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ORIGINAL ARTICLE

Abrogation of ionizing radiation-induced G_2 checkpoint and inhibition of nuclear export by *Cryptocarya* pyrones

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Abstract G_2 checkpoint inhibitors can force cells arrested in G_2 phase by DNA damage to enter mitosis. In this manner, several G_2 checkpoint inhibitors can enhance killing of cancer cells by ionizing radiation and DNA-damaging chemotherapeutic agents, particularly in cells lacking p53 function. All G_2 checkpoint inhibitors identified to date target protein phosphorylation by inhibiting checkpoint kinases or phosphatases. Using a phenotypic cell-based assay for G_2 checkpoint inhibitors, we have screened a large collection of plant extracts and identified Z-Cryptofolione and Cryptomoscatone D2 as highly efficacious inhibitors of the G_2 checkpoint. These compounds and related pyrones also inhibit nuclear export. Leptomycin B, a potent inhibitor of Crm1-mediated nuclear export, is also a very potent G_2 checkpoint inhibitor. These compounds possess a reactive Michael acceptor site and do not appear promising as a radiosensitizing agents because they are toxic to unirradiated cells at checkpoint inhibitory concentrations. Nevertheless, the results show that inhibition of nuclear export is an alternative to checkpoint kinase inhibition for abrogating the G_2 checkpoint and they should stimulate the search for less toxic nuclear export inhibitors.

Keywords DNA damage · Checkpoint inhibitor · Z-Cryptofolione · Leptomycin B · Nuclear export

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Introduction

DNA damage caused by ionizing radiation activates signal transduction pathways called checkpoints that temporarily halt cell cycle progression and are thought to promote cell survival by allowing more time for DNA repair. Two checkpoints have been identified that block cell cycle progression at the G_1-S and at G_2-M transitions, and the intra-S checkpoint that slows down S-phase traversal (reviewed in [1]). The G_1 checkpoint is completely dependent on p53 activation. In the majority of cancer cells, the p53 pathway is inactivated, leaving the G_2 checkpoint as the major remaining checkpoint. Therefore, the resistance of tumor cells to radiotherapy and DNA-damaging chemotherapeutic agents is likely to depend significantly on the integrity of the G_2 checkpoint.

The G_2 checkpoint links DNA damage sensors to the mitosis-inducing kinase CDK1 via a protein kinase signal transduction cascade. According to current, albeit incomplete models, DNA damage activates the PIKK family members ATM and ATR that phosphorylate a variety of downstream targets, including the effector kinases Chk1 and Chk2. Chk1 and Chk2 phosphorylate and inactivate CDC25C, preventing it from dephosphorylating and activating CDK1 (reviewed in [1]). Inhibition of this pathway has been proposed as a strategy to selectively enhance the sensitivity of tumor cells to DNA-damaging therapies (reviewed in [2]).

A number of chemical inhibitors of the G_2 checkpoint have been discovered in recent years. Most target protein kinases in the signalling cascade; caffeine inhibits ATM, UCN-01, isogranulatimide and others inhibit Chk1, and debromohymenialdesine and XL844 inhibit both Chk1 and Chk2 (reviewed in [2]). Several of these checkpoint kinase inhibitors are in preclinical development and the Chk1 inhibitory peptide CBP501 recently entered clinical trials [3]. It is not yet known, which other components of the G_2 checkpoint are targetable by drugs or whether their inhibition will result in the selective killing of cancer cells.

To identify new G_2 checkpoint inhibitors, we used a phenotypic cell-based screen [4] that does not require a priori knowledge of checkpoint mechanisms. In this study, we used this assay to identify highly efficacious checkpoint inhibitors from a plant extract. We show that Z-Cryptofolione and Cryptomoscatone D2 also inhibit nuclear export and that the highly selective nuclear export inhibitor leptomycin B also potently inhibits the G_2 checkpoint. Therefore, inhibition of nuclear export is an alternative approach to checkpoint kinase inhibition for abrogating the G_2 checkpoint.

