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Identification of potent cholecystokinin-B receptor antagonists: synthesis, molecular modeling and anti-cancer activity against pancreatic cancer cells†‡

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Advanced malignant stages of pancreatic cancer have poor prognosis and very few treatment strategies are available. Pancreatic cancer is known to possess unique growth-related receptors that when activated, stimulate tumour proliferation. Gastrin and its related peptide cholecystokinin (CCK) are also significantly involved in the growth of this cancer type as well as other malignancies through activation of the cholecystokinin-B receptor (CCK-BR). New treatment strategies with CCK-BR antagonists are being suggested that suppress the growth promoting effects of gastrin. In this paper, we report the development of two series of quinazolinone derivatives incorporating hydrazinecarbothioamide (compounds **3a–g**) and the hydrazino group (compounds **4a–e**) as linkers for developing CCK-BR antagonists. The affinities of the compounds were determined using docking into the CCK-BR homology modeled structure. The compounds were tested for *in vitro* CCK-BR binding and gastric acid secretion in an isolated lumen-perfused mouse stomach assay. The compounds exhibited CCK-BR binding activity (IC₅₀) in the range of 0.2–975 nM and showed good gastric acid secretion inhibitory activity. Molecular modeling of the compounds was done and pharmacophore mapping results showed good prediction of *in vitro* activity which correlated well with the experimental antagonistic activity. The compounds were further tested for their cytotoxicity on CCK-BR expressing pancreatic cancer cells. The results of the study provided two potent CCK-BR antagonists which also possess good to moderate growth inhibitory activities against pancreatic cancer cells.

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Introduction

Pancreatic cancer in advanced malignant stages has a poor prognosis^{1,2} and currently available therapeutic modalities have not markedly improved the survival rate.³ Pancreatic cancer is the twelfth most common cancer in the world. It is suggested that this malignancy will surpass colon and breast cancer in the next decade with the current rate of rise in incidence and therefore will become the second leading cause of cancer-related deaths in the USA.⁴

Gastrointestinal peptides such as gastrin and cholecystokinin (CCK) have been reported to have growth stimulatory ef-

fects on pancreatic cancer and the receptors for these peptides are markedly over-expressed in pancreatic cancer.^{5,6} These peptide hormones activate their receptors (CCK in this case) in pancreatic cancer through induction of the AKT pathway⁷ resulting in cell proliferation. There is sufficient evidence that the CCK-BR pathway is a key driver of pancreatic carcinogenesis, pancreatic cancer and other malignancies.⁸

Several studies have reported the presence of a mutated form of CCK-BR containing the translated 4th intron, termed CCK-C or CCK-cancer receptor (CCK-CR) in pancreatic cancer.⁹ Studies suggest that these additional 69 amino acids of the CCK-C receptor render the receptor constitutively active where it induces proliferation even in the absence of gastrin.¹⁰ Thus blocking this receptor in pancreatic cancer becomes even more important to slow down the proliferation. Moreover, it is also interesting to know that both CCK-BR and its splice variant CCK-CR bind to CCK and gastrin.¹¹

Several treatment strategies are currently being exploited to interrupt the CCK/gastrin:CCK-receptor (CCK-R) pathway in cancer. In the last decade, several highly selective CCK-A and CCK-B receptor antagonists have been developed.¹² This

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includes the use of oligonucleotides to gastrin,¹³ gastrin-specific antibodies,¹⁴ and gastrin vaccine, "Gastrimmune", or G17DT. A randomized placebo-controlled study using vaccine therapy demonstrated a significant survival benefit in pancreatic cancer patients that elicited neutralizing antibodies towards gastrin response to vaccination with G17DT.

In addition to the above strategies, studies to stop CCK-R activation through the use of specific antagonists have been reported though an initial clinical trial done with MK-329, a selective CCK-A receptor antagonist, failed.^{15,16} Since it is a well-known fact that the CCK-BR subtype is the primary type of receptor that mediates the cancer growth in pancreatic cancer patients, the research focus has thus been shifted to the use of CCK-BR-specific antagonists, *e.g.* a highly selective CCK-BR antagonist, netazepide (YF476), was used to treat patients with type 1 gastrin carcinoid tumour;¹⁷ further studies are needed using more potent and selective antagonists in pancreatic cancer. A phase Ib/Ia clinical trial is underway to evaluate CCK-BR antagonist Z-360 in combination with gemcitabine (a cytosine analogue and antineoplastic agent, used as an anti-cancer chemotherapy drug) in patients with advanced pancreatic cancer.¹⁸ Recently, there has also been a study on pancreatic ductal adenocarcinoma (PDAC) which are known to constitutively express the CCK-BR, using DNA aptamer AP1153,¹⁹ and it is anticipated that new CCK-BR AP-targeted nanocarriers will have a broad capability to deliver imaging agents or therapeutic cargos specifically to PDAC cells with minimal off-target effects.

There is a dire need for novel therapeutic agents which can target the gastrin:CCK-B-receptor pathways²⁰ which may help improve the survival of advanced gastric cancer patients. All the above studies provide sufficient evidence that new treatment regimens with CCK-BR antagonists could suppress the growth promoting effects of gastrin. In the present work, we demonstrate the synthesis, docking and *in vitro* evaluation of quinazolinone derivatives as potent CCK-BR antagonists and their cytotoxicity evaluation against pancreatic cancer cells (MiaPaca-2).

There are several distinct chemical classes of CCK-BR antagonists that have previously been identified through drug discovery programs. These include benzodiazepine-based compounds (L-365,260), (YM022), and (YF476), peptoids (PD-134,308) and indole (JB93182).²¹ However, despite the variety of CCK-BR targeted ligands, very few compounds are currently available for clinical use. This may be because many compounds in this class have been associated with poor or variable pharmacokinetics, with respect to both gastrointestinal absorption and blood-brain permeability.²¹ Since our compounds are targeted against pancreatic cancer, poor blood-brain permeability could be a good tool to distinguish between peripheral and central effects of CCK-BR antagonism as already reported in the case of S-0509,²² which is currently at phase I clinical trials for gastric secretion disorders. Hence, there is a need to design new CCK-BR antagonists.

Our lab is developing CCK-BR-specific antagonists which have been designed by taking the lead from the naturally occurring compound, asperlicin, a weak and non-selective CCK

ligand. In a previous communication, we have shown the design and synthesis of one such potent antagonist linked to the fluorescein moiety and used it as an imaging agent to study receptor trafficking in CCK-BR positive cells.²³ In another communication, we have shown the synthesis and *in vivo* biological activity of another quinazolinone derivative.²⁴ We have shown that the two key moieties, a quinazolinone ring and an aryl ring linked with a proper linker, are crucial for CCK-BR binding. Continuing our efforts, in the present paper, we report the synthesis and biological evaluation of several derivatives of these two series of compounds. We decided to explore the effect of two types of linkers. One series of compounds (3a-g) was prepared by incorporating hydrazinecarbothioamide (–NHNH–CS–NH–) as the linker between the quinazolinone and the aryl moiety and the other series (4a-e) was synthesized by keeping the hydrazino group (=N–NH–) as the linker between the quinazolinone and the indanone. (Scheme 1) and the effect of substitutions in the aryl ring was studied.

Results and discussion

Chemistry

The compounds described in this paper were prepared according to Scheme 2, incorporating either hydrazinecarbothioamide (compounds 3a-g) or a hydrazine linker (compounds 4a-e). In the former series, the quinazolinone ring was prepared by a one pot reaction of anthranilic acid with phenylisothiocyanates in glacial acetic acid under reflux conditions. The hydrazone derivatives (compounds 2a-g) were obtained by treatment of the thioxoquinazolinones (compounds 1a-f) with excess of anhydrous hydrazine in refluxing absolute ethanol. The isothiocyanates which were not commercially available were prepared by the standard methodology reported in the literature.

The hydrazinoquinazolinones (compounds 2a-g) were treated either with isothiocyanates or isatin to provide compounds (3a-g) and (4a-e), respectively. One such compound (3a) was attached with fluorescein in our previous communication and has been shown to have affinity in the nM range and we successfully used it as a fluorescent probe to image CCK-BR positive cells.²³ In order to improve the pharmacokinetic properties, we reported the synthesis of the derivatives of these series of compounds and their antagonistic activities. The cytotoxic activities of these compounds have also been evaluated against CCK-BR expressing human pancreatic cancer cells (MiaPaca-2).

