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CA224, a non-planar analogue of fascaplysin, inhibits Cdk4 but not Cdk2 and arrests cells at G_0/G_1 inhibiting pRB phosphorylation

Sachin Mahale,[a] Carine Aubry,[b] A. James Wilson,[b] Paul R. Jenkins,[b] Jean-Didier Maréchal,[c] Michael J. Sutcliffe[c] and 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*

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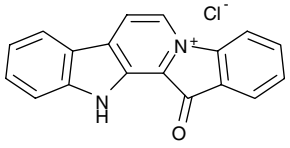
Abstract—Tryptamine derivatives, non-planar and potentially less toxic analogues of the anti-cancer agent fascaplysin, have been synthesised. They specifically inhibit Cdk4-D1 vis a vis Cdk2-A but, unlike fascaplysin, do not bind or intercalate DNA. CA224 is the most potent compound identified (Cdk4-D1 $IC_{50} \sim 5.5$ μM). As would be expected of a Cdk4 inhibitor that does not inhibit Cdk2, it maintains a G_0/G_1 block in synchronised cancer cells and inhibits Cdk4-specific phosphorylation of the retinoblastoma protein.

Fascaplysin (I), originally isolated from the Fijian sponge *Fascaplysinopsis* Bergquist sp.,[1] has been recently shown to block growth of cancer cells presumably through inhibition of cyclin-dependent kinase 4 (Cdk4), an early cell cycle enzyme misregulated in most cancers.[2]



(I)

Inhibition of Cdk4 concomitantly arrests cancer cells at the G_0/G_1 phase of the cell cycle and prevents phosphorylation of the retinoblastoma protein, pRB.[2] Hypophosphorylated pRB bound to the E2F family of transcription factors actively represses E2F-mediated gene transcription. When Cdk4 phosphorylates pRB, the hyperphosphorylated form dissociates so that E2Fs are free to induce a set of proteins that allow entry into the DNA synthesis (S) phase of the cell cycle. Thus, Cdk4 inhibition prevents entry of a cell into the S phase and thereby blocks its ability to multiply.[3]

Cdk4 is activated by D-type cyclins and negatively regulated by inhibitory proteins, one of them being $p16^{INK4A}$ (often referred to as p16). In most cancers, it is either that cyclin D1 is overproduced or that p16 is inactive. Sometimes, hyperactivating mutations in the catalytic part, Cdk4, are seen. Recent studies have provided compelling evidence that misregulation of Cdk4 activity can cause cancer and suggest that Cdk4-specific inhibition would be important for cancer therapy.[4–6] In contrast, it seems that Cdk2 which has often been proposed as a cancer target may not be at all suitable.[7] Hence, there is a great need for finding Cdk4-specific inhibitors that do not inhibit Cdk2.

Fascaplysin is one of the very few known Cdk4-specific inhibitors[8] that has shown efficacy in the NCI panel of 60 cancer cell lines[9] and it is being currently considered for therapeutic trials.[10] However, fascaplysin is quite toxic to normal cells, most likely because it binds and intercalates DNA.[11]

In order to identify non-toxic analogues of fascaplysin, we aimed to separate fascaplysin's ability to inhibit Cdk4 from its property of binding and intercalating

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* Corresponding author. Tel.: +44 0 116 250 7280; fax: +44 0 116 257 7287; e-mail: bchaudhuri@dmu.ac.uk

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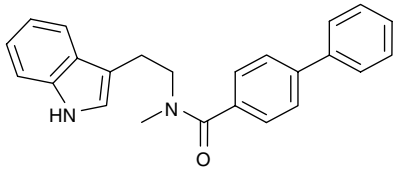
DNA. Therefore, we decided to synthesise three classes of non-planar tryptamine derivatives that would be structurally analogous to fascaplysin but would avoid intercalation with DNA.[12–14] Since the 3-D structure of Cdk4 is yet to be determined, the Cdk4 ATP-binding site was modelled on the basis of the known crystal structures of the homologous Cdk2 and Cdk6 enzymes.[12–14] Using this homology model, in silico studies suggested that inhibition of Cdk4 activity by fascaplysin arises from binding to the same amino acid residues to which ATP binds.[12–14] Non-planar compounds were then sought that maintained most of the key interactions thought to occur between fascaplysin and Cdk4. Preliminary SAR studies using Cdk4 and Cdk2 in vitro enzyme assays have corroborated that the predicted molecules inhibit Cdk4 and not Cdk2.[12–14] Here we describe more extensive studies on one class of tryptamine derivatives which prove that these fascaplysin analogues not only inhibit Cdk4 specifically but also fail to bind or intercalate DNA. The most potent compound in this series, CA224, was chosen to confirm that it also acts as a Cdk4 inhibitor in cancer cells.