Materials and methods

Checkpoint inhibition assay

The G_2 checkpoint assay [4] was used to identify checkpoint inhibitory activity in extract N29449 and to guide the isolation of active compounds. Briefly, MCF-7 mp53 cells were cultured as monolayers and exposed to 10 Gy γ-irradiation from a ^{60}Co source (1.2 Gy min^{-1}, Gammacell 220, Atomic Energy Commission of Canada). Sixteen hours later, when 90% of cells were arrested in G_2 [4], diluted crude plant extracts were added with 100 ng mL^{-1} nocodazole, and cells were cultured for another 8 h. The proportion of mitotic cells was determined using a modified ELISA assay [4]. The degree of checkpoint inhibition was measured by microscopy using either chromosome spreads or flow cytometry [5].

Isolation and chemical modification of *Cryptocarya* pyrones

A 500 mg sample of crude extract N29449 was suspended in 1 L of water and partitioned against ethyl acetate (EtOAc). The EtOAc fraction was concentrated in vacuo, suspended in 1 L of 9:1 methanol/H_2O, and then partitioned against hexanes. Next, water was added to the remaining methanolic fraction in order to achieve a 6:4 methanol/H_2O ratio. This fraction was partitioned against 2 L of dichloromethane. The G_2 checkpoint inhibitory activity was entirely present in the EtOAc, the 9:1 methanol/H_2O, and the CH_2Cl_2 fractions. Approximately 125 mg of the dichloromethane fraction was fractionated on a Sephadex LH-20 column using 80:20 methanol/dichloromethane as the eluent, yielding seven fractions. One fraction exhibited significant inhibitory activity and was further subjected to normal phase flash chromatography using an isocratic 75:25 EtOAc/hexanes elution. A final purification by reversed-phase HPLC using 48:52 H_2O/methanol as the eluent yielded pure Cryptomoscatone D2 (**1**), Z-Cryptofolione (**2**), Z-Cryptomoscatone D2 (**3**), Cryptofolione (**4**) and compounds **7** and **8** (see Fig. 1). Compounds **2** and **3** were isolated for the first time in this study. Their structures were confirmed by detailed NMR and mass spectrometric analyses. Cryptofolione acetonide (**5**) was prepared by adding 2 mL of 2,2-dimethoxypropane and a catalytic amount of pyridinium *p*-toluene sulfonate to approximately 2 mg of cryptofolione. Compound **5** was isolated as a white amorphous solid that gave an $[M + H]^+$ ion in the HRCIMS at *m/z* 355.19102 appropriate for a molecular formula of $C_{22}H_{26}O_4$ (ΔM −0.2 ppm). Its structure was confirmed by analysis of the 1D and 2D NMR spectra and it was in full agreement with previously published data [8]. The diacetylated analogue of Cryptofolione (**6**) was prepared by adding 0.5 mL of acetic anhydride and 1.5 mL of pyridine to approximately 2 mg of Cryptofolione. After workup, compound **6** was isolated as a white amorphous solid that gave an M^+ ion in the HREIMS at *m/z* 398.17274 appropriate for a molecular formula of $C_{23}H_{26}O_6$ (ΔM −0.51 ppm). Analysis of the 1D and 2D NMR spectra indicated the presence of two acetate groups in place of the two alcohol functionalities in cryptofolione. Cryptofolione was subjected to hydrogenation conditions using H_2(g) in the presence of catalytic Pd-C to yield compound **9**. After workup, the hydrogenation product **9** was isolated as a white amorphous solid that gave an $[M + H]^+$ ion in the HRCIMS at *m/z* 323.22162 appropriate for a molecular formula of $C_{19}H_{30}O_4$ (ΔM 1.9 ppm). Analysis of the 1D and 2D NMR spectra indicated the absence of proton and carbon resonances for the three olefins groups that were present in Cryptofolione. The absence of a third oxymethine proton resonance in the region around δ 4–5 ppm and a downfield shift in the carbonyl resonance at δ 177.6 ppm supported the opening of the lactone ring as first indicated by the molecular formula. The olefin and oxymethine resonances were replaced by seven new methylene group signals, with HMBC and COSY correlations consistent with the proposed structure of compound **9**.