Molecular modeling

Pharmacophore mapping and prediction of activity. In a previous paper, we developed a pharmacophore model based on six chemically diverse series of compounds taken from the literature.²⁵ The pharmacophore model was validated on a series of test compounds taken from the literature which resulted in a good prediction of antagonistic activities. The basic features essential for CCK-BR antagonistic activities

Scheme 1 Design of CCK-BR antagonists.

Scheme 2 Scheme for the synthesis of quinazolinone derivatives: (i) ACOH, reflux; (ii) anhy. hydrazine, abs. EtOH; (iii) isothiocyanates, anhy. DMF, 110 °C; (iv) isatin, abs. EtOH, reflux.

identified in the paper were used here for the synthesis of quinazolinone derivatives. The pharmacophore²⁵ was used in the present work to predict the antagonistic activities of the synthesized compounds which correlated very well with the *in vitro* results. All the newly synthesized compounds gave a pharmacophore fit value in the range of 7.83–5.33 with predicted activities in the range of 0.82–257 nM upon mapping to the features of the pharmacophore model (Table 1).

We also compared the fit values and estimated the activities with known CCK-BR antagonists YF476 and L365,260. Compound YF476, a highly potent and orally active CCK-BR antagonist, showed a fit value of 9.864 with an estimated activity of 0.0076 nM and was mapped to all the four features with a reported IC₅₀ of 0.10 nM using a similar assay.²⁶ Another compound, L-365,260, showed a fit value of 7.946 and an estimated activity of 0.06367 nM (actual activity, IC₅₀ = 3.8

nM) mapped to three features of the thepharmacophore.²⁷ As we have shown earlier,²⁵ a fit value from 6–8 bits suggests that a compound could be highly active and those with a fit value less than 6 bits might be moderately active or weakly active compounds. The compounds developed in this study showed high (fit value 7.83, 0.82 nM as the estimated activity) to moderate activity (fit value 5.33, 257 nM as the estimated activity) which correlated well with the corresponding actual activities (Table 1). The highly active compounds were mapped similar to the potent compound L-365,260 (Fig. 1).

Homology modeling of CCK-BR. The sequence alignment of human CCK-BR (ID: P32239) and the template human A2A adenosine receptor was generated using Clustal W and manually adjusted to avoid the insertions and deletions in the conserved transmembrane regions. The sequence similarity and sequence identity of the aligned sequence are 19.2 and 36.3%, respectively. The best modeled structure with the least DOPE score²⁸ (38262.156250) and PDF energy score²⁸ (−8161.8936 kcal mol^{−1}) was selected for molecular dynamics simulations.

Molecular dynamics simulation and validation. Molecular dynamics (MD) simulations were performed to improve the structure of CCK-BR and to study its bio-physical properties. The MD simulation results show that the model clearly diverged and the conserved disulphide bridge remained stable throughout the simulations. A root mean square deviation (RMSD) of less than 1 nm was obtained in the plot of backbone atoms.

The potential energy analysed during the 4 ns molecular dynamics simulation showed that the molecular system is stabilized and remains stable throughout the molecular dynamics simulations with the average of potential energy stabilized around -3.47×10^4 kJ mol^{−1}.

Model evaluation. The model was evaluated using the Verify3D score, ERRAT score and favourable residues in the Ramachandran plot (see the ESI†). The Verify3D score of the simulated protein was 77.83, which is greater than the expected low score as well as the verify score of the modelled protein before simulations. The phi and psi angles of the receptor were verified by the Ramachandran plot which showed 81.8% of the residues falling in the favoured region, 99% within the additionally allowed region and none of the binding site residue in the disallowed region. The ERRAT score was 95.68, suggesting ‘very good quality’ of the modelled protein structure.

Active site prediction. After obtaining the final model, the active sites of the protein were predicted using Discovery Studio based on the receptor cavity method and further modified manually using the active site residues namely Asn353A^{6.55}, Arg356A^{6.58} (TM6), His207 (EL2), and Tyr189A^{4.60} (TM4) which are known to be involved in CCK-BR antagonism for non-peptide antagonists through site-directed mutagenesis and docking studies reported earlier.^{29,30}

Molecular docking. The energy minimised structures of the compounds in the study were docked into the active site of the modeled CCK-BR structure. Both L365,260 and YF476, on being docked into the modelled CCK-BR active site, revealed H-bond interactions with Arg356A^{6.58} (L365,260), Asn353A^{6.55} and Arg356A^{6.58} (YF476). Both compounds

showed π -positive charge interaction with Arg356A^{6.58} through their respective aromatic rings (Fig. 2). YF476 showed hydrophobic alkyl interaction with Arg356A^{6.58} in addition to a π -sulphur bond with Met117 (EL1) and a π -alkyl hydrophobic bond with Ile375A^{7.38}, Leu113A^{2.63} and Pro114A^{2.64}. In the case of L365,260, the benzodiazepine core also showed a π -alkyl hydrophobic bond with His364 (EL3) and Ala366 (EL3) and a hydrophobic alkyl interaction with Ile375A^{7.38}. Both ligands were further stabilized by common van der Waals interactions to residues His376A^{7.39}, Pro114A^{2.64}, Ile375A^{7.38} and Ala352A^{6.54}.

Similarly, the common binding pocket for all our synthesized ligands is composed of residues Arg356A^{6.58}, Asn353A^{6.55}, Leu113A^{2.63}, Pro114A^{2.64}, His376A^{7.39}, Ser379A^{7.42}, Thr111A^{2.61}, Ile375A^{7.38} and Val349A^{6.51}, which are in the distance interval of 1.5 to 10 Å from the closest ligand atom. In addition to many hydrophobic contacts that contributed the most to the binding, ionic and hydrogen bonds could also be identified. The most active compound 3c showed π -bonding of the quinazolinone core with Arg356A^{6.58}, similar to that shown by L365,260 and YF476 (discussed above). 3c also interacted through a hydrophobic π - π T-shaped bond with His376A^{7.39} and two H-bonds with Asn353A^{6.55}. The residues, namely Leu113A^{2.63}, Pro114A^{2.64} and Arg356A^{6.58}, were also found to form π -alkyl hydrophobic bonds with 3c. Similar to YF476 and L365,260, it also showed van der Waals interactions with residues Leu113A^{2.63}, Pro114A^{2.64}, and Ile375A^{7.38} (Fig. 3). Compound 3f also showed π -bonding of the quinazolinone core with Arg356A^{6.58} as seen in L365,260 and YF476 along with a H-bond with Asn353A^{6.55}. It also showed π -alkyl hydrophobic bonds with His376A^{7.39} and Pro114A^{2.64} and was further stabilised by hydrophobic alkyl bonds with Ile372 and Met134.

In the case of 4a, there was one close contact between the carbonyl group of the quinazolinone core and Asn353A^{6.55} (TM6). Both compounds 4a and 4b showed π -bonding of the quinazolinone core and Arg356A^{6.58} as seen in L365,260 and YF476. Compound 4a also exhibited a H-bond with residue Arg213 (EL2) and van der Waals interactions with Pro114A^{2.64}, Ala352A^{6.54}, Ile375A^{7.38} and Thr111A^{2.61} (Fig. 3). Ligand 4a also showed hydrophobic π -alkyl interactions with Arg356A^{6.58}, Val349A^{6.51} and Ala352A^{6.54}, whereas Leu113A^{2.63}, Pro114A^{2.64} and Arg356A^{6.58} were found to form π -alkyl hydrophobic bonds with 4b, as also reported in the case of 3c. This is in agreement with reported interacting residues in earlier studies.^{29,30} Similar to YF476 and L365,260, both 4a and 4b were further stabilized by common van der Waals interactions to residues Pro114A^{2.64}, Ile375A^{7.38} and Val349A^{6.51}.

