Available online at www.sciencedirect.com
ScienceDirect
Bioorganic Chemistry 34 (2006) 287–297
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Preliminary Communication

Inhibition of cancer cell growth by cyclin dependent kinase 4 inhibitors synthesized based on the structure of fascaplysin

Sachin Mahale [a], Carine Aubry [b], Paul R. Jenkins [b,1], Jean-Didier Maréchal [c], Michael J. Sutcliffe [c,2], Bhabatosh Chaudhuri [a,*]

[a] *Leicester School of Pharmacy, De Montfort University, Leicester LE1 9BH, UK*
[b] *Department of Chemistry, University of Leicester, Leicester LE1 7RH, UK*
[c] *Departments of Biochemistry and Chemistry, University of Leicester, Leicester LE1 7RH, UK*

Received 5 May 2006
Available online 14 August 2006

Abstract

Tryptamine derivatives, a new structural class of cyclin dependent kinase 4 inhibitors, have been identified during extensive biological screening of synthetic molecules. The molecules were synthesized based on the structure of fascaplysin, which is not only a specific inhibitor of the Cdk4-cyclin D1 enzyme but also a relatively toxic molecule, probably because it binds and intercalates DNA. Interestingly, the new structural analogues of fascaplysin do not interact or intercalate with double-stranded DNA, although they inhibit Cdk4-cyclin D1 specifically. We found that compound CA199 was the most potent molecule, showing at least 25-fold specificity towards Cdk4-cyclin D1 (IC_{50} for Cdk4-cyclin D1 = 20 μM, Cdk2 > 500 μM). CA199 inhibits the growth of different cancer cell lines at concentrations ranging from 10–40 μM. It blocks growth of asynchronous cells at G_0/G_1 in a retinoblastoma protein (pRb) dependent manner. Moreover, CA199 blocks growth only at early G_1 in synchronised cells released from a mimosine-induced G_1/S block. These observations are reminiscent of a true Cdk4 inhibitor.

* Corresponding author. Fax: +44(0)116 257 7287.
E-mail addresses: kin@le.ac.uk (P.R. Jenkins), sjm@le.ac.uk (M.J. Sutcliffe), bchaudhuri@dmu.ac.uk (B. Chaudhuri).
[1] Fax: +44(0)116 252 3789.
[2] Fax: +44(0)116 252 3369.

0045-2068/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bioorg.2006.06.004

Keywords: G_0/G_1 arrest; Cyclin dependent kinases; pRb phosphorylation; Cell cycle; Cell proliferation; Cancer chemotherapeutics

1. Introduction

Genetic aberrations in the regulatory pathways which monitor the journey of a cell through the different phases of the cell cycle occur frequently in human cancers [1]. The link between the protein retinoblastoma (pRb) pathway and development of the cancer phenotype is well established [2–6]. Moreover, the abnormality in pRb/p16/cyclin D1/cyclin dependent kinase 4 (Cdk4) pathways seems to be a frequent event in human tumours [7]. For this reason, pharmacological inhibition of cyclin dependent kinases (Cdk-s) is possibly becoming one of the most productive strategies for the design and discovery of novel anticancer agents which specifically target the cell cycle. It is encouraging to note that a number of Cdk inhibitors are gradually entering clinical trials [8–11]. Although a large number of Cdk inhibitors have been identified, currently there is an intense interest in developing selective inhibitors of cyclin dependent kinase 4 (Cdk4) for potential use in cancer chemotherapeutics. Recent studies indicate that Cdk4 is an important target for intervention in cancer therapy since misregulation of Cdk4 activity can cause cancer [12]. In contrast, it seems that Cdk2 may not be a cancer target at all, although it has been proposed to be a strong candidate for some time [13].

Fascaplysin, a natural product originally isolated from a marine sponge, specifically inhibits Cdk4 enzyme [14,15]. The IC_{50} for Cdk4-cyclin D1 inhibition is approximately 0.35 μM and it blocks the growth of cancer cells at the G_0/G_1 phase of the cell cycle. An analogue of fascaplysin, 1-deoxysecofascaplysin A, has also been reported to inhibit cell growth of MCF-7 (human breast cancer) and OVCAR-3 (human ovarian cancer) cell lines *in vitro* [16]. However fascaplysin shows unusual toxicity at the cellular level. Further studies have shown that fascaplysin intercalates with double-stranded DNA molecules, a property similar to DNA intercalating agents like cryptolepine and ellipticine [17]. The ability of the planar structure of fascaplysin to intercalate DNA is very likely a contributing factor to its unusual toxicity at the cellular level.

