4B and α-pseudoequatorial positioning of H-4A. Consequently, the value of 2.0 Hz recorded for the coupling between H-4B and H-4a allowed the β-pseudoequatorial orientation of H-4a. These assignments were confirmed by the correlation observed in the NOESY spectrum (Table 2) of H-3 with H-4A and H-4B with H-4a. The same spectrum contained a significant correlation of H-4a with H-6a, which allowed the positioning of the latter to the β-face of the molecule. The coupling values of H-6a with H-6A and H-6B (11.1 and 7.6 Hz) allowed the positioning of H-6A and H-6B to the α- and β-sides of the molecule, respectively. In fact, a correlation was observed in the NOESY spectrum between H-6a and H-6B. Therefore, the relative stereochemistry of the junction between the B and D rings should be *trans*, similar to pretazettine (**5**) compared to the *cis*-junction in tazettine (**4**) [40]. The relative stereochemistry assigned to **6** was in full agreement with an inspection of its Dreiding model.

The absolute configuration of **6** was investigated by comparing its ECD spectrum with that of **4** (Fig. 2) as well as that reported in the literature for **5** [40] both recorded under the same conditions. The dominant chromophore in all three alkaloids is the methylenedioxybenzene moiety. The CD spectrum was characterized by two significant bands centered at approximately at 291 and 242 nm, typical for the alkaloids of the [2]benzopyrano[3,4-*c*]indole subgroup. Alkaloid **4**, which belongs to this subgroup with the *cis* B/D ring junction, exhibited the −/+ order (*ca.* 290 nm/240 nm) of the corresponding Cotton effect related to the methylenedioxybenzene chromophore, while alkaloid **5**, its 6a-epimer, gave the opposite order (+/− at *ca.* 290 nm/240 nm). Jonquailine had the +/+ order (*ca.* 290 nm/240 nm). These data are consistent with jonquailine's absolute stereochemistry being similar to that of **5**, with the difference in the ECD spectra possibly arising due to the different configuration at C-8, which remains to be determined for both alkaloids.

Jonquailine is closely related to 6-*O*-methylpretazettine isolated by Bastida and coworkers from *Eucharis amazonica* [39]. The configuration at C-3, C-4a and C-11 (our C-6a) of 6-*O*-methylpretazettine was assigned using ROESY correlations and coupling constants measured in the ^{1}H NMR spectrum. Furthermore, the β configuration at C-6 (our C-8) was assigned on the basis of the fragmentation recorded in its mass spectrum, although no

explanation for this unusual method for the assignment of stereochemistry was provided [39]. However, jonquailine, which has the same configuration as 6-*O*-methylpretazettine at C-3, C-4a and C-11 (our C-6a), as assigned using NOESY (a technique which gave similar results to the ROESY used by Bastida) correlations and coupling constants measured in the ^{1}H NMR spectrum, appears to be epimeric to the above mentioned natural product at C-8 (C-6 in 6-*O*-methylpretazettine). Key evidence for this conclusion comes from its CD spectrum (Fig. 2), which exhibited a +/+ order (*ca.* 290 and 240 nm), compared to a +/−order (291 and 242 nm) reported by Bastida and coworkers [39].

The C-8 epimeric purity of jonquailine also rules out its generation as an artifact from pretazettine by reaction with methanol used in the extraction or during the chromatographic purification of the crude plant extracts. This transformation was achieved by Wildman and Bailey [49], who treated pretazettine with methanol under acidic conditions and obtained an epimeric mixture at C-8. Indeed, besides generation of an epimeric mixture, such a transformation necessitates acid catalysis in contrast to the slightly basic alkaloid-rich conditions utilized in our isolation procedure. Furthermore, if in any case this reaction should occur, neither the precursor pretazettine nor the epimeric 6-*O*-methylpretazettine, which should be in equilibrium with jonquailine working in anhydrous condition, was found in our extract. Pretazettine and jonquailine were also not found by Bastida and co-workers [39] in the extract of *E. amazonica*. In addition it has been previously shown that similar isolation conditions resulted in a complete conversion of pretazettine to tazettine and the current consensus view of the natural product community is that tazettine is indeed an isolation artifact of pretazettine [50]. A final proof of the natural origin of jonquailine was found by extracting the bulbs of *N. jonquilla quail* described above but using ethanol and analyzing the crude extract by GC/MS as detailed in the experimental for alkaloids and corresponding isocarbostyril content. Lycorine, haemanthamine and narciclasine, the main components, as well as the new alkaloid jonquailine (t_R = 24.59 min) were identified also by co-injection with their standards and by comparing the mass spectral fragmentation with standard reference spectra reported in literature [41,42]. Minor components were identified as deoxytazettine, epi-macronine, trisphaeridine and tazettine [41,42]. In addition no peak corresponding to the 8-*O*-ethylpretazettine was observed and thus the acetalization of the lactol at C-8 did not occur during the extraction process. This result was in agreement with the isolation of the 6-*O*-methylpretazettine and not its corresponding ethyl acetal when *Eucharis amazonica* as cited above was extracted with ethanol [39].