Therefore, the two amino acids Asn353A^{6.55} and Arg356A^{6.58} (TM6) appear to be much more involved in the antagonistic activity of all ligands through H-bonds and several residues with hydrophobic interactions. The pharmacophore mapping results of L365,260 and YF476 also exhibited four features, namely two hydrogen bond donors and two hydrophobic features. These features/groups were found to interact within the binding site of the CCK-BR receptor through the similar groups in the ligands. Hence the

Table 1 CCK-BR antagonists 3a–g and 4a–e produced via Scheme 1

Comp. no.	Substitution	Potential energy (kcal mol ⁻¹)	Fit value	Features mapped	Estimated activity		Actual activity	Activity scale ^b (estimated)	Activity scale (actual)	CDOCKER energy (kcal mol ⁻¹)	CDOCKER interaction energy (kcal mol ⁻¹)	Gastric acid secretion inhibition (%) at 100 nM	Cytotoxicity evaluation	
					IC ₅₀ (nM)	IC ₅₀ (nM)							IC ₅₀ (μM)	
YF476	—	—	9.864	1111	0.0076	0.10 ^a	+++	+++	+++	-22.52	-37.96	ND	ND	
L365,260	—	0.1167	7.946	1011	0.0636	5.2 ^a	+++	+++	+++	-19.09	-33.84	ND	0.0018	
R														
3a	<i>p</i> -OC ₂ H ₅	<i>m</i> -COOH	7.75	1011	0.985	0.66	+++	+++	+++	-19.33	-36.16	58	280	
3b	<i>m</i> -Br	<i>m</i> -COOH	7.49	1011	1.74	8.64	+++	+++	+++	-18.38	-37.46	62	150	
3c	<i>p</i> -Br	<i>m</i> -COOH	7.17	1011	3.79	0.2001	+++	+++	+++	-17.73	-37.85	39	200	
3d	H	<i>m</i> -COOH	5.33	1001	257	975.4	+	+	+	-20.87	-35.01	48 at 1 μM	180	
3e	<i>p</i> -Cl	<i>p</i> -OC ₂ H ₅	7.72	1011	1.05	9.715	+++	+++	+++	-17.91	-37.05	53	80	
3f	<i>p</i> -OC ₂ H ₅	<i>p</i> -Br	7.81	1011	0.863	2.005	+++	+++	+++	-21.37	-34.33	39	10	
3g	<i>p</i> -OC ₂ H ₅	<i>p</i> -Cl	7.83	1011	0.82	11.48	+++	+++	+++	-16.09	-37.12	58	250	
4a	<i>p</i> -OC ₂ H ₅	—	6.78	1011	9.37	17.36	+++	+++	+++	4.78	-31	48	80	
4b	<i>p</i> -CH ₃	—	5.48	1011	185.5	56.49	++	++	++	2.26	-28.26	67 at 300 nM	10	
4c	<i>p</i> -Cl	—	5.55	1011	158.2	ND	++	++	ND	5.3	-33.43	58	100	
4d	<i>m</i> -Cl	—	5.74	1011	100.8	384.2	++	++	+	4.5	-28.41	58	92	
4e	<i>m</i> -Br	—	6.38	1011	23.1	19.46	++	++	+++	1.9	-35.36	62	200	

^a Activities were taken from the literature determined through a similar assay (*J. Med. Chem.*, 1993, 36(26), 4276–4292 & G. S. Baldwin, *PNAS*, 1994, 91, 7593–7597). ^b Activity scale: +++ (0–20 nM, highly active), ++ (30–200 nM, moderately active), + (>200 nM, poorly active) (*J. Chem. Inf. Model.*, 2005, 45, 1934–1942)).

Fig. 1 Mapping of the CCK-BR antagonists onto the pharmacophore model (hypothesis 1). The blue contour represents the HY-ALI features, the cyan contour represents the HY-AR features, and the dark pink contour represents the HBD features.

ligand-based approach used in our previous study²⁵ could be utilized here to optimize the compounds and docking of these compounds showed important binding interactions.

Biological evaluation

Receptor binding assay and SAR. The affinity of the compounds for the CCK-BR was determined by a competitive binding assay with a Bolton–Hunter labelled CCK-8 radioligand, ¹²⁵I-BH-CCK-8 (Perkin Elmer). Compounds in both series showed good binding activities in the range of 0.2–975 nM (Table 1). Compound 3c with *p*-bromo substitution in ring A and *m*-COOH in ring B showed the highest affinity (IC₅₀ 0.200 nM) followed by 3a (ref. 23) (*p*-OC₂H₅, ring A; *m*-COOH, ring B; IC₅₀ 0.66 nM). Compound 3g (*p*-OC₂H₅, ring A; *p*-Cl, ring B) showed an IC₅₀ of 11.48 nM, whereas 3e (*p*-Cl, ring A; *p*-OC₂H₅) and 3b (*m*-Br, ring A; *m*-COOH, ring B) showed an IC₅₀ of 9.715 and 8.64 nM, respectively. Compound 3f (*p*-OC₂H₅, ring A; *p*-Br, ring B) also showed good affinity (2.005 nM) whereas the unsubstituted ring A in com-

pound 3d resulted in an IC₅₀ of 975 nM. Therefore, the substitution in ring A is important for improving the activity and a polar substituent in ring B improves the activity.

The other series of compounds with a hydrazino linker and substitution in only ring A and with ring B being indanone resulted in overall less active compounds. In this series, the most active compound is 4a with *p*-OC₂H₅ substitution in ring A (IC₅₀ 17.36 nM) favoured over *p*-CH₃ (4b, IC₅₀ 56.49 nM). *m*-Br substitution in compound 4e (IC₅₀ 19.46 nM) in ring A resulted in better affinity over *m*-Cl (IC₅₀ 384 nM). Overall, the electron donating group improved the activity in this series of compounds.

To summarize the SAR, we suggest that overall the substitution in ring A is important for CCK-BR antagonistic activity with *p*-OC₂H₅ substitution (3a) and *p*-Br (3c) in ring A along with a *m*-COOH group in ring B being favourable in the series with the hydrazinecarbothioamide linker. Both these compounds were also predicted highly active through a pharmacophore mapping exercise and their actual activities correlated well with the estimated activities with fit values greater than 7 (designated as +++ in Table 1). The most favoured substitution in the compounds with the hydrazino group as the linker and indanone as ring B was *p*-OC₂H₅ (4a) which was found to be active in the *in vitro* binding experiment. Compounds 3c and 4a also exhibited favourable interactions in the CCK-BR binding site as discussed in the docking results above and shown in Table 1.

Gastric acid secretion assay. To further establish the ability of the compounds in the two series, they were tested to antagonize pentagastrin-induced gastric acid secretion in an isolated lumen perfused mouse stomach assay. All the compounds produced concentration dependent, non-parallel shifts to the right of the concentration response curves to Boc-pentagastrin^{12,31} and decreased the maximum response. Therefore, pA₂ (the negative algorithm of the molar concentration of a dose of the agonist to that of half the dose) could not be calculated. The compounds were capable of inhibiting the gastric acid secretion (Table 1) at 100 nM. The compounds showed almost 50–60% inhibition and one compound with no substitution in ring A showed an inhibition of 39% at a much higher concentration (1 μM), again suggesting that the substitution in ring A is important for specific CCK-BR antagonistic activity in both series of compounds.

Cytotoxicity evaluation. All the compounds synthesized in this study were then tested for their anti-proliferative activity against pancreatic cancer cells (MiaPaca-2) using the MTT assay.³² The growth inhibition effect evaluated through dose-response graphs showed that all the quinazolinone derivatives exhibited a growth inhibitory effect and an IC₅₀ in the range of 10–280 μM was obtained. The positive control for the experiment L365,260 showed an IC₅₀ of 0.0018 μM. Compound 3f with a hydrazine linker and *p*-OC₂H₅ (ring A) and *p*-Br (ring B) substitution exhibited the best IC₅₀ (10 μM) whereas compound 4b showed a similar activity (IC₅₀ 10 μM) to the indole series. Therefore, our results provided two potent CCK-BR

Fig. 2 Docked structures of L365,260 and YF476 into the homology modelled CCK-B structure.

antagonists exhibiting good CCK-BR antagonistic activity as well as cytotoxicity against CCK-BR expressing pancreatic cancer cells (MiaPaca-2) (Table 1).