The main goal of the current study was to develop potent, non-planar Cdk4-specific analogues of fascaplysin which do not intercalate or interact with the minor groove of double-stranded DNA, with the aim that these non-planar molecules would avoid toxicity at the cellular level. Using a homology model of Cdk4, based on the X-ray crystallographic structures of Cdk2 and Cdk6, we have designed and synthesized non-planar tryptamine derivatives analogous to the structure of fascaplysin which show remarkably specific activities against Cdk4-cyclin D1 [18–20]. The compound CA199 proved to be the most potent in this series of tryptamines.

2. Materials and methods

2.1. Chemical synthesis of CA199

Detailed synthetic routes to the tryptamine derivatives including CA199 have been reported earlier [20].

2.2. Kinase assays

Compounds were screened in Cdk4-cyclin D1 and Cdk2-cyclin A kinase assays which were based on chemiluminescences detection rather than radioactivity. The detailed procedure has been described previously [20].

2.3. MTT assay to measure cell growth inhibition in vitro

The four cancer cell lines chosen for screening the fascaplysin analogues are relatively resistant to known chemotherapeutic compounds. They were the non-small cell lung carcinoma lines A549 (pRb^+, $p53^+$), Calu-1 (pRb^+, p53-null), the colon carcinoma line LS174T (pRb^+, $p53^+$) and the prostate carcinoma line PC3 (pRb^+, p53-null). The genotypes within brackets indicate the status of the tumor suppressor proteins pRb and p53.

All four cell lines were maintained at 37 °C in 5% CO_2 in RPMI-1640 medium, supplemented with 10% fetal calf serum and 100 μg/ml Normocin™. Exponentially growing cells, representing an asynchronous population, were seeded in 96-well plates, at densities of 5000–10,000 cells per well (depending on the doubling time of the individual cell line) in 180 μl of complete growth medium. The wells at the extreme four corners were omitted in order to avoid the edge effect and variations in the assay. The cells were allowed to stabilize on plates by incubating for 24 h. Serial dilutions of compounds (diluted in medium without serum) from a 10 mM stock (prepared in DMSO) were prepared. Twenty microliters of 10× concentrated compounds were added into the wells in triplicates while equivalent amounts of DMSO were added to the control wells after 24 h of stabilization of cells. The cells were mixed gently and incubated further for 48 h. After exposure to drug compounds, 50 μl of 2 mg/ml MTT (Sigma) was added and the plates were incubated for 2–3 h at 37 °C in the dark. The medium containing MTT was removed. The blue-coloured formazan that formed was dissolved in 150 μl of DMSO per well. The absorbance was measured at 540 nm. The IC_{50} concentration of compounds was calculated as the concentration at which 50% of cell growth was inhibited as compared to the control wells which did not contain any drug.

2.4. Ethidium bromide displacement assay

The DNA binding affinities of fascaplysin and its non-planar analogues were measured using an ethidium bromide fluorescence quenching assay. This assay is based on the displacement of DNA intercalating agent ethidium bromide from a closed circular plasmid DNA in the presence of compound [21,22]. The assay involves addition of 10 μl of 10× concentrated stock solution of compounds (dissolved in DMSO) to 90 μl of reaction mixture containing 6 μg of purified pBlueScript DNA (Stratagene) and 1.3 μM ethidium bromide in a buffer (20 mM NaCl, 2 mM Hepes and 10 μM EDTA) with final pH 7.4. Equivalent amounts of DMSO were added to the vehicle controls. The reduction in relative fluorescence units (RFU) was monitored ($\lambda_{excit} = 260$ nM, $\lambda_{emiss} = 600$ nM) and recorded after 1 min equilibration time. Fascaplysin and actinomycin D, which are known to intercalate double-stranded DNA molecules, were used as standard compounds in the assay [17].

2.5. Topoisomerase I DNA unwinding assay

The ability of fascaplysin and its analogues to intercalate into plasmid DNA was determined by a topoisomerase I unwinding assay [23]. Reactions contained 5 nM supercoiled pBlueScript plasmid DNA and 10 U of topoisomerase I (Invitrogen). Assays were performed in the presence or absence of compounds in 40 μl of DNA unwinding buffer (50 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA and 30 μg/ml bovine serum albumin). Following a 15 min incubation at 37 °C, reaction mixtures were treated with 3 μl of 250 mM EDTA and extracted with phenol/chloroform. Aqueous samples (20 μl) were treated with 2 μl of 2.5% SDS, mixed with 2.5 μl agarose gel-loading buffer (10×) and subjected to electrophoresis on a 0.9% Tris–acetate (pH7.4)–agarose gel. DNA bands were stained with 1 μg/ml ethidium bromide and visualised using a UV illuminator.

2.6. Flow cytometric analysis

2.6.1. Serum starvation experiment

Calu-1 cells were serum starved by incubating in the medium containing 0.1% FBS for 24 h. Then they were released in the complete medium in the presence or absence of CA199 for further 24 h.