Inhibition of Cdk4/Cdk2 kinases. The chemical syntheses of these fascaplysin analogues (Table 1) have been described earlier.[14] They were initially screened in the Cdk4 and Cdk2 enzyme assays based on chemiluminescence detection rather than radioactivity. The kinase assays measure the IC_{50}s of the compounds (i.e., concentrations at which 50% enzyme activity is inhibited) through the depletion in ATP concentrations occurring as a result of phosphorylation by Cdks of GST-pRB152 (a substrate for both Cdk4-cyclin D1 and Cdk2-cyclin A).[2,14] The IC_{50}s of all compounds in this series in the Cdk4-cyclin D1 and Cdk2-cyclin A assays are shown in Table 1.

CA224 was identified as the most active compound that inhibits Cdk4-cyclin D1 with an IC_{50} of approximately 5.5 μM (Table 1). CA224 showed approximately 100-fold greater specificity towards Cdk4-cyclin D1 than the Cdk2-cyclin A enzyme. All results in Table 1 show means and standard deviations from three independent experiments.



CA224

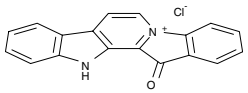
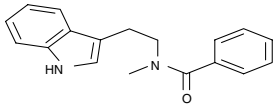
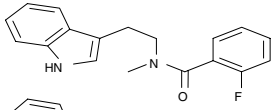
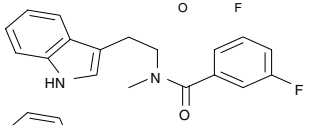
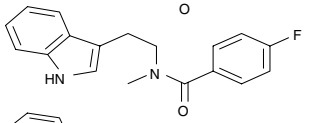
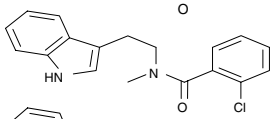
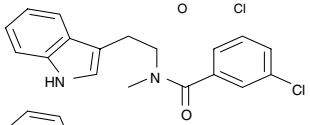
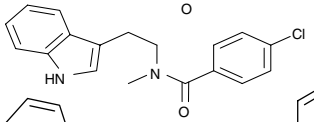
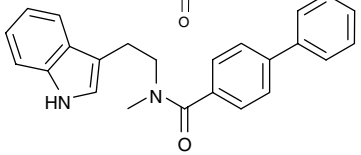
Inhibition of cell proliferation. All the non-planar analogues of fascaplysin were tested for their ability to inhibit cancer cell growth. The four cancer cell lines that were used are the non-small cell lung carcinoma lines A549 (pRB^+, $p53^+$), Calu-1 (pRB^+, p53-null), colon carcinoma LS174T (pRB^+, $p53^+$) and the prostate carcinoma PC3 (pRB^+, p53-null) lines. All lines were chosen for their relative resistance to chemotherapeutic agents. The genotypes within brackets indicate the status of the tumour suppressor proteins pRB and p53. The 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™.