Inhibition of proliferation of MiaPaca-2 cells induced by Boc-pentagastrin. Smith *et al.*³³ reported that the growth responses of several human pancreatic cancer cell lines are stimulated by CCK in serum-free medium, and its tropic effect can be blocked by a specific antagonist.⁵ They also demonstrated that gastrin exerts a growth-stimulating effect on pancreatic cancer *in vivo* and *in vitro*.³⁴ Therefore, we checked whether the cytotoxicity shown by compounds 3f and 4b in MiaPaca-2 cells is specific towards gastrin induced proliferation or not. The cellular proliferation induced by one of the potent and specific CCK-BR agonists, Boc-pentagastrin, was undertaken. Boc-pentagastrin showed a proliferative effect in the MiaPaca-2 cells from a concentration of 1 pM to 10 μ M tested for a time period of 48 h to 120 h. No cellular growth was observed in the first 24 h. Maximum cellular growth was observed at 10 nM at 96 h (also reported earlier).³³ Both compounds 3f and 4b were tested at 10 μ M (IC_{50} concentration as determined from the MTT assay) and at 1 μ M to evaluate their inhibitory effect on specific growth induced by Boc-pentagastrin. The compounds exhibited anti-

proliferative action against MiaPaca-2 cells at both tested concentrations (Fig. 4a and b), exhibiting maximum inhibition at 72 h when given alone. The compounds also inhibited the proliferative effects of Boc-pentagastrin when used together. Several studies have been performed to show the presence of the CCK-C receptor subtype in pancreatic cancer cells but not in normal tissues.³⁵ Our results suggest that compounds 3f and 4b might show some kind of selectivity towards CCK-C receptors in the MiaPaCa-2 cell line which has been shown to express this splice variant.⁹

ADMET prediction. The intestinal absorption and blood-brain barrier penetration were predicted by developing an ADME model using descriptors 2D PSA and AlogP98 that include 95% and 99% confidence ellipses. These ellipses define regions where well-absorbed compounds are expected to be found. The results of DS 4.0-ADME model screening showed that both our active compounds 3f and 4b possess 99% confidence levels for human intestinal absorption and show low blood-brain barrier (BBB) penetration. Since we are targeting pancreatic cancer, the low BBB penetration of our active compounds is advantageous. Also, TOPKAT results predicted that both 3f and 4b are non-toxic, non-mutagen and non-irritant to the skin, which makes our active compounds competitive

Fig. 4 Time and concentration dependent effect of antagonists **3f** (a) and **4b** (b) on the cell viability of MiaPaCa-2 cells. The lines depict various concentrations of antagonists (alone), Boc-pentagastrin (alone) and/or combination of antagonist and Boc-pentagastrin. DMSO control at all the time points is taken as 100%.

inhibited the growth stimulatory effects of gastrin in MiaPaCa-2 cells and exhibited the best computed ADMET properties.

Experimental section

Analytical grade materials were purchased from commercial suppliers and were used without purification, unless otherwise noted. Melting points were determined on a Thomas Hoover apparatus and are uncorrected. The ^1H NMR spectra were obtained using a Bruker 300 MHz spectrometer at ambient temperature. IR spectroscopy was performed with a FTIR apparatus (Model 1600, Perkin Elmer). Mass spectra were recorded on an AP1, PE SCEIX system. Fast atom bombardment (FAB) mass spectra were recorded with an SX 102/DA-6000 mass spectrometer (JEOL) with *m*-nitrobenzyl alcohol as the matrix; elemental analysis was done using a Carlo Erba 1108 (Heraeus, Hanau, Germany). All experiments involving animals were approved by the Institutional Animal Ethics Committee under the Committee for the Purpose of Control and Supervision and Experimentation on Animals (CPCSEA), Ministry of Environment, Government of India.

General method for synthesis of 3-aryl-2-thioxo-2,3-dihydroquinazolinone-4-(1H)-one (1a-f)

Anthranilic acid (3.0 g, 0.021 M) and appropriately substituted aryl isothiocyanate (0.022 M) were refluxed in glacial acetic acid (75 mL) for 16 h. The white lustrous crystals were separated by filtration and washed with water until the washings showed neutral pH. The crystalline product was dried and checked for purity using TLC (ethyl acetate-petroleum ether in 50%).

3-(4-Methylphenyl)-2-thioxo-2,3-dihydroquinazoline-4-(1H)-one (1a). Yield, 4.5 g (85%); mp > 300 °C; IR (KBr) ν_{max} /cm $^{-1}$; 3245.02 (NH), 1662.43 (C=O), 1200.05 (C=S); ^1H NMR (DMSO- d_6); δ 12.965 (Brs, 1H, D $_2$ O exchangeable), 7.953 (d, 1H, J = 7.8 Hz), 7.797 (t, 1H, J = 7.65 Hz), 7.450 (d, 1H, J = 8.1 Hz), 7.359 (t, 1H, J = 6.0 Hz), 7.276 (d, 2H, J = 6.6 Hz), 7.140–7.120 (m, 1H), 2.364 (s, 3H).

3-(4-Ethoxyphenyl)-2-thioxo-2,3-dihydroquinazoline-4-(1H)-one (1b). Yield, 4.914 g (80%); mp 285 °C; IR (KBr) ν_{max} /cm $^{-1}$; 3223.54 (NH), 1662.59 (C=O), 1205.10 (C=S). ^1H NMR (DMSO- d_6); δ 11.987 (Brs., 1H, D $_2$ O exchangeable), 7.942 (d, 1H, J = 8.7 Hz), 7.688–7.637 (m, 2H), 7.339 (t, 1H, J = 5 Hz), 7.122 (d, 2H, J = 8.7 Hz), 6.809 (d, 2H, J = 9.0 Hz), 4.223 (q, 2H, J = 6.8 Hz), 1.392 (t, 3H, J = 6.9 Hz).

3-Phenyl-2-thioxo-2,3-dihydroquinazoline-4-(1H)-one (1c). Yield, 1.930 g (90%); mp > 300 °C, IR (KBr) ν_{max} /cm $^{-1}$; 3219 (NH), 1662.5 (C=O), 1197.7 (C=S), ^1H NMR (DMSO- d_6); δ 13.037 (Brs, 1H, D $_2$ O exchangeable), 7.958 (d, 2H, J = 7.8 Hz), 7.798 (t, 2H, J = 5.0 Hz), 7.472–7.253 (m, 5H).

3-(4-Bromophenyl)-2-thioxo-2,3-dihydroquinazoline-4-(1H)-one (1d). Yield, 1.608 g (99%); mp > 320 °C; IR (KBr) ν_{max} /cm $^{-1}$; 3235.35 (NH), 1663 (C=O), 1199.05 (C=S), ^1H NMR (DMSO- d_6); δ 13.097 (Brs, 1H, D $_2$ O exchangeable), 7.973 (d, 1H, J = 7.8 Hz), 7.839 (t, 1H, J = 5.1 Hz), 7.695 (d, 2H, J = 7.2 Hz), 7.461 (d, 1H, J = 8.1 Hz), 7.383 (t, 1H, J = 5.0 Hz), 7.290 (d, 2H, J = 7.2 Hz).

3-(4-Chlorophenyl)-2-thioxo-2,3-dihydroquinazoline-4-(1H)-one (1e). Yield, 1.783 g (100%); mp > 320 °C; IR (KBr) ν_{max} /cm $^{-1}$; 3246.0 (NH), 1662.5 (C=O), 1199.6 (C=S), 759.9 (C-Cl), ^1H NMR (DMSO- d_6); δ 11.971 (Brs, 1H, D $_2$ O exchangeable), 7.964 (d, 1H, J = 6.3 Hz), 7.815 (t, 1H, J = 7.6 Hz), 7.557 (d, 1H, J = 5.0 Hz), 7.445–7.376 (m, 4H), 7.350–7.267 (m, 1H).

3-(3-Chlorophenyl)-2-thioxo-2,3-dihydroquinazoline-4-(1H)-one (1f). Yield, 1.67 g (98%); mp 298 °C; IR (KBr) ν_{max} /cm $^{-1}$; 3205 (NH), 1664.78 (C=O), 1199.21 (C=S), 761 (C-Cl), ^1H NMR (DMSO- d_6); δ 13.105 (Brs, 1H, D $_2$ O exchangeable), 7.974 (d, 1H, J = 7.2 Hz), 7.824 (t, 1H, J = 7.6 Hz), 7.548–7.468 (m, 3H), 7.439 (s, 1H), 7.388 (d, 1H, J = 7.5 Hz), 7.338 (t, 1H, J = 7.0 Hz).