2.6.2. Mimosine block experiments

Mimosine (Calbiochem) was prepared as a 10 mM stock solution in 100% DMSO and added to cell cultures at a final concentration of 200 μM. Cells were incubated at 37 °C in 5% CO_2 for 32 h and were then washed and further incubated in fresh medium in the absence or presence of compounds either for 18 or 36 h.

The untreated (control) and treated (with compounds) Calu-1 cells were harvested by trypsinization, washed once with PBS and then fixed in 70% chilled (−20 °C) ethanol for 1 h. After the fixation step, cells were centrifuged for 5 min at 3000*g* at room temperature and then the pellet was suspended in PBS containing 50 μg/ml propidium iodide (Sigma) and 0.5 mg/ml DNase free Ribonuclease (Sigma). The cells were stained for 1 h in dark at 4 °C. The cell cycle analysis was performed on a Beckman–Coulter (Epics® Altra™) fluorescence activated cell sorter (FACS). In order to gate all the events which represent single cells, and not cell doublets or cell clumps, the following analyses were performed on the samples. Cytograms of propidium iodide fluorescence peak signals versus integrated fluorescence or the linear signals were plotted. All data points on the straight line were isolated in a single gate and the gated data further used for plotting a histogram that represents a complete cell cycle. The total number of events was not allowed to exceed 200 events/s. Data acquisition was stopped after a minimum of 10,000 events had been collected.

2.7. Western blot analysis

Calu-1 cells were seeded in 25 cm^2 tissue culture flasks in complete medium. When the culture flasks reached 40–50% confluency, the cells were treated with the compound for 24 h. After treatment, the cells were harvested by trypsinization, washed in ice-cold PBS and then lysed in a buffer that contains a cocktail of protease inhibitors. The lysates were centrifuged at 14,000*g* for 10 min at 4 °C and the amount of proteins in the clear

supernatant was estimated using the Bradford method (Bio-Rad). 50 μg of protein from each sample were subjected to SDS–PAGE separation. The proteins were transferred to a PVDF membrane (Millipore) and blocked with 5% milk. Membranes were probed with polyclonal antibodies raised against the full-length pRb protein, and the phospho-specific pRb epitopes, pRb (Ser780-P), pRb (Ser795-P) and pRb (Ser807/811-P) (New England BioLabs). After overnight incubation at 4 °C, membranes were exposed to appropriate HRP-conjugated secondary antibody at room temperature for 1 h. Immuno-reactivity was visualized with the enhanced chemiluminescence Western blot detection reagents (Santa Cruz Biotechnology).

3. Results and discussion

The effect of fascaplysin analogues on the activity of enzymes Cdk4 cyclin D1 and Cdk2-cyclin A has been reported earlier [20]. CA199 is the most potent molecule in the series and shows more than 10-fold specificity for Cdk4-D1 than Cdk2-A.

3.1. Cancer cell growth inhibition

All the novel tryptamine derivatives in this series (based on the structure of fascaplysin) were tested in four different cancer cell lines for their ability to inhibit cancer cell growth *in vitro*. The inhibitory effects of compounds were quantified using the MTT assay and IC_{50}s for cell growth inhibition (i.e. the concentration of a compound at which 50% cell growth is inhibited) were determined. The results of cell proliferation assay (Table 1, columns 6–9) confirm that CA199 is the most potent molecule also at the cellular level. It inhibits the growth of cancer cell lines at low micro molar concentrations (10–40 μM, Table 1).

3.2. Ethidium bromide displacement assay

Results obtained from ethidium bromide displacement assays indicate that none of the structural analogues of fascaplysin interact with the minor groove of double-stranded DNA since they do not displace the plasmid-bound ethidium bromide (Table 1). Graphs that depict the interactions of fascaplysin, CA199 and actinomycin D with closed circular pBlueScript plasmid DNA are shown in Fig. 1.

It is apparent that 100 μM of CA199 is incapable of displacing 1.3 μM ethidium bromide from pBlueScript DNA. Less than 5% displacement of bound ethidium bromide is observed at 100 μM. In contrast, the DNA intercalative drug actinomycin D readily dislodged the bound ethidium bromide ($IC_{50} = 35$ μM). Fascaplysin dislodged the DNA bound ethidium bromide at lower concentrations, $IC_{50} = 5.5$ μM.