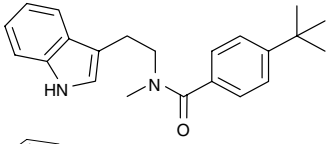
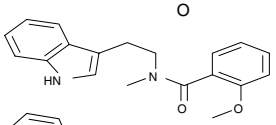
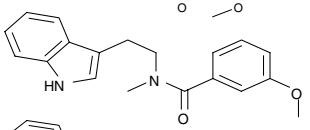
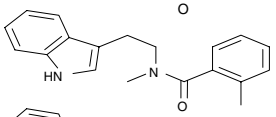
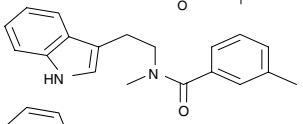
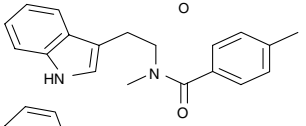
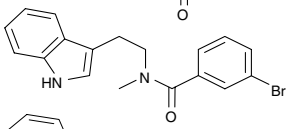
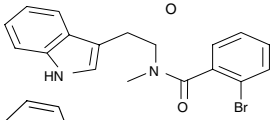
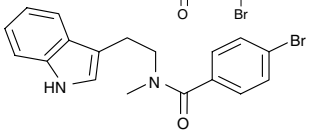
Five thousand to 10,000 cells were seeded in 96-well plates in 180 μl of complete growth medium and incubated for 24 h. Ten millimolar stock solutions of drug compounds in DMSO were serially diluted in medium without serum. Twenty microlitres of 10× concentrated compounds was added into the wells in triplicate, while equivalent amount of DMSO was added to the control wells. The contents of the wells were mixed gently and incubated further for 48 h. After exposure to compound, 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 DMSO per well. The absorbance was measured at 540 nm. The IC_{50}s of the compounds were calculated as the concentrations at which 50% of cell growth was inhibited as compared to the control wells which did not contain any drug. The results are depicted in Table 1 and indicate that CA224 is also most potent at the cellular level in inhibiting the growth of cancer cells at low micromolar concentrations (3–12 μM). The tryptamine derivatives CA225 and CA223 which are moderately active in the Cdk4 assay inhibit cell growth in the range of 10–20 and 20–40 μM, respectively.

Displacement of ethidium bromide from DNA. The DNA-binding affinities of fascaplysin and the new tryptamine derivatives, structurally analogous to fascaplysin, were then investigated using an ethidium bromide fluorescence quenching assay. It measures a compound's ability to displace the DNA intercalating agent ethidium bromide from closed circular plasmid DNA.[15,16] Ten microlitres of 10× concentrated serially diluted stock solutions of compounds (dissolved in DMSO) was added to 90 μl of a reaction mix that contains 6 μg of purified pBlue-Script DNA (Stratagene) and 1.3 μM ethidium bromide in a buffer (20 mM NaCl, 2 mM Hepes and 10 μM EDTA, pH 7.4). Equivalent amounts of DMSO were added to the vehicle controls. The decrease in fluorescence is monitored (λ_{excit} = 260 nM, λ_{emiss} = 600 nM) and recorded after a 1 min equilibration time. Fascaplysin and actinomycin D, which are known to intercalate double-stranded DNA molecules, were used as controls in the assay.[11] The results show that none of the analogues displace bound ethidium bromide from double-stranded DNA (Table 1). Results of the representative compound CA224 are shown graphically in Fig. 1. As expected, both actinomycin D and fascaplysin dislodge ethidium bromide bound to DNA (IC_{50} = 35 and 5 μM, respectively), but CA224 is incapable of doing so; less than 5% displacement of bound ethidium bromide is observed even at 100 μM concentration of CA224.

Inhibition of DNA unwinding initiated by topoisomerase I. The ability of fascaplysin and its analogues to intercalate plasmid DNA was determined by a topoisomerase I

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

Compound	Structures	Cdk4-cyclin D1	Cdk2-cyclin A	EtBr displacement from DNA	Cell growth inhibition			
					LS174T	A549	Calu-1	PC3
Fascaplysin		0.41 ± 0.04	>250	5 ± 0.4	0.88 ± 0.04	0.69 ± 0.03	1.3 ± 0.1	0.92 ± 0.06
CA192		88 ± 6.1	1523 ± 27	Does not displace	83 ± 3	95 ± 2.5	104 ± 5	86 ± 3.5
CA218		103 ± 8.5	1230 ± 28	Does not displace	110 ± 4.5	103 ± 4	145 ± 5	108 ± 7
CA219		88 ± 9	765 ± 26	Does not displace	97 ± 4	106 ± 2.5	136 ± 3.4	98 ± 5.5
CA220		59 ± 7	850 ± 34	Does not displace	87 ± 2	102 ± 6	141 ± 4.5	94 ± 3.6
CA221		46 ± 3.5	784 ± 20	Does not displace	99 ± 4	96 ± 7	147 ± 8.5	92 ± 6.2
CA222		109 ± 7.5	1120 ± 38	Does not displace	118 ± 5	95 ± 4.2	123 ± 5.9	94 ± 4.5
CA223		38 ± 6	731 ± 26	Does not displace	42 ± 2.5	27 ± 2.5	78 ± 3	47 ± 3
CA224		6.2 ± 0.9	521 ± 11.5	Does not displace	3.5 ± 0.9	3.5 ± 0.6	11.5 ± 2.5	6.2 ± 1.1