Z-Cryptofolione (2). Isolated as a clear oil; HRCIMS *m/z* 314.15226 $C_{19}H_{22}O_4$ (ΔM –1.5 ppm); $[\alpha]^{20}_D$ +120.5 (*c* 1.2, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$) δ7.33 (t, J = 7.5 Hz, H-3″/H-5″), 7.25 (d, J = 7.5 Hz, H-2″/H-6″), 7.25 (d, J = 7.5 Hz, H-4″), 6.85 (m, H-4), 6.53 (d, J = 11.7 Hz, H-8′), 6.02 (d, J = 9.8 Hz, H-3), 5.85 (dt, J = 15.5, 7.1 Hz, H-2′),

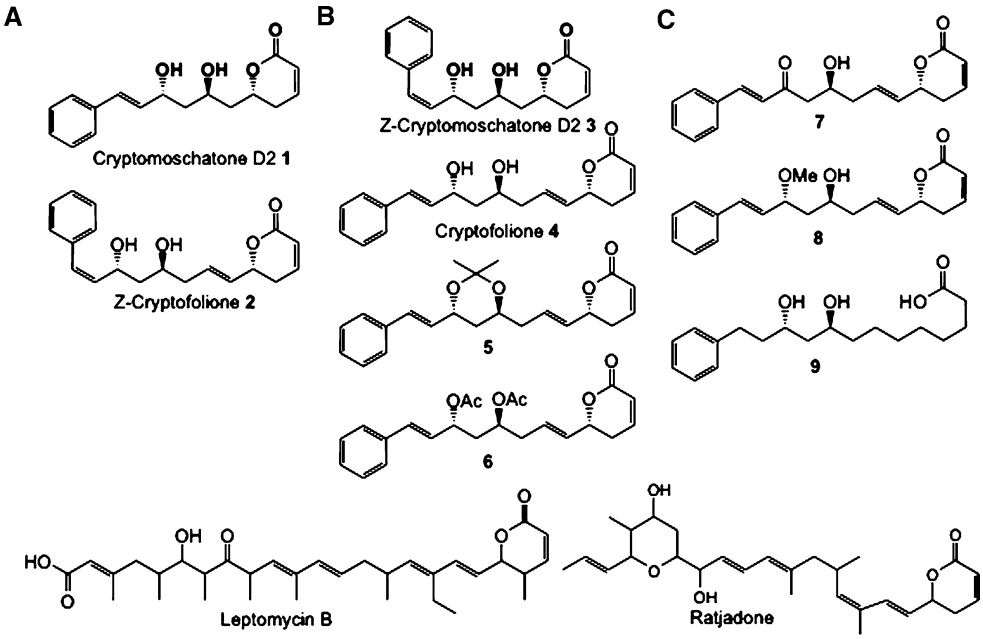

Fig. 1 Structural formulae of *Cryptocarya* pyrones with strong G_2 checkpoint inhibitory activity (left column), moderate activity (middle column) or no activity (right column). Structures of leptomycin B and ratjadone are shown for comparison

5.76 (t, J = 11.7, 9.8 Hz, H-7′), 5.65 (dd, J = 15.5, 6.4 Hz, H-1′), 4.93 (m, H-6′), 4.87 (m, H-6), 4.06 (m, H-4′), 2.54 (s, -OH), 2.40 (m, H-5), 2.28 (t, J = 6.6 Hz, H-3′), 1.79 (m, H-5′); ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.9 (C-2), 144.6 (C-4), 136.4 (C-1″), 133.6 (C-7′), 131.0 (C-2′), 130.8 (C-8′), 130.0 (C-1′), 128.7 (C-2″/ C-6″), 128.4 (C-3″/ C-5″), 127.4 (C-4″), 121.6 (C-3), 77.7 (C-6), 68.3 (C-4′),65.7 (C-6′), 42.3 (C-5′), 4.03 (C-3′), 29.7 (C-5).