3.3. Topoisomerase I catalysed DNA unwinding assay

Interactions between CA199 and double-stranded DNA molecules were studied using topoisomerase I catalysed DNA unwinding assays (Fig. 2). In the first set of experiments unwinding/relaxation of supercoiled pBlueScript plasmid DNA was carried out in the presence of CA199, fascaplysin and camptothecin. Fascaplysin showed inhibition of DNA unwinding/relaxation catalysed by the enzyme topoisomerase I indicating its intercalating

Table 1
Activity of fascaplysin analogues in different *in vitro* assays and their chemical structures (IC_{50} values are in μM)

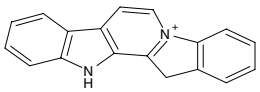
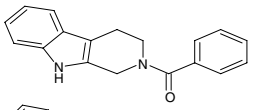
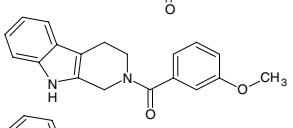
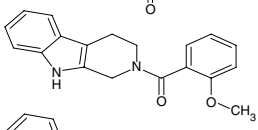
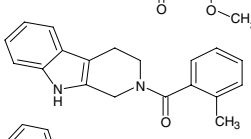
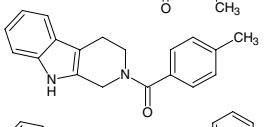
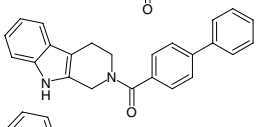
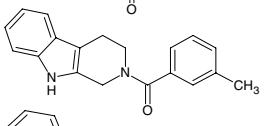
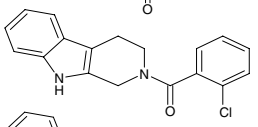
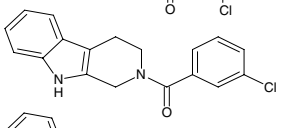
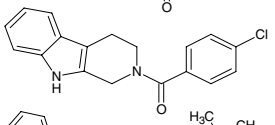
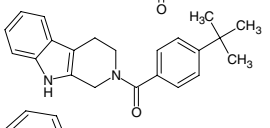
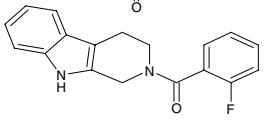
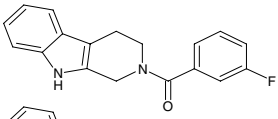
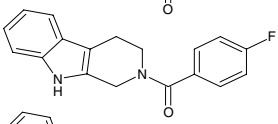
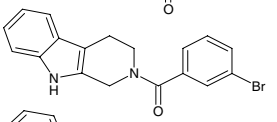
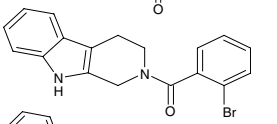
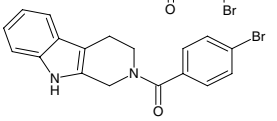
Compound	Structure	EtBr displacement from DNA	Cell growth inhibition determined by MTT assay			
			LS174T	A549	Calu-1	PC3
Fascaplysin		5 ± 0.4	0.88 ± 0.04	0.69 ± 0.03	1.3 ± 0.1	0.92 ± 0.06
CA161		Does not displace	97 ± 3.5	89 ± 6	120 ± 8	86 ± 3.5
CA199		Does not displace	7.1 ± 1.3	8 ± 2	47 ± 3.5	13.1 ± 1.8
CA198		Does not displace	33 ± 2.5	46 ± 2	92 ± 4.5	42 ± 3
CA204		Does not displace	81 ± 6	77 ± 3.8	110 ± 5	51 ± 4.2
CA206		Does not displace	110 ± 8	93 ± 4	105 ± 7	98 ± 4.4
CA205		Does not displace	100 ± 5.5	85 ± 3	104 ± 3.5	98 ± 4.4
CA207		Does not displace	56 ± 3	54 ± 3.6	75 ± 4	42 ± 2.5
CA208		Does not displace	119 ± 4.8	72 ± 4.5	114 ± 7	71 ± 5
CA209		Does not displace	53 ± 3	52 ± 4	72 ± 4.5	41 ± 3.2
CA210		Does not displace	137 ± 6	74 ± 5.5	79 ± 4	105 ± 6
CA211		Does not displace	29 ± 2	31 ± 3.5	54 ± 4	32 ± 1.5
CA212		Does not displace	96 ± 4.1	82 ± 3.5	122 ± 4	73 ± 3

Table 1 (*continued*)

Compound	Structure	EtBr displacement from DNA	Cell growth inhibition determined by MTT assay			
			LS174T	A549	Calu-1	PC3
CA213		Does not displace	92 ± 5	80 ± 3.5	112 ± 7	70 ± 3.6
CA214		Does not displace	88 ± 2.5	79 ± 4.4	132 ± 6	74 ± 3
CA215		Does not displace	62 ± 2.8	37 ± 3	48 ± 3.5	57 ± 3
CA235		Does not displace	90 ± 4	82 ± 2	74 ± 2.5	65 ± 3.7
CA236		Does not displace	75 ± 4	46 ± 1.5	63 ± 3	44 ± 3.5