3-(3-Bromophenyl)-2-thioxo-2,3-dihydroquinazoline-4-(1H)-one (1g). Yield: 0.50 g (38%); mp 295 °C, IR (KBr) ν_{max} /cm $^{-1}$; 3210 (NH), 1672.42 (C=O), 1197.74 (C=S), ^1H NMR (DMSO- d_6); δ 13.105 (Brs, 1H, D $_2$ O exchangeable), 7.973 (d, 1H, J = 8.1 Hz), 7.824 (t, 1H, J = 7.6 Hz), 7.636–7.612 (m, 2H), 7.462 (d, 2H, J = 7.5 Hz), 7.386–7.334 (m, 2H).

General procedure for synthesis of 2-hydrazino-3-arylquinazoline-4-(3*H*)-one (2a–g)

A mixture of 1 (0.016 M) and anhydrous hydrazine (5.87 mL, 0.16 M) in anhydrous methanol was refluxed for 18 h. The solid was separated by filtration, washed with cold methanol and dried.

2-Hydrazino-3-(4-methylphenyl)quinazoline-4-(3*H*)-one (2a). Yield, 2.2 g (71%); mp 193–194 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3497.67, 3311.65, 3209.05 (NH's), 1673.02 (C=O); ^1H NMR (DMSO- d_6); δ 7.925 (d, 1H, J = 7.8 Hz), 7.787–7.761 (d, 1H, J = 7.8 Hz), 7.670 (t, 1H, J = 7.0 Hz), 7.389 (m, 1H), 7.361 (d, 2H, J = 7.5 Hz), 7.187 (d, 2H, J = 7.8 Hz), 6.787 (Brs, 1H, D₂O exchangeable), 4.391 (Brs, 2H, D₂O exchangeable), 2.393 (s, 3H).

2-Hydrazino-3-(4-ethoxyphenyl)quinazoline-4-(3*H*)-one (2b). Yield, 3.4 g (79%); mp 222 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3423.4 (NH–NH₂), 1706.9 (C=O), ^1H NMR (DMSO- d_6); δ 7.924 (d, 1H, J = 7.8 Hz), 7.669 (t, 1H, J = 7.6 Hz), 7.359 (d, 1H, J = 7.2 Hz), 7.211 (d, 2H, J = 8.7 Hz), 7.158 (t, 1H, J = 5.0 Hz), 7.070 (d, 2H, J = 9.0 Hz), 6.906 (Brs, 1H, D₂O, exchangeable), 4.458 (Brs, 2H, D₂O exchangeable), 4.122–4.053 (q, 2H, J = 6.9 Hz), 1.392–1.346 (t, 3H, J = 6.9 Hz).

2-Hydrazino-3-phenylquinazoline-4-(3*H*)-one (2c). Yield, 2.5 g (80%); mp 205 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3487.67, 3229.05 (NH's), 1673.02 (C=O), ^1H NMR (DMSO- d_6); δ 8.220 (Brs, 1H, D₂O exchangeable), 7.910 (m, 2H), 7.655 (d, 1H, J = 7.2 Hz), 7.557 (m, 2H, J = 8.4 Hz), 7.399 (d, 1H, J = 8.1 Hz), 7.372–7.327 (m, 2H), 7.161 (d, 1H, J = 7.8 Hz), 4.419 (Brs, 2H, D₂O exchangeable).

2-Hydrazino-3-(4-bromophenyl)quinazoline-4-(3*H*)-one (2d). Yield, 0.185 g (37%); mp 210 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3291.67, 3330.86 (NHs), 1676.23 (C=O), ^1H NMR (DMSO- d_6); δ 8.225 (Brs, 1H, D₂O exchangeable), 8.011 (d, 1H, J = 7.8 Hz), 7.962 (d, 2H, J = 8.1 Hz), 7.694 (t, 1H, J = 7.5 Hz), 7.543 (d, 2H, J = 8.4 Hz), 7.413 (d, 1H, J = 8.1 Hz), 7.278 (t, 1H, J = 7.5 Hz), 4.386 (Brs, 2H, D₂O exchangeable).

2-Hydrazino-3-(4-chlorophenyl)quinazoline-4-(3*H*)-one (2e). Yield, 0.58 g (58%); mp 190 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3210.33, 3306.27 and 3328.41 (NHs), 1675.57 (C=O), ^1H NMR (DMSO- d_6); δ 8.352 (Brs, 1H, D₂O exchangeable), 8.022 (d, 1H, J = 9.9 Hz), 7.940 (d, 1H, J = 9.0 Hz), 7.693 (t, 1H, J = 7.5 Hz), 7.422 (d, 2H, J = 9.0 Hz), 7.331 (d, 2H, J = 8.4 Hz), 7.251 (t, 1H, J = 7.5 Hz), 4.41 (Brs, 2H, D₂O exchangeable).

2-Hydrazino-3-(3-chlorophenyl)quinazoline-4-(3*H*)-one (2f). Yield, 0.267 g (26%); mp 170 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3312.35 (NHs) 1674.79 (C=O), 759.19 (C–Cl), ^1H NMR (DMSO- d_6); δ 8.823 (Brs, 1H, D₂O exchangeable), 8.025 (d, 1H, J = 7.8 Hz), 7.290 (d, 1H, J = 5.0 Hz), 7.131 (d, 1H, J = 7.8 Hz), 7.404 (d, 1H, J = 8.1 Hz), 7.686 (t, 1H, J = 4.0 Hz), 7.574–7.493 (m, 2H), 4.370 (Brs, 2H, D₂O exchangeable).

2-Hydrazino-3-(3-bromophenyl)quinazoline-4-(3*H*)-one (2g). Yield, 0.289 g (28%); mp 190 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3306.60 (NHs), 1672.42 (C=O), ^1H NMR (DMSO- d_6); δ 8.248 (Brs, 1H, D₂O exchangeable), 4.382 (Brs, 2H, D₂O exchangeable), 8.025 (d, 1H, J = 7.8 Hz), 7.971 (d, 1H, J = 7.8 Hz), 7.822 (t, 1H, J = 7.6 Hz), 7.711–7.661 (t, 1H, J = 7.5 Hz), 7.61 (s, 1H), 7.461–7.266 (m, 3H).

General method for synthesis of {*N*-aryl-2-[3,4-dihydro-3 or 4-substituted aryl]-4-oxo-2 quinazonyl]} hydrazinecarbothioamide (3a–g)

An equimolar mixture of appropriate compound 2 (0.878 mM) and 3- or 4-substituted aryl isothiocyanate (0.878 mM) was stirred in DMF under an inert atmosphere at 110 °C for 12 h. DMF was evaporated by using a rotavapor. The residue obtained was washed with cold methanol, filtered and dried to give 3a–g.

3-(2-(3-(4-Ethoxyphenyl)-4-oxo-3,4-dihydroquinazolin-2-yl)-hydrazinecarbothioamido)benzoic acid (3a). Yield, 1.90 g (60%); mp 239 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3442.5 (NH), 1662.5 (C=O), 1249.8 (C=S), 2924.52 (–OC₂H₅); ^1H NMR (DMSO- d_6); δ 12.995 (s, 1H, D₂O exchangeable), 11.500 (Brs, 1H, D₂O exchangeable), 9.520 (Brs, 1H, D₂O exchangeable), 8.234 (Brs, 1H, D₂O exchangeable), 8.207 (s, 1H), 7.955 (d, J = 7.56, 1H), 7.796 (t, 1H, J = 7.6 Hz), 7.686 (t, 1H, J = 7.5 Hz), 7.446 (d, 1H, J = 8.34 Hz), 7.381 (t, 1H, J = 7.652 Hz), 7.204–7.314 (m, 1H), 7.204 (d, 2H, J = 8.1 Hz), 7.094 (d, 1H, J = 7.8 Hz), 6.994 (d, 2H, J = 7.9 Hz), 4.101 (q, 2H, J = 6.4 Hz), 1.380 (t, 3H, J = 6.6 Hz); MASS (m/z) = 474 (C₂₄H₂₁N₅SO₄[M – 1]⁺). Anal. calcd. for C₂₄H₂₁N₅SO₄: C, 60.62; H, 4.45; N, 14.73. Found: C, 60.76; H, 4.52; N, 14.89.