CA225		49 ± 6.5	658 ± 23	Does not displace	18 ± 1	12 ± 1.8	52 ± 3.5	15 ± 1.5
CA226		79 ± 9	849 ± 30	Does not displace	106 ± 4	103 ± 9	146 ± 8	89 ± 5
CA228		81 ± 4	938 ± 25	Does not displace	109 ± 6.4	95 ± 5.7	139 ± 7	85 ± 5
CA229		113 ± 7.5	1125 ± 23	Does not displace	105 ± 6.5	110 ± 8	141 ± 11	92 ± 4
CA230		78 ± 8	830 ± 31	Does not displace	88 ± 3.8	96 ± 5.7	136 ± 9.5	81 ± 3.2
CA233		63 ± 6	790 ± 27	Does not displace	82 ± 4.3	92 ± 2.5	103 ± 4.8	55 ± 3
CA234		95 ± 7	584 ± 24	Does not displace	76 ± 4	95 ± 6.2	122 ± 4.4	59 ± 4
CA237		74 ± 6.5	635 ± 21	Does not displace	89 ± 3.8	91 ± 4.6	144 ± 7	66 ± 3.5
CA238		37 ± 5	580 ± 18	Does not displace	52 ± 2.9	55 ± 3.2	64 ± 4.1	36 ± 2.2

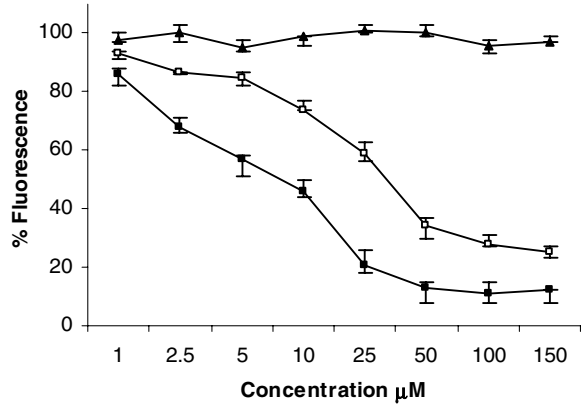


Figure 1. CA224, like all tryptamine derivatives structurally analogous to fascaplysin, does not displace ethidium bromide from the minor groove of double-stranded DNA. The assay was performed with increasing concentrations of fascaplysin (filled squares), actinomycin D (unfilled squares) and CA224 (filled triangles) to see if the compounds could displace ethidium bromide from the minor groove of double-stranded DNA. The results represent means and standard deviations from three independent experiments.

unwinding assay.[17] Reaction mixtures contained 5 nM supercoiled pBlueScript (Stratagene) plasmid DNA and 10 units of topoisomerase I (Invitrogen) that allows relaxation of supercoiled DNA. 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 the DNA was 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 1% Tris-acetate (pH 7.4)–agarose gel. DNA bands were stained with 1 μg/ml ethidium bromide and visualised using a UV illuminator.