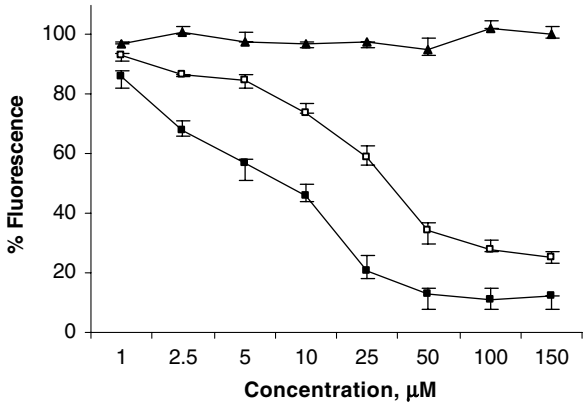


Fig. 1. CA199 does not displace ethidium bromide from the minor groove of double-stranded DNA molecules. The ability of fascaplysin, non-planar analogues including CA199 and actinomycin D to interact with the minor groove of DNA was determined by a fluorescence based ethidium bromide displacement assay. The assays were performed as described in Section 2. This figure show representative curves with increasing concentration of fascaplysin (filled squares) actinomycin D (unfilled squares) and CA199 (filled triangles). The results represent means and standard deviations from three independent experiments.

nature which is likely to be the consequence of its planar structure. Interestingly, CA199 which is a non-planar analogue of fascaplysin does not show any inhibition of DNA relaxation even at high concentration up to 100 μM (Fig. 2). To ensure that these results reflect a lack of DNA intercalation rather than an inhibition of topoisomerase I enzyme activity, a

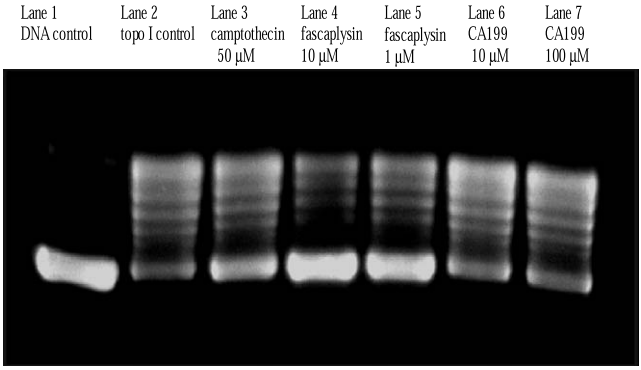


Fig. 2. CA199 does not intercalate into pBlueScript DNA. The ability of CA199 to intercalate DNA was investigated using a topoisomerase I catalysed DNA unwinding assay and compared with fascaplysin and the DNA intercalating agent camptothecin. The unwinding/relaxation assays were carried out as described in Section 2. Lane 1, Supercoiled pBlueScript DNA. Lane 2, Relaxed supercoiled pBlueScript DNA unwound after reaction with topoisomerase I enzyme, in the absence of compound. Lanes 3–7, Topoisomerase I relaxation reaction carried out in the presence of camptothecin, fascaplysin and CA199 at the concentrations indicated.

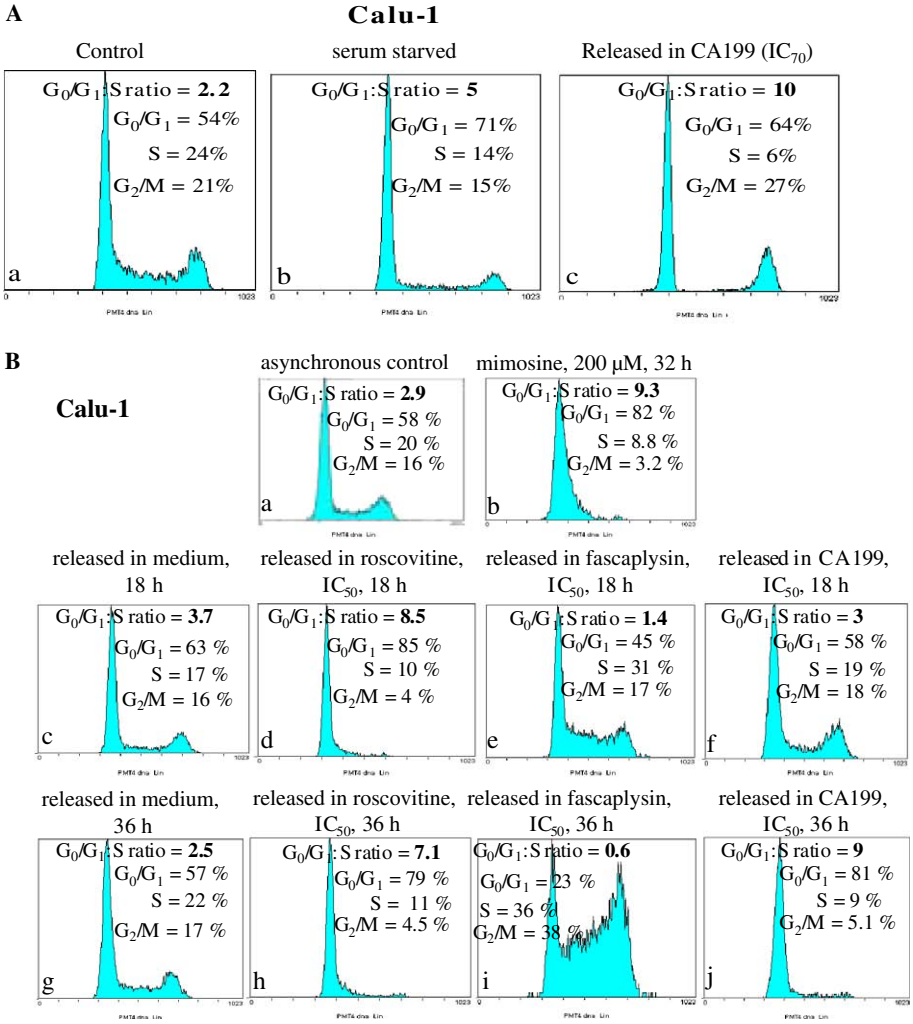
second set of experiments were performed using relaxed (negatively supercoiled) pBlueScript DNA as an initial substrate (prepared by the treatment of supercoiled plasmid DNA with Topoisomerase I) for the assay (data not shown). The DNA remained relaxed after treatment with 100 μM of CA199, confirming its non-intercalative nature.