As a consequence of its anticancer activity-guided isolation, jonquailine showed anticancer effects in cell cultures *in vitro*. The pH of the media used to carry out these biological assays (*e.g.* MEM) is neutral. So that, the possible hydrolysis of **6** into pretazettine, which occurs in acid conditions as previously reported for the epimeric mixture of 6-*O*-methylpretazettine [49], was ruled out. Due to its structural similarity with the protein biosynthesis inhibitor pretazettine (**5**), which exhibited promising activity against drug resistant murine cancer as discussed above, jonquailine was evaluated in a human cancer cell line panel containing models for drug resistant cancers with different mechanisms of resistance (Table 3). The panel contained human U87 [51,52] and U373 [51] glioblastoma, human A549 non-small cell lung cancer (NSCLC) [53] and human SKMEL-28 melanoma [54], all exhibiting

various degrees and mechanisms of apoptosis resistance, as well as an apoptosis-sensitive tumor model, Hs683 anaplastic oligodendroglioma [51]. Likely reflecting the different mechanisms of resistance, the potency of jonquailine against these cells ranged from GI_{50} = 1 μM to 51 μM, with apoptosis-sensitive Hs683 cells being the least responsive.

Our previous experience working with cells resistant to various proapoptotic stimuli shows that although a sensitive population of cells is rapidly eliminated with proapoptotic agents, generally a significant portion of the cells in the culture resist the effects of the proapoptotic agents, even at concentrations 100- or 1000-fold of their GI_{50} values [55]. Fig. 3A shows that there are no significant resistant populations remaining after the treatment of U373, A549 and SKMEL-28 cultures with jonquailine. Indeed, although jonquailine was less potent at eradicating the sensitive population in the U87 cell culture than such powerful proapoptotic agents as paclitaxel or podophyllotoxin, it affected a significantly larger proportion of cells overall (Fig. 3B).

Furthermore, jonquailine was evaluated against two lung cancer cell lines H1993 and H2073. These two cell lines were derived from the same patient, who underwent chemotherapy, but relapsed with tumor regrowth at the primary site. H1993 was derived from a lymph node metastasis isolated prior to chemotherapy, while H2073 was derived from the lung tumor regrowth months after chemotherapy [56]. The H2073 cell line is significantly more drug-resistant as can be seen in the difference of several orders of magnitude in the GI_{50} values for paclitaxel (Table 3). Although jonquailine's potency also decreases against the H2073 cells, the drop is significantly less pronounced than what was observed with paclitaxel (Table 3). Most encouragingly however, it was found that jonquailine synergizes with paclitaxel in its antiproliferative action against both of these cell lines (Fig. 3C and D), indicating a potential use of jonquailine as an adjuvant to conventional chemotherapy for drug-resistant cancers.

In a final experiment to assess jonquailine's potential efficacies against drug-resistant cancer, it was evaluated against MDR cells. The MDR uterine sarcoma cell line MES-SA/Dx5 was established by growing the parent uterine sarcoma MES-SA in the presence of increasing concentrations of doxorubicin and is known to be resistant to multiple functionally and structurally unrelated molecules [53]. Indeed, paclitaxel (Table 3) and vinblastine (not shown) [20] displayed more than a thousand fold drop in potency when tested for antiproliferative activity against the MDR cell line as compared with the parent line. In contrast, there was little variation in the sensitivities of the two cell lines toward jonquailine (Table 3).

The antitumor activity clearly depends on the functionalities of the carbon skeleton of the pretazettine Amaryllidaceae alkaloid subgroup. In fact, the closest tazettine did not show antitumor activity but pretazettine, its hydroxy derivative at C-8, showed this activity, while for 6-*O*-methylpretazettine, that is the 8-epimer of jonquailine, any biological activity is reported. So that although the stereochemistry at C-8 of pretazettine as well as that of jonquailine, which seems to be a natural epimer at C-8, is not yet determined, the hydroxylation at C-8 appeared an important feature for the anticancer activity. Furthermore,

the stereochemistry of the lactol group as well as its acetalization did not affect the anticancer activity.