Z-Cryptomoscatone D2 (3). Isolated as a clear oil; positive ion HRESIMS m/z $[M + Na]^+$ 311.1258 (calcd for $C_{17}H_{20}O_4Na$ 311.1259); $[\alpha]^{22}_D$ + 121.0 (c 0.1, CH_2Cl_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.36 (t, J = 7.5 Hz, H-3″/H-5″), 7.28 (m, H-2″/H-6″), 7.28 (m, H-4″), 6.90 (m, J = 9.4, 3.9 Hz, H-4), 6.58 (d, J = 11.6 Hz, H-6′), 6.02 (d, J = 9.4 Hz, H-3), 5.82 (dd, J = 11.3, 9.4 Hz, H-5′), 4.99, (m, H-4′), 4.77 (m, H-6), 4.43 (m, H-2′), 2.38 (m, H-5), 1.85 (m, H-1′); ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.5 (C-2), 145.5 (C-4), 136.5 (C-1″), 133.7 (C-5′), 131.3 (C-6′), 128.9 (C-2″/ C-6″), 128.6 (C-3″/ C-5″), 127.7 (C-4″), 121.6 (C-3), 75.1 (C-6), 66.2 (C-4′), 65.1 (C-2′), 43.2 (C-3′), 42.5 (C-1′), 30.1 (C-5).

Nuclear export assay

The effect of chemicals on nuclear export was determined as described [6]. Briefly, HeLa cells stably transfected with an HIV-Rev/glucocorticoid receptor (GR)/GFP construct were treated with 1 μM dexamethasone for 30 min. Chemicals were then added and the cells were cultured for an additional 30 min. The cells were then washed twice in phosphate-buffered saline (PBS) and then cultured for 60 min in the presence of test compounds, without dexamethasone. Cells were then visualized by fluorescence microscopy after fixation and staining of DNA with Hoechst 33342. Export inhibition was assessed as the presence or absence of nuclear/nucleolar retention of GFP marker by visual examination.

Results and discussion

Isolation and identification of Cryptomoscatone D2 and Z-Cryptofolione

A cell-based assay for G_2 checkpoint inhibition was used to screen 20,000 plant extracts from the NIH/National Cancer Institute Natural Products Repository. A crude extract of *Cryptocarya concinna* (N29449), a tree of the laurel family, showed strong G_2 checkpoint inhibitory activity. Assay-guided fractionation and analysis of nuclear magnetic resonance (NMR) and mass spectroscopy (MS) data led to the identification of Cryptomoscatone D2 (**1**) and Z-Cryptofolione (**2**) as the most active checkpoint inhibitors in the extract (Fig. 1, left column). Cryptomoscatone D2 was previously isolated from a plant of the same genus [7], while Z-Cryptofolione is a previously undescribed isomer of Cryptofolione (**4**) [8].

Inhibition of ionizing radiation-induced G_2 arrest by Cryptomoscatone D2 and Z-Cryptofolione

G_2 checkpoint inhibition by compounds **1** and **2** was examined in human breast carcinoma MCF-7 cells expressing

dominant-negative p53. Cells were exposed to 10 Gy ionizing radiation to induce 90% G_2 arrest [4] and then were incubated for 8 h with dimethylsulfoxide (DMSO) (negative control), **1**, **2** or caffeine (positive control). The drug carrier DMSO did not overcome G_2 arrest (data not shown), but exposure to **1** (1–30 μg mL^{-1}) or **2** (10–30 μg mL^{-1}) caused a reduction in the number of G_2-arrested cells accompanied by a large increase in the number of mitotic cells, demonstrating G_2 checkpoint abrogation (Fig. 2 a). These two compounds were as highly efficacious as the well-characterized checkpoint inhibitor caffeine, which averages approximately 60% checkpoint abrogation in these assay conditions. However, **1** and **2** were about 30-fold more potent than caffeine. Interestingly, cells treated with these compounds were unable to exit mitosis (not shown), as is the case for the structurally unrelated checkpoint inhibitors 13-hydroxy-15-oxozoapatlin and psilostachyin [5, 9], but unlike other checkpoint inhibitors described to date, including UCN-01, debromohymenialdisine, caffeine and isogranulatimide. This aspect was not explored further.