These results indicate that CA199 neither intercalates nor interacts with the minor groove of double-stranded DNA molecules.

3.4. Flow cytometric analysis

The *in vitro* Cdk4-cyclin D1 and Cdk2-cyclin A enzyme assays had confirmed that CA199, the non-planar analogue of fascaplysin, selectively inhibits Cdk4-cyclinD1. This led us to believe that, if this compound inhibits Cdk4-cyclin D1 specifically, then it should maintain a serum starvation induced G_0/G_1 block since Cdk4 activity is essential for the transition of cells from G_0/G_1 to S phase of the cell cycle.

Fig. 3. (A) FACS analysis of serum starved Calu-1 cells. (a) Untreated or control cells, (b) cell serum starved for 24 h and (c) serum starved cells released in the presence of CA199 at IC_{70} concentration for 24 h. (B) FACS analysis showing CA199 does not prevent late G_1 blocked Calu-1 cells from entering into cell cycle. (a) Untreated or control cells (b) treatment with 200 μM mimosine for 32 h (c) released in fresh medium for 18 h, (d) released in the presence of roscovitine (IC_{50}), 18 h, (e) released in the presence of fascaplysin (IC_{50}), 18 h, (f) released in the presence of CA199 (IC_{50}), 18 h, (g) released in the fresh medium, 36 h, (h) released in the presence of roscovitine (IC_{50}), 36 h, (i) released in the presence of fascaplysin (IC_{50}), 36 h and (j) released in the presence of CA199 (IC_{50}), 36 h. Two to three fold increase in the G_0/G_1:S ratio indicates the G_0/G_1 arrest of cells. (C) Western blotting of the pRb protein in Calu-1 cells showing its phosphorylation status. Asynchronous Calu-1 cells were treated for 24 h with fascaplysin and CA199. The antibodies Ser780, Ser795 and Ser807/811 detect phosphorylation of pRb at Serine residues 780, 795 and 807/811, respectively. Antibody pRb (4H) detects the total pRb protein (phosphorylated and unphosphorylated). (C) Untreated Calu-1 cells, 24 h after seeding, (T1) cells treated with IC_{50} concentration of fascaplycin, 24 h, (T2) cells treated with IC_{50} concentration of CA199, 24 h, and (T3) cells treated with IC_{70} concentration of CA199, 24 h.