In conclusion, a new pretazettine-type alkaloid named jonquailine was isolated from bulbs of *N. jonquilla quail* containing a replacement of the hemiacetal functionality present in pretazettine with an acetal group. Acetal functionality makes jonquailine stable, which is important in view of its discovered anticancer potential. The results obtained compared with literature data also showed that the hydroxylation at C-8 is an important feature for the anticancer activity but this seems unaffected by the stereochemistry or the acetalization of the lactol. Jonquailine was demonstrated to be effective against drug-resistant human tumor models with diverse mechanisms of resistance including various forms of apoptosis resistance and MDR. It also displayed synergy with paclitaxel, indicating its potential use as an adjuvant to conventional chemotherapy for drug-resistant cancers. Because pretazettine was previously shown to be a translation inhibitor, this mode of action could also be postulated for jonquailine. It is noteworthy that translation inhibitor omacetoxine was recently approved for the treatment of tyrosine kinase inhibitor-resistant chronic myelogenous leukemia and is the first agent with this mode of action in the clinic [14]. However, the mechanisms underlying jonquailine's effectiveness against drug-resistant tumor cells are yet to be elucidated and efforts toward this goal are underway in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2015.01.009.

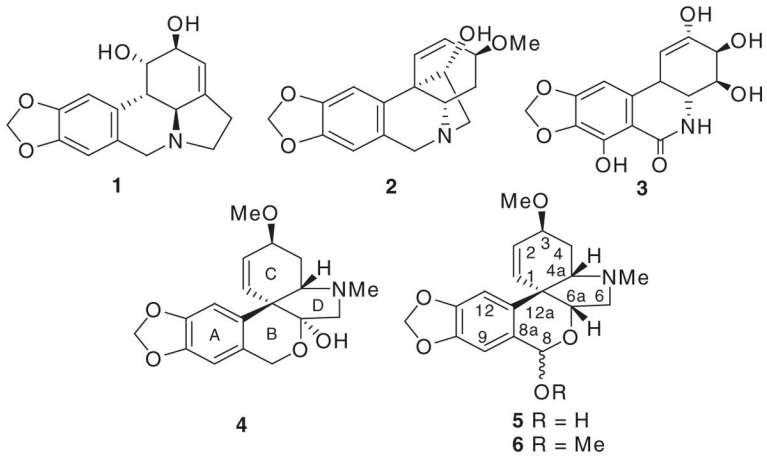

Fig. 1.
Structures of Amaryllidaceae anticancer constituents.

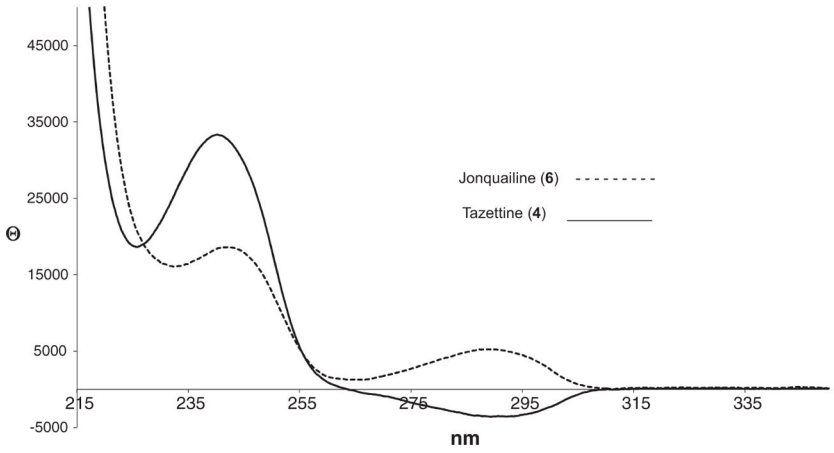

Fig. 2.

ECD spectrum of jonquailine (**6**) compared to that of tazettine (**4**) recorded in MeOH.