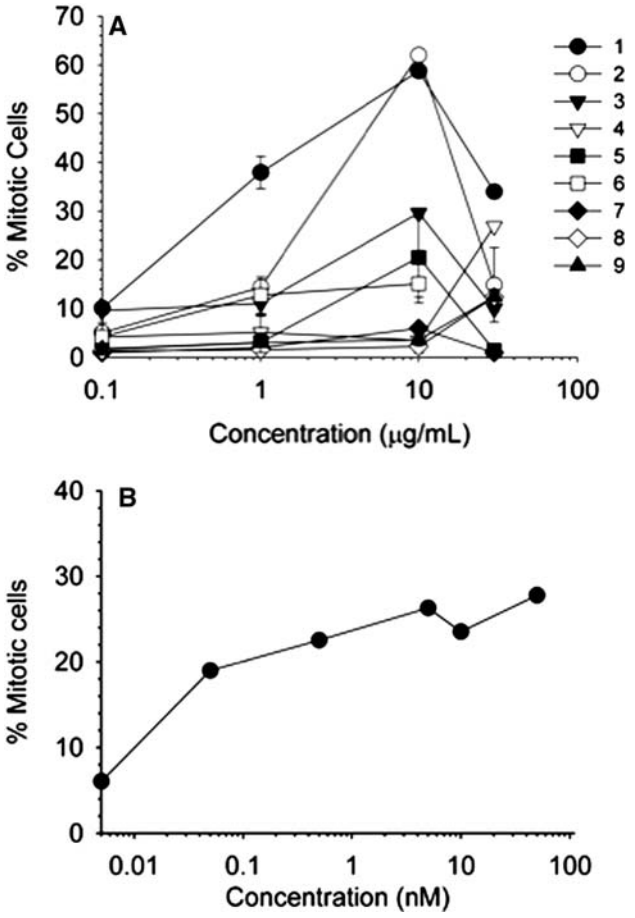

Fig. 2 **a** G_2 checkpoint inhibition by *Cryptocarya* pyrones. Cells arrested in G_2 phase after exposure to ionizing radiation were treated with different compound concentrations and the percentage of cells forced to enter mitosis (checkpoint inhibition) was determined. Results shown are means of duplicate experiments (due to limiting amounts of compound) or mean ± SD ($n \geq 3$). **b** G_2 checkpoint inhibition by leptomycin B. Results shown are means of duplicate experiments

The plant extract also contained additional related compounds that were less active as checkpoint inhibitors. Z-Cryptomoscatone D2 (**3**) is a novel compound with similar potency to **1** and **2** but lower efficacy. Cryptofolione (**4**) was both less potent and active than **1** and **2** (Fig. 2 a). Compound **7**, with a carbonyl at position 6′, and compound **8**, with a methoxy substituent at position 6′, showed little or no activity (Fig. 2 a). We also prepared compounds **5**, **6** and **9** by chemical modification of cryptofolione and measured their activity as checkpoint inhibitors. The acetonide compound **5** showed moderate activity at 10 μg mL^{-1} (Fig. 2 a). Compound **6**, which is acetylated at positions 4′ and 6′, showed little or no activity while compound **9**, which has an open lactone ring and no α,β unsaturated carbonyl, and in which carbons C1′-C2′ and C7′-C8′ are saturated, was inactive (Fig. 2 a).

These results provide some indication of the structural requirements for checkpoint inhibition by this class of agents. The lactone ring and at least one hydroxyl group appear to be important, as compounds **5**, **6**, **7** and **9** exhibit reduced activity or no activity at all. The effect of isomerization at the C7′-C8′double bond is interesting. One the one hand, the E isomer of Cryptomoscatone D2 **1** is more active than its Z isomer **3** while the opposite is seen for cryptofolione, whose Z isomer **2** is more active than the E isomer **4**. Therefore, there is no absolute requirement for either a Z or E configuration. Rather, the observation that the extended E form of the shorter Cryptomoscatone and the condensed Z form of the longer Cryptofolione are more active may mean that Z-Cryptomoscatone is too short and E-Cryptofolione is too long for optimal activity. Finally, the observation that **1**, **2**, **3** and **5** show reduced activity at concentrations above 10 μg mL^{-1} (Fig. 2 a) probably indicates that higher concentrations of these compounds inhibit cellular proteins required for cell survival or entry into mitosis.