A
Calu-1
Control
serum starved
Released in CA199 (IC_{70})
G_0/G_1:S ratio = 2.2
G_0/G_1 = 54%
S = 24%
G_2/M = 21%
a
G_0/G_1:S ratio = 5
G_0/G_1 = 71%
S = 14%
G_2/M = 15%
b
G_0/G_1:S ratio = 10
G_0/G_1 = 64%
S = 6%
G_2/M = 27%
c
B
Calu-1
asynchronous control
mimosine, 200 μM, 32 h
G_0/G_1:S ratio = 2.9
G_0/G_1 = 58 %
S = 20 %
G_2/M = 16 %
a
G_0/G_1:S ratio = 9.3
G_0/G_1 = 82 %
S = 8.8 %
G_2/M = 3.2 %
b
released in medium, 18 h
released in roscovitine, IC_{50}, 18 h
released in fascaplysin, IC_{50}, 18 h
released in CA199, IC_{50}, 18 h
G_0/G_1:S ratio = 3.7
G_0/G_1 = 63 %
S = 17 %
G_2/M = 16 %
c
G_0/G_1:S ratio = 8.5
G_0/G_1 = 85 %
S = 10 %
G_2/M = 4 %
d
G_0/G_1:S ratio = 1.4
G_0/G_1 = 45 %
S = 31 %
G_2/M = 17 %
e
G_0/G_1:S ratio = 3
G_0/G_1 = 58 %
S = 19 %
G_2/M = 18 %
f
released in medium, 36 h
released in roscovitine, IC_{50}, 36 h
released in fascaplysin, IC_{50}, 36 h
released in CA199, IC_{50}, 36 h
G_0/G_1:S ratio = 2.5
G_0/G_1 = 57 %
S = 22 %
G_2/M = 17 %
g
G_0/G_1:S ratio = 7.1
G_0/G_1 = 79 %
S = 11 %
G_2/M = 4.5 %
h
G_0/G_1:S ratio = 0.6
G_0/G_1 = 23 %
S = 36 %
G_2/M = 38 %
i
G_0/G_1:S ratio = 9
G_0/G_1 = 81 %
S = 9 %
G_2/M = 5.1 %
j
C
Calu-1
C
T1
T2
T3
pRb (4H)
Ser780
Ser795
Ser807/811

3.5. CA199 maintains the G_0/G_1 block in serum starved p53 null Calu-1 cells

Treatment of Calu-1 cells, after release from cell synchronization (serum starvation for 24 h using 0.1% FBS), with IC_{70} concentrations of CA199 resulted in full maintenance of the G_0/G_1 block. Since the continuation of G_0/G_1 block after serum starvation requires Cdk4 enzyme to be inactive, these results indicate that CA199 probably inhibits cellular Cdk4 at these concentrations (Fig. 3A) and thereby maintains the G_0/G_1 block induced by serum starvation. The higher G_0/G_1:S percentage ratios in the cells released from serum starvation in the presence of compound compared to serum starved cells is because nearly all cells in the S phase, present during serum starvation, enter G_2/M phase after release whereas CA199 treated cells are prevented from entering the S phase of the cell cycle from the G_0/G_1 phase (Fig. 3A, compare b and c).

3.6. CA199 does not prevent late G_1 (i.e. G_1/S) phase blocked cells from progressing in the cell cycle but arrests cells at the following G_0/G_1 phase

Mimosine, a non-essential amino acid, inhibits DNA polymerase α in eukaryotic cells and thereby blocks them at the G_1/S boundary of the cell cycle [24]. Since the function of Cdk4 enzyme is crucial while cells progress through the early G_1 phase of the cell cycle, we hypothesised that if CA199 selectively inhibits Cdk4, it would not affect the progression of cells which have already gone beyond early G_1 and are blocked at the G_1/S boundary. We studied the effects of CA199 on mimosine treated/blocked cells. Calu-1 cells were blocked with mimosine for 32 h and were then released either in the presence of fresh medium or in the presence of one of the following compounds: CA199, roscovitine (a Cdk2-specific inhibitor) and fascaplysin (a Cdk4-specific inhibitor that also intercalates DNA). The cell cycle status of these cells were elucidated using FACS analyses at 18 and 36 h from the time of release from mimosine block. At the IC_{50} concentration of CA199, cells progressed from the G_1/S phase of the cell cycle after 18 h of release from mimosine block and were only arrested at the following G_0/G_1 phase after 36 h of release (Fig. 3B, compare histograms f and j). In the presence of the Cdk2-specific inhibitor, roscovitine, the cells remained blocked, as expected, at the G_1/S phase after 18 and 36 h of release from mimosine block (Fig. 3B, compare histograms d and h), while cells released in the presence of fascaplysin progressed from the G_1/S phase and underwent partial blocks at the S phase (after 18 h of release from mimosine block) and G_2/M phase (after 36 h of release from mimosine block) of the cell cycle possibly because of fascaplysin's innate DNA intercalating ability (Fig. 3B, compare histograms e and i). These results further suggest that CA199 (a) is very likely to be a Cdk4-specific inhibitor, (b)has no effect on the Cdk2 enzyme that is active at the late G_1 (i.e. G_1/S) phase of the cell cycle, and (c) unlike fascaplysin, is unable to intercalate DNA.

3.7. Western blot analysis: effects on the Cdk4 specific pRb phosphorylation status in Calu-1 cells

The phosphorylation status of pRb in Calu-1 cells was tested. Western blot results obtained after treatment of Calu-1 cells with CA199 (IC_{70}) for 24 h show that total cellular pRb levels (i.e. phosphorylated plus unphosphorylated) remain unchanged after treatment with CA199. It is also clear that CA199 prevents phosphorylation at the residues Ser780, Ser795 and Ser807/811 which are known to be phosphorylated specifically by the Cdk4

enzyme (Fig. 3C) [25,26]. As reported earlier, fascaplysin treatment of a pRb-positive cancer cell line (i.e. Calu-1 cells) results in the inhibition of pRb phosphorylation at Cdk4 specific serine residues (Fig. 3C) [15].