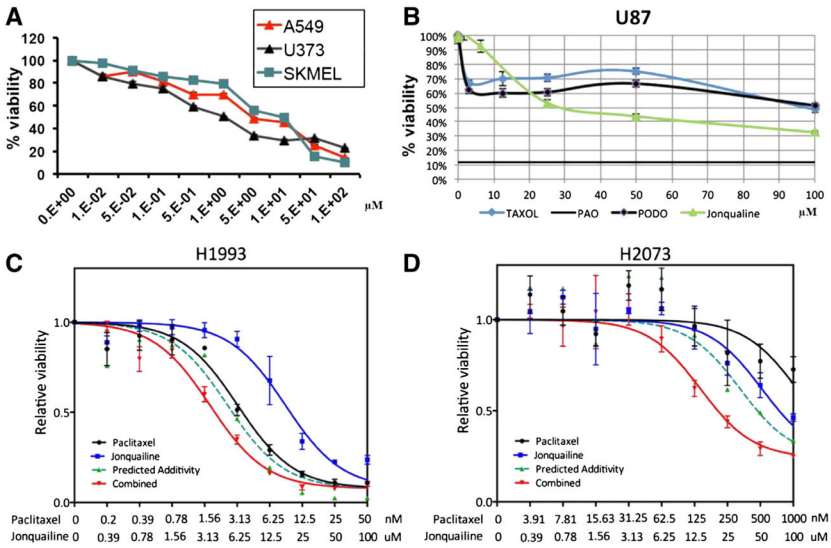

Fig. 3.

A. Growth inhibition curves illustrating the lack of resistant populations in U373, A549 and SKMEL-28 cultures treated with jonquailine. B. Growth inhibition curves of U87 cells illustrating that jonquailine affected a significantly larger proportion of cells overall than paclitaxel (Taxol) and podophyllotoxin (PODO). Phenyl arsine oxide (PAO), a non-discriminate general cytotoxic agent, was used as a positive control. C and D. Assessment of a combination treatment of paclitaxel with jonquailine illustrating their synergism. H1993 is an NSCLC cell line and H2073 is its drug-resistant counterpart.

Table 1

^{1}H, ^{13}C NMR and HMBC data of jonquailine (**6**)[a,b].

No.	δ_C[c]	δ_H	HMBC
1	129.8 d	5.54 dt (10.9, 1.6)	H-4a, H-6a
2	129.3 d	5.87 br d (10.9)	
3	72.8 d	4.16 br ddd (9.5, 6.8, 1.6)	H-4B, OMe
4	29.7 t	2.50 m	H-2, H-3
		1.76 ddd (11.9, 9.5, 2.0)	
4a	63.9 d	2.93 m	H-4A, H-6B, *N*-Me
6	54.0 t	3.00 dd (11.1, 9.3)	*N*-Me
		2.66 dd (9.3, 7.6)	
6a	73.4 d	4.27 dd (11.1, 7.6)	H_2-6
8	100.5 d	5.59 s	H-6a, H-9, 8-OMe
8a	135.3 s		H-6a, H-8, H-9
9	108.1 d	6.78 s	H-8
10	146.4 s		O–CH_2–O, H-8, H-9
11	147.7 s		H-12
12	105.3 d	6.76 s	H-9
12a	126.8 s		H-8, H-12
12b	46.1 s		H-2, H-12
O–CH_2–O	101.1 t	5.91 d (1.3 Hz)	
		5.90 d (1.3 Hz)	
3-OMe	56.2 q	3.45 s	H-3
8-OMe	55.7 q	3.55 s	H-8
N–Me	43.1 q	2.49 s	

[a] 2D ^{1}H, ^{1}H (COSY) and ^{13}C, ^{1}H (HSQC) NMR experiments confirmed the correlations of all the protons and the corresponding carbons.

[b] Coupling constants (J) are given in parenthesis.

[c] Multiplicities were assigned with DEPT.

Table 2

NOESY data of jonquailine (**6**).

Irradiated	Observed
H-2	3-OMe
H-3	H-4A
H-4A	H-3, H-4B
H-4B	H-4a
H-4a	H-6a
H-6A	H-6B
H-6a	H-6B

Table 3

Antiproliferative properties of jonquailine (**6**) against cancer cell lines possessing various types of drug resistance and representing cancers with dismal prognoses.

	GI_{50} *in vitro* values (μM)[a]								
	Glioma			Melanoma	Lung carcinoma			Uterine sarcoma	
	U87	**U373**	**Hs683**	**SKMEL-28**	**A549**	**H1993**	**H2073**	**MES-SA**	**MES-SA/Dx5**
6	28	1	51	11	5	17	85	25	29
Paclitaxel	96	nd	nd	nd	nd	0.003	N1	0.007	10

[a]Average concentration required to reduce the viability of cells by 50% after a 48–72 h treatment relative to a DMSO control from at least two independent experiments, each performed in multiple replicates, as determined by MTT assay.