Inhibition of nuclear export by *Cryptocarya* pyrones

The structures of these compounds are unlike those of other checkpoint inhibitors described to date (reviewed in [2]). Similar to psilostachyin [9], cryptofolione does not inhibit the checkpoint kinases ATM, ATR, Chk1 or Chk2 or the protein phosphatases PP1 and PP2A in vitro (not shown). The *Cryptocarya* pyrones bear some structural resemblance to the inhibitors of Crm1-mediated nuclear export leptomycin B and ratjadone (Fig. 1), in that they possess a lactone-ring followed by a long carbon chain with hydroxyl groups [10]. In addition, when a COMPARE analysis [11] was carried out

of correlations between gene expression levels and sensitivity to cryptofolione for different cancer cell lines, NLP-1 (*n*ucleoporin-*l*ike *p*rotein *1*) was found as the gene whose expression levels correlated most highly (Pearson Correlation Coefficient = 0.616) with the sensitivity to cryptofolione of cancer cell lines with mutated p53. NLP-1 is a protein that interacts with Crm1 and with nuclear export sequences in proteins [12]. These observations prompted us to examine whether these compounds affect nuclear export.

We used a cellular system, in which the HIV-1 Rev protein, which contains a nuclear export sequence, is fused to the hormone-responsive element of the glucocorticoid receptor and to GFP [6]. Addition of dexamethasone causes nuclear accumulation of Rev/GR/GFP in nucleoli at glucocorticoid response elements (GRE). Removal of dexamethasone causes Rev/GR/GFP to relocalize to the cytoplasm, allowing visualization of nuclear export signal-mediated nuclear export.

Rev/GR/GFP was localized mostly in the cytoplasm in the absence of dexamethasone (Fig. 3 a) and in the nucleus in the presence of dexamethasone (Fig. 3 b), as previously described [6]. Most of the protein was exported out of the nucleus by 60 min after dexamethasone removal, a process that was not affected by the drug carrier DMSO (Fig. 3 h). Leptomycin B prevented export of the protein to the cytosol (Fig. 3 c), as previously observed [13]. At concentrations that inhibited the checkpoint, the *Cryptocarya* pyrones inhibited nuclear export (Fig. 3 e–g for examples, and Table 1). The compounds that showed little or no checkpoint inhibition also showed little or no nuclear export inhibition (Table 1). Compound **6** inhibited nuclear export at 30 μg mL^{-1} but its checkpoint inhibition was not tested at concentrations higher than 10 μg mL^{-1} because of limited compound availability (Table 1). Overall, there was a fairly consistent correlation between checkpoint inhibition and export inhibition.

Table 1 Checkpoint and nuclear export inhibition by *Cryptocarya* pyrones and Leptomycin B

Compound	G_2 checkpoint inhibition	Nuclear export inhibition
1	+++ (1)	+ (10)
2	+++ (10)	+ (10)
3	++ (1)	+ (10)
4	+ (30)	+ (10)
5	+ (10)	+ (10)
6	–[a]	+ (30)
7	–	ND
8	–	–
9	–	–
Leptomycin B	++	+

ND Not determined

+++ very efficacious inhibition, – no inhibition

Numbers in brackets represent lowest concentration (μg mL^{-1}) required for inhibition

[a] activity could not be determined at concentrations higher than 10 μg mL^{-1}

Potent G_2 checkpoint inhibition by leptomycin B

The observations described above prompted us to ask whether leptomycin B, the most potent and selective export inhibitor described to date [10], can also abrogate the G_2 checkpoint. Indeed, leptomycin B showed very potent inhibition of G_2 arrest induced by ionizing radiation, with significant activity at concentrations as low as 0.05 nM (Fig. 2 b).