In conclusion, this report describes the elucidation of the biological properties of a novel, non-toxic class of Cdk4-specific inhibitors that are derived from the structure of the natural product, fascaplysin, a known toxic Cdk4-specific inhibitor. We believe that these observations strongly indicate that the early lead molecule CA199 could be the basis for further development of more potent Cdk4 specific inhibitors.

Acknowledgment

The work in the authors' laboratory was supported by Cancer Research UK.

References

[1] D. Fabbro, C. Garcia-Echeverria, Curr. Opin. Drug. Discov. Devel. 5 (2002) 701–712.
[2] L. Zhu, Eur. J. Cancer 41 (2005) 2415–2427.
[3] J. Bartek, J. Bartkova, J. Lukas, Curr. Opin. Cell Biol. 8 (1996) 805–814.
[4] A.S. Lundberg, R.A. Weinberg, Mol. Cell. Biol. 18 (1998) 753–761.
[5] R.A. Weinberg, Cell 81 (1995) 323–330.
[6] L. Yamasaki, Cancer. Treat. Res. 115 (2003) 209–239.
[7] S. Ortega, M. Malumbres, M. Barbacid, Biochem. Biophys. Acta 1602 (2002) 73–87.
[8] A.M. Senderowicz, E.A. Sausville, J. Natl. Cancer Inst. 92 (2000) 376–387.
[9] M. Knockaert, P. Grrengard, L. Meijer, Trends Pharmacol. Sci. 23 (2002) 417–425.
[10] E.A. Sausville, Curr. Med. Chem. Anticancer Agents 3 (2003) 47–56.
[11] P.M. Fischer, A. Gianella-Borradori, Expert Opin. Investig. Drugs 14 (2005) 457–477.
[12] M. Malumbres, M. Barbacid, Cancer Cell 9 (2006) 2–4.
[13] A.B. Gladden, J.A. Diehl, Cancer Cell 4 (2003) 160–162.
[14] N.L. Segraves, S.J. Robinson, D. Garcia, S.A. Said, X. Fu, F.J. Schmitz, H. Pietraszkiewicz, F.A. Valeriote, P. Crews, et al., J. Nat. Prod. 67 (2004) 783–792.
[15] R. Soni, L. Muller, P. Furet, J. Schoepfer, C. Stephan, S. Zumstein-Mecker, H. Fretz, B. Chaudhuri, Biochem. Biophys. Res. Commun. 275 (2000) 877–884.
[16] R.D. Charan, T.C. McKee, M.R. Boyd, Nat. Prod. Res. 18 (2004) 225–229.
[17] A. Hormann, B. Chaudhuri, H. Fretz, Bioorg. Med. Chem. 9 (2001) 917–921.
[18] C. Aubry, P.R. Jenkins, S. Mahale, B. Chaudhuri, J.D. Marechal, M.J. Sutcliffe, Chem. Commun. 7 (2004) 1696–1697.
[19] C. Aubry, A. Patel, S. Mahale, B. Chaudhuri, J.D. Marechal, M.J. Sutcliffe, P.R. Jenkins, Tetrahedron Lett. 46 (2005) 1423–1425.
[20] C. Aubry, A.J. Wilson, P.R. Jenkins, S. Mahale, B. Chaudhuri, J.D. Marechal, M.J. Sutcliffe, Org. Biomol. Chem. 4 (2006) 787–801.
[21] A.J. Geall, M.A. Eaton, T. Baker, C. Catterall, I.S. Blagbrough, FEBS Lett. 45 (1999) 337–342.
[22] H. Brotz-Oesterhelt, I. Knezevic, S. Bartel, T. Lampe, U. Warnecke-Eberz, K. Ziegelbauer, D. Habich, H. Labischinski, J. Biol. Chem. 278 (2003) 39435–39442.
[23] J.M. Fortune, N. Osheroff, J. Biol. Chem. 273 (1998) 17643–17650.
[24] C. Ji, L.J. Marnett, J.A. Pietenpol, Oncogene 15 (1997) 2749–2753.
[25] L. Connell-Crowley, J. Wade Harper, D.W. Goodrich, Mol. Biol. Cell 8 (1997) 287–301.
[26] M. Kitagawa, H. Higashi, H.K. Jung, I. Suzuki-Takahashi, M. Ikeda, K. Tamai, J. Kato, K. Segawa, E. Yoshida, S. Nishimura, Y. Taya, EMBO J. 15 (1996) 7060–7069.