Figure 2 compares CA224's ability to intercalate DNA molecules with camptothecin (a known intercalator of

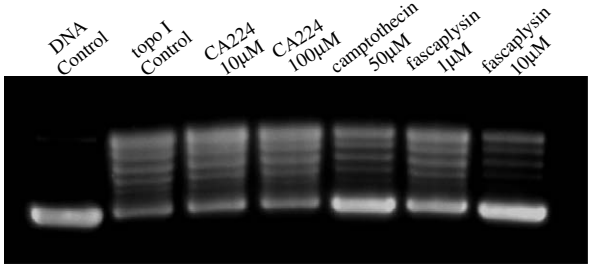


Figure 2. CA224 does not intercalate DNA. The ability of fascaplysin's structural analogue CA224 to intercalate DNA was investigated after DNA was unwound/relaxed using topoisomerase I and the results were compared with that from fascaplysin and camptothecin. Lane 1, control supercoiled form of pBlueScript plasmid DNA. Lane 2, supercoiled pBlueScript plasmid DNA unwound/relaxed with topoisomerase I enzyme, in the absence of any compound. Lanes 3–7, pBlueScript plasmid DNA unwound/relaxed by topoisomerase I in the presence of CA224, camptothecin and fascaplysin at the concentrations indicated.

DNA, as control) and fascaplysin. Fascaplysin shows inhibition of DNA relaxation catalysed by the enzyme topoisomerase I indicating its intercalating nature, possibly manifested because of its planar structure. The non-planar compound CA224 did not show any inhibition of DNA relaxation even at a high concentration of 100 μM. To ensure that these results truly reflected a lack of DNA intercalation rather than an inhibition of topoisomerase I, a second set of experiments (data not shown) were performed using relaxed DNA as substrate (prepared first by treating supercoiled pBlueScript plasmid DNA with topoisomerase I). The DNA remained relaxed after treatment with 100 μM CA224, confirming the non-intercalative nature of the compound which may indicate that other compounds in this series are also likely to behave in the same way.

The results from the ethidium bromide displacement and topoisomerase I catalysed DNA unwinding assays indicate that CA224 neither interacts nor intercalates with the minor groove of double-stranded DNA molecules (Figs. 1 and 2).

Flow cytometric analysis. The in vitro enzyme assays had confirmed that the fascaplysin analogue CA224 inhibits Cdk4-cyclin D1 and not Cdk2-cyclin A. In proliferative cells, Cdk4 is activated at the G_0/G_1 phase of the cell cycle. Therefore, we queried if CA224 would maintain a G_0/G_1 block induced by serum starvation. Calu-1 (p53-null) cells were used to test the effect of CA224 on the cell cycle.

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 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. Cell cycle analysis was performed on the Beckman-Coulter (Epics® Altra™) fluorescence-activated cell sorter (FACS). In order to gate all events representing single cells, and not cell doublets or cell clumps, cytograms of propidium iodide fluorescence peak signals versus integrated or linear fluorescence signals were plotted. All data points on the straight line were isolated in a single gate and the gated data were 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.

Calu-1 cells were starved of serum for 24 h using 0.1% FBS. When these G_0/G_1 synchronised cells were released in the presence of [IC_{70}] of CA224, the G_0/G_1 block was fully maintained (Fig. 3a) indicating that CA224 most likely inhibits cellular Cdk4. A higher G_0/G_1:S ratio is observed in cells released from serum starvation, in the presence of CA224, compared to serum-starved cells. This is because nearly all cells in the S phase, present during serum starvation, enter the G_2/M phase after release, while cells in G_0/G_1 phase are prevented from