3-(2-(3-(3-Bromophenyl)-4-oxo-3,4-dihydroquinazolin-2-yl)-hydrazinecarbothioamido)benzoic acid (3b). Yield: 0.658 g (65%); mp 195 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3327.92 (NH), 1670.16 (C=O), 1197.92 (C=S), 759.96 (C–Br); ^1H NMR (DMSO- d_6); δ 12.564 (s, 1H, D₂O exchangeable), 11.545 (Brs, 1H, D₂O exchangeable), 9.296 (Brs, 1H, D₂O exchangeable), 8.503 (Brs, 1H, D₂O exchangeable), 8.384 (d, 1H, J = 7.6 Hz), 7.976 (d, 1H, J = 8.3 Hz), 7.984 (t, 1H, J = 5.0 Hz), 7.892 (d, 1H, J = 7.2 Hz), 7.836–7.829 (m, 3H), 7.908 (d, 2H, J = 7.8 Hz), 7.743–7.712 (m, 1H), 6.923 (d, 2H, J = 5.0 Hz); MASS (m/z) M⁺ = 509 (C₂₂H₁₆BrN₅O₃S). Anal. calcd. for C₂₂H₁₆BrN₅O₃S: C, 51.77; H, 3.16; N, 13.72. Found: C, 51.89; H, 3.28; N, 13.83.

3-(2-(3-(4-Bromophenyl)-4-oxo-3,4-dihydroquinazolin-2-yl)-hydrazinecarbothioamido)benzoic acid (3c). Yield, 0.352 g (50%); mp 220 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3330.73 (NH), 1676.02 (C=O), 1223.82 (C=S), 762.19 (C–Br), ^1H NMR (DMSO- d_6); δ 12.539 (s, 1H, D₂O exchangeable), 11.691 (Brs, 1H, D₂O exchangeable), 9.295 (Brs, 1H, D₂O exchangeable), 8.399 (Brs, 1H, D₂O exchangeable), 8.359 (d, 1H, J = 6.3 Hz), 7.975 (d, 1H, J = 9.4 Hz), 7.940 (t, 1H, J = 7.2 Hz), 7.892 (d, 1H, J = 7.3 Hz), 7.836–7.819 (m, 3H), 7.808 (d, 2H, J = 7.8 Hz), 7.753–7.712 (m, 1H), 6.823 (d, 2H, J = 5.1 Hz); MASS (m/z) M⁺ = 509 (C₂₂H₁₆BrN₅O₃S). Anal. calcd. for C₂₂H₁₆BrN₅O₃S: C, 51.77; H, 3.16; N, 13.72. Found: C, 51.88; H, 3.29; N, 13.85.

3-(2-(4-Oxo-3-phenyl-3,4-dihydroquinazolin-2-yl)-hydrazinecarbothioamido)benzoic acid (3d). Yield, 1.354 g (50%); mp 263 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$; 3435.92 (NH), 1675.93 (C=O), 1298.66 (C=O); ^1H NMR (DMSO- d_6); δ 12.784 (s, 1H, D₂O exchangeable), 11.504 (Brs, 1H, D₂O exchangeable), 9.534 (Brs, 1H, D₂O exchangeable), 8.292 (Brs, 1H, D₂O exchangeable), 8.334 (d, 1H, J = 7.8 Hz), 7.956 (d, 1H, J = 9.4 Hz), 7.924–7.909 (t, 1H, J = 5.0 Hz), 7.892 (d, 1H, J = 7.2 Hz), 7.836–7.829 (m, 3H), 7.908 (d, 2H, J = 9.2 Hz), 7.743–7.712 (m, 2H), 6.723 (d, 2H, J = 5.0 Hz); MASS (m/z) M⁺ = 431

(C₂₂H₁₇N₅O₃S). Anal. calcd. for C₂₂H₁₇N₅O₃S: C, 61.24; H, 3.97; N, 16.23. Found: C, 61.39; H, 3.90; N, 16.10.

2-(3-(4-Chlorophenyl)-4-oxo-3,4-dihydroquinazolin-2-yl)-N-(4-ethoxyphenyl)hydrazinecarbothioamide (3e). Yield, 0.798 g (65%); mp 242–244 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3330 (NH), 1698.94 (C=O), 1296.55 (C=S), 2967.2 (C-H); ¹H NMR (DMSO-d₆); δ 10.775 (Brs, 1H, D₂O exchangeable), 9.932 (Brs, 1H, D₂O exchangeable), 8.659 (Brs, 1H, D₂O exchangeable), 7.955 (d, 1H, *J* = 7.2 Hz), 7.797 (t, 1H, *J* = 7.6 Hz), 7.544 (t, 1H, *J* = 9.3 Hz), 7.450 (d, 1H, *J* = 8.7 Hz), 7.395 (d, 2H, *J* = 9.0 Hz), 7.164 (d, 2H, *J* = 8.7 Hz), 7.078 (d, 2H, *J* = 8.7 Hz), 6.994 (d, 2H, *J* = 8.7 Hz), 4.131 (q, 2H, *J* = 7.0 Hz), 1.391 (t, 3H, *J* = 6.8 Hz). MASS (*m/z*) *M*⁺ = 465 (C₂₃H₂₀ClN₅O₂S). Anal. calcd. for C₂₃H₂₀ClN₅O₂S: C, 59.29; H, 4.33; N, 15.03. Found: C, 59.10; H, 4.25; N, 15.21.

N-(4-Bromophenyl)-2-(3-(4-ethoxyphenyl)-4-oxo-3,4-dihydroquinazolin-2-yl)hydrazinecarbothioamide (3f). Yield, 0.285 g (57%); mp > 250 °C; I.R. (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3400 (NH), 1662.83 (C=O), 1250.86 (C=S), 2967.06 (C-H); ¹H NMR (DMSO-d₆); δ 10.945 (Brs, 1H, D₂O exchangeable), 10.235 (Brs, 1H, D₂O exchangeable), 9.198 (Brs, 1H, D₂O exchangeable), 8.246 (d, 1H, *J* = 6.6 Hz), 7.918 (d, 2H, *J* = 7.8 Hz), 7.699 (d, 2H, *J* = 7.5 Hz), 7.363–7.298 (m, 4H), 7.198 (d, 1H, *J* = 7.2 Hz), 6.860 (d, 2H, *J* = 7.5 Hz), 3.992 (q, 2H, *J* = 6.2 Hz), 1.342 (t, 3H, *J* = 6.1 Hz); MASS (*m/z*) *M*⁺ = 509 (C₂₃H₂₀BrN₅O₂S). Anal. calcd. for C₂₃H₂₀BrN₅O₂S: C, 54.12; H, 3.95; N, 13.72. Found: C, 54.28; H, 3.86; N, 13.91.

N-(4-Chlorophenyl)-2-(3-(4-ethoxyphenyl)-4-oxo-3,4-dihydroquinazolin-2-yl)hydrazinecarbothioamide (3g). Yield, 0.82 g (72%); mp 260 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3435.26 (NH), 1616.47 (C=O), 1225.79 (C=S), 2973.73 (C-H); ¹H NMR (DMSO-d₆); δ 13.355 (Brs, 1H, D₂O exchangeable), 10.450 (Brs, 1H, D₂O exchangeable), 10.303 (Brs, 1H, D₂O exchangeable), 8.123 (d, 1H, *J* = 8.1 Hz), 7.990–7.921 (m, 1H), 7.831 (d, 1H, *J* = 7.8 Hz), 7.588 (d, 2H, *J* = 7.6 Hz), 7.399–7.324 (m, 4H), 7.270 (t, 1H, *J* = 7.5 Hz), 7.106 (d, 2H, *J* = 8.4 Hz), 4.116 (q, 2H, *J* = 6.6 Hz), 1.388 (t, 3H, *J* = 6.4 Hz); MASS (*m/z*) *M*⁺ = 465 (C₂₃H₂₀ClN₅O₂S). Anal. calcd. for C₂₃H₂₀BrN₅O₂S: C, 59.29; H, 4.33; N, 15.03. Found: C, 59.12; H, 4.40; N, 15.18.

