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Ligand/PTC-free intramolecular Heck reaction: synthesis of pyrroloquinoxalines and their evaluation against PDE4/luciferase/oral cancer cell growth *in vitro* and zebrafish *in vivo*†

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A series of 1,3-disubstituted pyrrolo[2,3-*b*]quinoxalines has been designed for the potential inhibition of PDE4 without inhibiting luciferase. A ligand/PTC (phase transfer catalyst) free intramolecular Heck cyclization strategy was used to prepare these compounds, some of which showed significant inhibition of PDE4B (IC_{50} ≈ 5–14 μM) and growth inhibition of oral cancer cells (CAL 27) but not inhibition of luciferase *in vitro*. They also showed acceptable safety profiles but no apoptosis in zebrafish embryos.

Phosphodiesterases (PDEs), a superfamily of enzymes that degrade cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), are classified into 11 families *e.g.* PDE1–PDE11.[1] One of them, *e.g.* the cAMP-specific enzyme PDE4 has been targeted to treat several inflammatory diseases including COPD, asthma and CNS related disorders. However, recent literature has indicated that PDE4 is widely expressed in tumour cells and thus inhibition of PDE4 in cancerous cells could be a new therapeutic target for cancer. For example, PDE4 inhibitors have been reported to inhibit brain tumor cell growth,[2,3] reduce proliferation and angiogenesis of lung cancer cell lines[4] and display the ability for selective apoptosis of malignant cells without affecting normal cells.[5] Thus, targeting PDE4 can be beneficial in treating cancer. Due to our long term interest in PDE4 inhibitors,[6] we now report the design and synthesis of 1,3-disubstituted pyrrolo[2,3-*b*]quinoxalines as new and potential inhibitors of PDE4. To the best of our knowledge this class of heterocycles has not previously been explored as PDE4/cancer cell growth inhibitors.

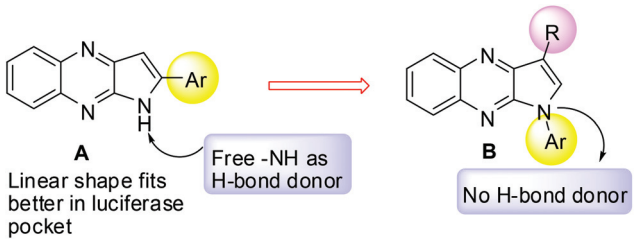

Fig. 1 Design of PDE4 inhibitor **B** *via* modification of known luciferase inhibitor **A**.

Recently, we reported the luciferase inhibiting properties of a series of 2-substituted pyrrolo[2,3-*b*]quinoxalines **A** (Fig. 1) that were originally designed for potential inhibition of PDE4.[7] These molecules were identified as false positive hits when tested against PDE4B *in vitro* by using a luciferase reporter gene assay and were found to interact with luciferase preferentially over the enzyme PDE4B. A subsequent docking study revealed that factors governing the binding of these molecules into the luciferase pocket include (i) the linear shape of **A** which helped them to fit effectively inside the luciferase pocket and (ii) the free pyrrole nitrogen which formed strong H-bonds with the surrounding amino acid residues inside the pocket. Based on this and to prevent the inhibition of luciferase we decided to make two major structural modifications *i.e.* (i) changing the linear shape of **A** by shifting the substituent from C-2 to the N-1 of the pyrrolo[2,3-*b*]quinoxaline ring, thereby blocking the H-bonding property of the NH and (ii) introduction of a small group at C-3 to disrupt the linearity of the molecule further. We anticipated that these changes might help the newly designed molecules **B** (Fig. 1) derived from **A** to inhibit PDE4 effectively but not luciferase. Our hypothesis was

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†Electronic supplementary information (ESI) available: Experimental procedures, spectral data for all new compounds, results of *in vitro*/*in vivo* and docking study. See DOI: 10.1039/c3ob41504j

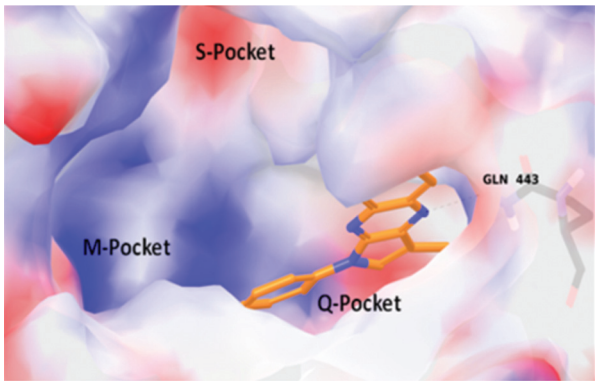
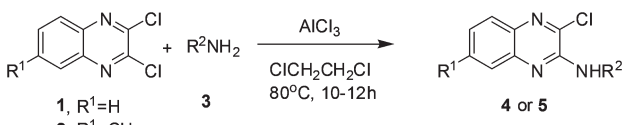


Fig. 2 Binding of designed molecule **B** (R = Me, Ar = Ph) in PDE4B pocket.

further supported by the docking studies, which showed that **B** (R = Me, Ar = Ph) interacted more strongly with the PDE4B pocket (Fig. 2) (Glide score −8.0) than luciferase (Glide score −3.3).