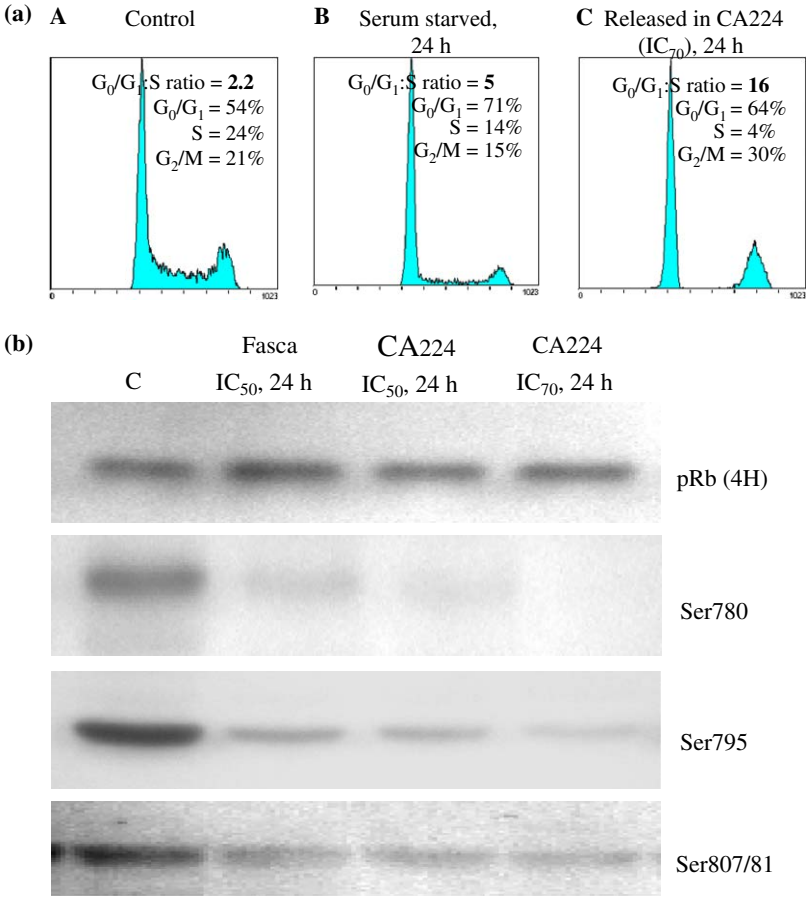


Figure 3. (a) FACS analysis of serum-starved Calu-1 cells released in the presence of CA224. (A) Untreated or control cells; (B) cells starved of serum for 24 h; (C) serum-starved cells released in the presence of CA224 at IC_{70} concentration for 24 h. (b) Western blotting of proteins obtained from asynchronous Calu-1 cells treated with CA224. Asynchronous Calu-1 cells were treated with CA224 for 24 h. The antibodies to Ser780, Ser795 and Ser807/811 detect pRB proteins phosphorylated at Ser780, Ser795 and Ser807/811, respectively, while pRb (4H) detects both phosphorylated and unphosphorylated forms of the pRB protein. The lane 'C' indicates proteins from untreated cells; 'fasca' is an abbreviation of fascaplysin.

entering into the S phase of the cell cycle (Fig. 3a, B and C) suggesting inhibition of Cdk4 enzyme at the cellular level.

Western blot analysis. Cdk4 phosphorylates pRB at specific serine residues, Ser780, Ser795 and Ser807/811, when cells progress from G_0/G_1 to G_1/S phases of the cell cycle. If CA224 were to be a cellular Cdk4 inhibitor, it should prevent phosphorylation at these serine residues when asynchronously growing cells are treated with CA224. Calu-1 cells were seeded in tissue culture flasks. When cells reached 40–50% confluency, they were treated with IC_{50} and IC_{70} concentrations of CA224, or IC_{50} concentration of fascaplysin 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 amounts of proteins in the clear supernatant were estimated using the Bradford method (Bio-Rad). Fifty micrograms of protein from each sample was 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 antibodies at room temperature for 1 h. Immuno-reactivity was visualised with the enhanced chemiluminescence Western blot detection reagents (GE-Amersham).

The Western blot analyses (Fig. 3b) show that, after treatment of Calu-1 cells with CA224 (IC_{50} and IC_{70}) for 24 h, pRB remains unphosphorylated at Ser780, Ser795 and Ser807/811 which are specifically phosphorylated by the Cdk4 enzyme. The total pRB levels in CA224-treated cells remain unchanged. As reported earlier,[2] it is observed that fascaplysin treatment of cancer cells also prevents pRB phosphorylation at the same Cdk4-specific serine residues.

In conclusion, novel tryptamine derivatives, which were designed on the basis of the fascaplysin structure, have been shown to be inhibiting Cdk4 selectively vis a vis Cdk2 and their cellular relevance has been confirmed using CA224, the most potent compound of this series. With this proof of concept in hand, we think it would be possible to generate more potent CA224 analogues

using our fascaplysin structure-based chemical biological approach.

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