General procedure for synthesis of (Z)-3-aryl-2-(2-(2-oxoindolin-3-ylidene)hydrazinyl)quinazolin-4(3H)-one (4a–e)

An equimolar mixture of 2,3-dioxindole (0.499 g, 0.0034 M) and appropriate 2-hydrazino-3-arylquinazolin-4(3H)-one (0.0034 M) was refluxed in 50 mL of absolute ethanol for 2 h. The precipitate thus formed was filtered, washed with cold ethanol, dried and crystallized from ethanol to give compounds 4a–e.

(Z)-3-(4-Ethoxyphenyl)-2-(2-(2-oxoindolin-3-ylidene)hydrazinyl)quinazolin-4(3H)-one (4a). Yield, 2.5 g (90%); mp 275–280 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3064.7, 3184.3 (NH's), 1708.8 and 1622 (C=O); ¹H NMR (DMSO-d₆); δ 11.51 (s, 1H, D₂O exchangeable), 10.4696 (s, 1H, D₂O exchangeable), 8.006 (d, 1H, *J* = 7.5 Hz), 7.856 (d, 1H, *J* = 8.4 Hz), 7.767 (t, 1H, *J* = 7.2 Hz), 7.340 (d, 2H, *J* = 8.7 Hz), 7.311 (t, 1H, *J* = 8.6 Hz), 7.176 (t, 1H, *J* = 7.2 Hz), 7.128 (d, 2H, *J* = 8.7 Hz), 6.980 (d, 1H, *J* =

7.5 Hz), 6.755 (d, 1H, *J* = 7.5 Hz), 6.520 (t, 1H, *J* = 7.5 Hz), 4.112–4.06 (q, 2H, *J* = 6.8 Hz), 1.380 (t, 3H, *J* = 6.6 Hz); MASS (*m/z*) *M*⁺ = 425 (C₂₄H₁₉N₅O₃). Anal. calcd. for C₂₄H₁₉N₅O₃: C, 67.76; H, 4.50; N, 16.46. Found: C, 67.51; H, 4.62; N, 16.30.

(3Z)-1H-Indole-2,3-dione 3-[[3-(4-methylphenyl)-4-oxo-3,4-dihydroquinazolin-2-yl]hydrazone] (4b). Yield, 0.6 g (68%); mp 280 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3184.2 (NH), 1708.7 (C=O); ¹H NMR (DMSO-d₆); δ 11.53 (Brs, 1H, D₂O exchangeable), 10.43 (s, 1H, D₂O exchangeable), 8. (d, 1H, *J* = 7.5 Hz), 7.879 (d, 1H, *J* = 9.2 Hz), 7.768 (t, 1H, *J* = 6.6 Hz), 7.407 (d, 2H, *J* = 8.1 Hz), 7.310 (t, 2H, *J* = 8.2 Hz), 7.154 (t, 2H, *J* = 6.2 Hz), 6.839 (d, 1H, *J* = 7.5 Hz), 6.740 (d, 1H, *J* = 7.5 Hz), 6.501–6.451 (t, 1H, *J* = 7.47 Hz), 2.45 (s, 3H). MASS (*m/z*) *M*⁺ = 395 (C₂₃H₁₇N₅O₂). Anal. calcd. for: C, 69.86; H, 4.33; N, 17.71. Found: C, 69.69; H, 4.44; N, 17.57.

(3Z)-1H-Indole-2,3-dione 3-[[3-(4-chlorophenyl)-4-oxo-3,4-dihydroquinazolin-2-yl]hydrazone] (4c). Yield, 0.269 g (70%); mp 280 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3313.58 (NH's), 1691.14 (C=O); ¹H NMR (DMSO-d₆); δ 11.632 (Brs, 1H, D₂O exchangeable), 10.622 (Brs, 1H, D₂O exchangeable), 8.455 (d, 1H, *J* = 8.4 Hz), 8.027 (d, 1H, *J* = 7.5 Hz), 7.818 (d, 2H, *J* = 8.7 Hz), 7.739–7.641 (m, 1H), 7.421–7.291 (m, 4H), 7.061 (t, 2H, *J* = 7.5 Hz), 6.896 (d, 1H, *J* = 7.8 Hz); MASS (*m/z*) *M*⁺ = 415 (C₂₂H₁₄ClN₅O₂). Anal. calcd. for C₂₂H₁₄ClN₅O₂: C, 63.54; H, 3.39; N, 16.84. Found: C, 63.39; H, 3.48; N, 16.70.

(3Z)-1H-Indole-2,3-dione 3-[[3-(3-chlorophenyl)-4-oxo-3,4-dihydroquinazolin-2-yl]hydrazone] (4d). Yield, 0.052 g (34%); mp 335 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3434.84 (NH), 1623.32 (C=O), 756.94 (C-Cl); ¹H NMR (DMSO-d₆); δ 11.70 (Brs, 1H, D₂O exchangeable), 10.58 (Brs, 1H, D₂O exchangeable), 8.018 (d, 1H, *J* = 8.1 Hz), 6.91 (d, 1H, *J* = 6.9 Hz), 6.777 (d, 1H, *J* = 7.8 Hz), 6.600 (t, 1H, *J* = 6.6 Hz), 7.322 (t, 1H, *J* = 8.1 Hz), 7.188 (t, 1H, *J* = 8.2 Hz), 7.810–7.762 (m, 3H), 7.677–7.641 (m, 3H); MASS (*m/z*) *M*⁺ = 415 (C₂₂H₁₄ClN₅O₂). Anal. calcd. for C₂₂H₁₄ClN₅O₂: C, 57.41; H, 3.07; N, 15.22. Found: C, 57.27; H, 3.22; N, 15.39.

(3Z)-1H-Indole-2,3-dione 3-[[3-(3-bromophenyl)-4-oxo-3,4-dihydroquinazolin-2-yl]hydrazone] (4e). Yield, 0.050 g (38%); mp 295 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3332.73 (NH), 1710.51 (C=O); ¹H NMR (DMSO-d₆); δ 13.141 (Brs, 1H, D₂O exchangeable), 11.644 (Brs, 1H, D₂O exchangeable), 8.012–7.943 (m, 1H), 7.876 (d, 1H, *J* = 8.4 Hz), 7.805–7.758 (m, 2H), 7.609–7.433 (m, 2H), 7.385–7.266 (m, 2H), 7.236 (t, 1H, *J* = 7.5 Hz), 6.907 (d, 1H, *J* = 7.5), 6.769 (d, 1H, *J* = 7.5 Hz), 6.612 (t, 1H, *J* = 7.5 Hz); MASS (*m/z*) *M*⁺ = 459 (C₂₂H₁₄BrN₅O₂). Anal. calcd. for C₂₂H₁₄BrN₅O₂: C, 63.54; H, 3.39; N, 16.84. Found: C, 63.36; H, 3.29; N, 16.98.

Molecular modeling

All molecular modeling work was performed using Discovery Studio 2.5.5 and Discovery Studio 4.0 (Accelrys Inc.). The molecules were built using the “Builder” module. The energies were minimized using the “CHARMM” force field in a step wise manner by using “steepest descent” followed by “conjugate gradient” till a RMSD derivative of 0.001 was achieved. Conformational models of the molecules were generated

using the “best quality” conformational search option in “Catalyst” using a constraint of 20 kcal mol⁻¹ energy threshold above the global energy minimum and “CHARMM” force field parameters. A maximum of 250 conformations were generated using the “best fit method” to ensure maximum coverage in the conformational space. The pharmacophore mapping exercise was performed using the “Best mapping” protocol while keeping the “flexible method” and “maximum omitted features” at 2. All the activities were predicted as nM and fit values were calculated by the software.

Homology modeling

The sequence of human CCK-BR (ID: P32239) was retrieved from SwissProt database.³⁶ The template structure and sequence of human A2A adenosine receptor X-ray structure (3EML)³⁷ were downloaded from PDB (www.rcsb.org). The sequence alignment was generated with the Clustal W multiple sequence alignment tool and manually adjusted to avoid insertions and deletions in the transmembrane regions.

The intracellular loop (IL3) of CCK-BR was deleted as the template 3EML lacked this region. Most of the third cytoplasmic loop (Leu209^{5,70}–Ala221^{6,23}) of the template was replaced with the lysozyme from T4 bacteriophage and the carboxyl terminal tail (Ala317–Ser412) was deleted in the template 3EML.pdb to improve the likelihood of crystallization. Moreover, it has also been reported that most of the ligands bind to the extracellular region and the conserved transmembrane region of the CCK-BR.³⁸ Hence coordinates for this region could not be generated for this study.