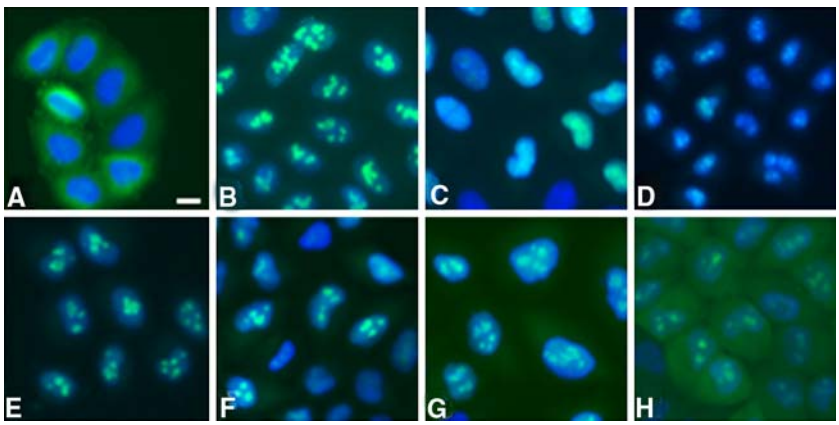

Fig. 3 Inhibition of NES-mediated nuclear export by *Cryptocarya* pyrones. **a**, untreated cells. **b**–**h**, the cells were treated with dexamethasone to induce nuclear accumulation of Rev/GR/GFP, the dexamethasone was washed away and cells were further incubated in the absence of dexamethasone for 0 min (**b**) or 60 min (**c**–**h**) in the presence of leptomycin B (**c**), **2** (**d**), **3** (**e**), **5** (**f**), **4** (**g**), or DMSO (**h**). The cells were then fixed and Rev/GR/GFP was visualized by fluorescence microscopy. The oval shaped structures are individual nuclei and the nucleoli are the bright dots within the nuclei. Bar = 10 μm

A distinct checkpoint causing G_2 arrest is the decatenation checkpoint, which is activated by agents such as ICRF-193 that inhibit DNA topoisomerase II without inducing DNA damage, which leptomycin B has been shown to inhibit [14]. However, in HeLa cells, leptomycin B was not observed to overcome G_2 arrest induced by etoposide, a topoisomerase II inhibitor that induces DNA damage [15].

The demonstration that Cryptomoscatone D2, Z-Cryptofolione and leptomycin B abrogate G_2 arrest induced by ionizing radiation is strong evidence that nuclear export is required to prevent premature mitosis in checkpoint-arrested cells. A number of proteins involved in control of the G_2/M transition shuttle in and out of the nucleus via a Crm1-dependent mechanism [16]. Human cyclin B1 shuttles between the nucleus and the cytoplasm during interphase and CDK1/cyclin B accumulates in the nucleus at the G_2/M transition. This nuclear accumulation is thought to be important for initiating and coordinating mitotic events [16]. Cdc25B and Cdc25C are also exported from the nucleus via Crm1 and leptomycin B causes the nuclear accumulation of cyclin B1, Cdc25B and Cdc25C in interphase cells [17]. However, mutation of nuclear export sequences in Cdc25B or Cdc25C is not sufficient to induce entry into mitosis in HeLa cells [18, 19]. It is possible that the nuclear translocation of several proteins, rather than a single one, is required for G_2 checkpoint abrogation.

G_2 checkpoint abrogation in combination with radiotherapy or DNA-damaging chemotherapy can selectively enhance the killing of cancer cells with mutated p53. Does abrogation by nuclear export inhibitors have therapeutic potential for cancer therapy? On the one hand, a large number of proteins and complexes such as the ribosomal subunits are subjected to Crm1-dependent export [20, 21], indicating that inhibitors of Crm-1-mediated nuclear export might be toxic to normal cells. Leptomycin B has potent activity in vitro against a range of tumors and it has undergone a Phase I clinical trial under the name elactocin [22]. However, elactocin produced serious dose-limiting toxicity and further clinical trials were not recommended [22]. These results indicated that Crm-1 is not a viable drug target for cancer therapy. However, it is possible that some of the toxic effects of leptomycin B arise from its chemical reactivity. Leptomycin B binds covalently to cysteine 529 in Crm1 through Michael addition via its α,β-unsaturated carbonyl (reviewed in [20]). Compounds with Michael acceptor functionalities, such as Cryptomoscatone D2, Z-Cryptofolione and leptomycin B, are generally considered undesirable drug candidates. Thus, it is possible that non-covalent reversible Crm-1 inhibitors might show fewer toxic side effects and a therapeutic window. Unfortunately, our attempts to inactivate the α,β-unsaturated carbonyl of *Cryptocarya* pyrones by conjugation with β-mercaptoethanol did not produce stable compounds (data not shown). Therefore it was not possible to test whether this functional group is required for checkpoint or nuclear export inhibition.

The *Cryptocarya* pyrones are cytotoxic at concentrations that abrogate the checkpoint and therefore do not appear promising as checkpoint inhibitors for therapeutic intervention. Nevertheless, the demonstration that nuclear export inhibitors can abrogate the G_2 checkpoint induced by ionizing radiation should stimulate the search for compounds with a similar mechanism of action but with markedly less toxicity.

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