Among the various methods reported for the synthesis of pyrroloquinoxalines, the Pd-catalysed pyrrole ring formation on the pyrazine motif of the quinoxaline ring appeared to be attractive. Thus, Pd[8,9] or Pd/C-mediated[10] coupling–cyclization of *N*-alkyl or *N*-aryl-3-chloroquinoxalin-2-amine with terminal alkynes has been used to afford 1,2-disubstituted pyrrolo[2,3-*b*]-quinoxalines. The synthesis of 1,3-disubstituted analogues has also been reported *via* intramolecular Heck coupling of allyl-3-haloquinoxalin-2-ylamines under Jeffery's "ligand-free" conditions.[11] However, this method was limited to the synthesis of *N*-alkyl substituted pyrroloquinoxalines only and its application towards *N*-aryl derivatives has not been explored. We adopted a similar but suitably modified strategy to synthesize our target compounds *e.g.* 1,3-disubstituted *N*-aryl/alkyl pyrrolo[2,3-*b*]quinoxalines **B**. Our strategy was based on Pd-catalyzed intramolecular Heck coupling of allyl-3-chloroquinoxalin-2-ylamines in the absence of any ligand or phase transfer catalyst (*vide infra*, Table 2).

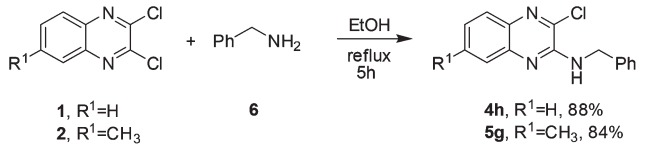
The starting amines **4a–g** and **5a–f** were synthesized *via* the reaction of 2,3-dichloroquinoxalines **1** or **2** with aromatic amines **3** in the presence of $AlCl_3$ (Scheme 1) whereas the *N*-benzyl-3-chloroquinoxalin-2-amine derivatives **4h** and **5g** were prepared by reacting **1** and **2** with benzyl amine **6** (Scheme 2).[9a] The amines **4a–h** and **5a–g** were then allylated to



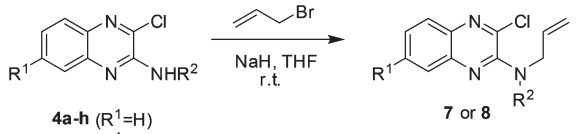
4 (R^1=H)
4a R^2 = Ph, 72%
4b R^2 = *p*-Me-C_6H_4, 75%
4c R^2 = *p*-*OMe*-C_6H_4, 72%
4d R^2 = *p*-*F*-C_6H_4, 71%
4e R^2 = *p*-*Cl*-C_6H_4, 77%
4f R^2 = *p*-*Br*-C_6H_4, 82%
4g R^2 = *m*-*F*-C_6H_4, 74%

5 (R^1=CH_3)
5a R^2 = Ph, 73%
5b R^2 = *p*-Me-C_6H_4, 72%
5c R^2= *p*-*OMe*-C_6H_4, 74%
5d R^2 = *p*-*F*-C_6H_4, 73%
5e R^2 = *p*-*Br*-C_6H_4, 78%
5f R^2 = *m*-*F*-C_6H_4, 76%

Scheme 1 Synthesis of *N*-aryl-3-chloroquinoxaline-2-amines (**4a–g**, **5a–f**).



Scheme 2 Synthesis of *N*-benzyl-3-chloroquinoxalin-2-amine derivatives.