A total of 10 models of the CCK-BR along with 30 loop models were built using the “MODELER” module present in Discovery Studio 4.0 at a low optimization level. The loop modeling was performed for the modeling of gaps created during the sequence alignment. For the specific case of CCK-BR, the conserved disulphide bridge between Cys127 and Cys205 was manually introduced according to experimental data.³⁹ This disulphide bridge is of importance in the ligand binding interaction and the structure stability of the protein. All the models were sorted on the basis of their PDF total energy as well as DOPE score. A model is better optimized against the homology restraint when it has a lower PDF total energy.

For accessing the validity of the homology model with the sequence of residues it contain, the verify score of each model was calculated by using the “Profile-3D” module present in Discovery Studio 4.0. Finally the model having the lowest PDF total energy score as well as the highest verify score was selected for molecular dynamics simulations.

Molecular dynamics simulation

The modelled structure of the CCK-BR generated above was used as the starting structure for molecular dynamics simulations. The stability of the structure was verified through molecular dynamics (MD) simulations using GROMACS⁴⁰ in an explicit membrane aqueous system. The total system consisted of 123 molecules of DPPC lipids, 31 444 water mol-

ecules, 358 amino acids, Na⁺ and Cl⁻ ions. Energy minimization was carried out using steepest descent algorithms applying Berger lipids parameters⁴¹ for the lipid component in combination with the GROMOS⁴² representation of the protein. Energy minimization was carried out using the steepest descent algorithm with strong position restrained force on the protein, so that the protein position does not change during energy minimization. The steepest descent algorithm converged to $F_{\max} < 1000 \text{ kJ mol}^{-1} \text{ nm}^{-1}$ in 1248 steps. Parinello–Rahman coupling,⁴³ Berendsen⁴⁴ and Nosé–Hoover thermostat coupling⁴⁵ were used to equilibrate the system in a stable environment (323 K, 1 bar). Electrostatic energy was computed by the Particle Mesh Ewald (PME) method⁴⁶ and the linear constraint solver (LINCS) algorithm⁴⁷ was applied to fix all bonds involving hydrogen. The structure in the medium was equilibrated for 100 ps in NVT followed by 1000 ps in NPT due to membrane simulation. Finally, a 4 nanosecond molecular dynamics simulation was carried out for the structure. The RMSD (root-mean-square deviation) from the initial structure was calculated during the 4 ns simulation run. After the MD simulation, the stereochemistry quality of the structures was validated with PROCHECK⁴⁸ and Verify 3D⁴⁹ and the quality factors of the protein models were calculated using ERRAT2,⁵⁰ available in SAVES (Structural Analysis and Verification Server) [http://nihserver.mbi.ucla.edu/SAVES/]. The Ramachandran plot⁵¹ was created by submitting the model to PDBsum.⁵²

Molecular docking

All molecular docking work was done using Discovery Studio 4.0 software packages (Accelrys, California, USA). All the simulations were performed on Intel(R) Pentium(R) IV 2.4GHz, running Windows XP professional version 2006 operating system. The modelled structure was defined as the receptor molecule and was chosen for the ‘input receptor molecule’ parameter in the C-DOCKER protocol for molecular docking. All the molecules YF476, L365,260, and the synthesized compounds (3a–g and 4a–e) were drawn and energy minimized in separate molecular windows and saved as .dsv file. The saved structures were then used as ‘input ligands’ in the CDOCKER protocol and docked into the active site of CCK-BR.

ADME parameters screening for drug-likeness

The compounds were subjected to ADMET calculations. Parameters such as aqueous solubility, absorption, plasma protein binding, cytochrome P450 2D6 inhibition, and hepatotoxicity were determined using the ADMET protocol in DS4.0 (Table TS1 and Fig. S6 in the ESI†). Moreover, the toxicity potential (*i.e.*, carcinogenicity and mutagenicity) of the compounds was also predicted using the TOPKAT (TOXicity Prediction by Komputer Assisted Technology) protocol in DS4.0 (Table TS2 in the ESI†).

Biological evaluation

Receptor binding assay. The affinity of the test compounds for the CCK-B/gastrin receptor, solubilized in

detergent, was determined by a competitive binding assay with a Bolton–Hunter-labelled CCK-8 radioligand as previously described.⁵³ For this, the plasma membrane prepared from rat cerebral cortex (100 µg per tube) was mixed with a 125I-Bolton Hunter-CCK-8 (Perkin Elmer) radio-ligand (20 pM) in the absence and presence of increasing concentrations of the test compounds. Non-specific binding was determined in the presence of 1 µM unlabeled CCK-8s peptide (Bachem, Bubendorf, Switzerland). The tubes were incubated at 25 °C for 1 h to achieve steady state binding. The bound radio-ligand was rapidly separated from the free radio-ligand using Millipore receptor binding filter mats. Bound radioactivity was quantified with a Microbeta liquid scintillation counter from Perkin Elmer. Data was analysed using the Graphpad software using the non-linear least squares curve fitting routine in the Prism 5 software of the program.

Gastric acid secretion inhibition assay. For determination of acid secretion, the whole stomach was excised from albino mice (25–30 g) and was placed in a tissue organ bath purchased from local suppliers and perfused by the methods described by Black *et al.*⁵⁴ The effluent from the perfused stomach was monitored for a change in pH using a continuous probe (Ecoscan pH meter from Eutech Instruments) inserted into the organ bath. The basal acidic pH and increase in acid output were produced by a sub-maximal concentration of Boc-pentagastrin (10 nM) before and 45 min after incubation with various concentrations of the test compound and the resultant percentage inhibition was calculated.

In vitro cytotoxicity assay. Cell survival was measured by using the MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] micro-culture tetrazolium assay, according to the method described by Mosmann.³² A total of 1000 cells per well were seeded in a 96-well plate. After 24 h of incubation in a 5% humidified CO₂ incubator at 37 °C, varying concentrations of compounds were added to a final volume of 200 µL of standard growth medium per well. After 24 h of incubation at 37 °C, 20 µL of MTT (Invitrogen) (5 mg mL⁻¹ in PBS) was added to each well and incubated for 4 h at 37 °C. The medium was removed and formazan crystals thus formed were dissolved in DMSO. The plates were read immediately in a microplate reader (Tecan, GENios-Pro, Austria) operating at 540 nm.

Exogenous gastrin assay. The tropic effects of gastrin to promote tumour growth can be blocked by specific antagonists.³³ To show whether the stimulatory effect of gastrin was blocked by 3f and 4b, the MiaPaca-2 cells were incubated with Boc-pentagastrin (Bachem) for 24 h and then treated with 3f, 4b and Boc-pentagastrin either alone or in combination. Cellular proliferation was studied using the MTT assay as described above. Briefly, the cells were seeded in a 96 well plate (4000 cells per well) in 10% FCS-DMEM. Cells were then incubated in incomplete medium for 24 h to attain the quiescent stage. Boc-pentagastrin and the antagonists were then added in the incomplete medium. The MTT assay was carried out after 48–120 h of incubation by replacing the medium with fresh medium with the same concentration of Boc-

pentagastrin and/or the antagonist every day. The absorbance was measured as described above.

Conclusions

To summarize, the quinazolinone ring system has been employed as a template for the synthesis of two series of CCK-BR antagonists. The study resulted in the development of potent CCK-BR antagonists. All the compounds were predicted for activities using pharmacophore mapping exercises and a good correlation was found between the experimentally determined and predicted antagonistic activities. The compounds showed favorable interactions within the binding site of the homology modeled CCK-BR structure. The two best compounds 3c and 4a exhibited remarkable antagonistic activities of 0.2 nM and 17.36 nM, respectively. The compounds also showed promising results in a functional gastric acid secretion assay using a lumen-perfused isolated mouse stomach and inhibited the growth of pancreatic cancer cells (MiaPaca-2) exhibiting good cytotoxicity with compounds 3f (hydrazinecarbothioamide linker) and 4b (hydrazine linker, indanone series) as the most active compounds. Compound 3f, being the best overall compound, exhibited good receptor binding affinity and cytotoxicity activity. Thus the optimized compounds in this study can be taken further for pre-clinical evaluation in drug discovery processes to develop anticancer compounds against pancreatic cancer.

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