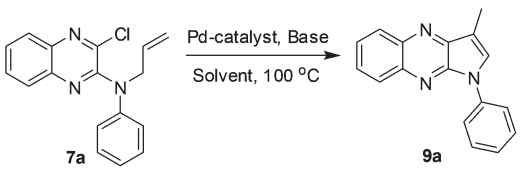


7 (R^1=H)
7a R^2 = Ph, 85%
7b R^2 = *p*-Me-C_6H_4, 90%
7c R^2 = *p*-*OMe*-C_6H_4, 95%
7d R^2 = *p*-*F*-C_6H_4, 85%
7e R^2 = *p*-*Cl*-C_6H_4, 80%
7f R^2 = *p*-*Br*-C_6H_4, 95%
7g R^2 = *m*-*F*-C_6H_4, 85%
7h R^2 = -CH_2Ph, 80%

8 (R^1=CH_3)
8a R^2 = Ph, 90%
8b R^2 = *p*-Me-C_6H_4, 90%
8c R^2 = *p*-*OMe*-C_6H_4, 80%
8d R^2 = *p*-*F*-C_6H_4, 85%
8e R^2 = *p*-*Br*-C_6H_4, 85%
8f R^2 = *m*-*F*-C_6H_4, 85%
8g R^2 = -CH_2Ph, 80%

Scheme 3 Synthesis of *N*-allyl-3-chloroquinoxalin-2-amines (**7** and **8**).

Table 1 Effect of reaction conditions on Pd-mediated cyclisation of **7a**[a]

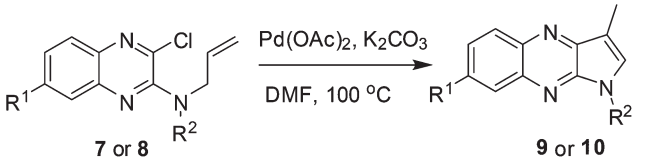


Entry	Pd-catalysts	Base	Solvent	Time (h)	Yield[b] (%)
1	$Pd(OAc)_2$	Et_3N	DMF	2	88
2	$Pd(OAc)_2$	Et_3N	DMF	2	65[c]
3	$Pd(OAc)_2$	K_2CO_3	DMF	2	91
4	$Pd(OAc)_2$	K_2CO_3	DMF	2	90[c]
5	$Pd(OAc)_2$	K_2CO_3	1,4-Dioxane	5	66
6	$Pd(PPh_3)_4$	Et_3N	DMF	5	32
7	$Pd(PPh_3)_4$	K_2CO_3	DMF	6	30
8	$Pd(PPh_3)_4$	Et_3N	1,4-Dioxane	6	0
9	10% Pd/C	Et_3N	DMF	5	0
10	10% Pd/C	K_2CO_3	DMF	6	0

[a] Reactions were carried out by using **7a** (1.0 mmol), Pd-catalyst (0.015 mmol), XPhos (0.15 mmol) and a base (3.0 mmol) in a solvent (10 mL) at 100 °C under nitrogen. [b] Isolated yields. [c] Reaction was carried in the absence of XPhos.

give *N*-allyl substituted amines **7a–h** and **8a–g** (Scheme 3). The cyclization of a representative compound **7a** was initially studied by varying the reaction conditions (Table 1). The reaction was performed using $Pd(OAc)_2$ and Et_3N in DMF in the presence of XPhos as a ligand at 100 °C. The reaction was completed within 2 h affording the desired pyrrolo[2,3-*b*]quinoxaline **9a** in 88% yield (entry 1, Table 1). While the product yield was decreased considerably in the absence of XPhos (entry 2, Table 1), an improvement in the product yield (91%)

Table 2 Synthesis of 1,3-disubstituted pyrrolo[2,3-*b*]quinoxalines[a]



Entry	Amines	Products; R^2 =	Yield[b] (%)
1	**7a**	**9a**, R^2 = Ph	82
2	**7b**	**9b**, R^2 = *p*-Me-C_6H_4	90
3	**7c**	**9c**, R^2 = *p*-OMe-C_6H_4	85
4	**7d**	**9d**, R^2 = *p*-F-C_6H_4	81
5	**7e**	**9e**, R^2 = *p*-Cl-C_6H_4	85
6	**7f**	**9f**, R^2 = *p*-Br-C_6H_4	92
7	**7g**	**9g**, R^2 = *m*-F-C_6H_4	90
8	**7h**	**9h**, R^2 = $PhCH_2$	80
9	**8a**	**10a**, R^2 = Ph	89
10	**8b**	**10b**, R^2 = *p*-Me-C_6H_4	90
11	**8c**	**10c**, R^2 = *p*-OMe-C_6H_4	80
12	**8d**	**10d**, R^2 = *p*-F-C_6H_4	85
13	**8e**	**10e**, R^2 = *p*-Br-C_6H_4	84
14	**8f**	**10f**, R^2 = *m*-F-C_6H_4	90
15	**8g**	**10g**, R^2 = $PhCH_2$	91

[a] Reactions were carried out by using amines **7** or **8** (1.0 mmol), $Pd(OAc)_2$ (0.015 mmol), and K_2CO_3 (3.0 mmol) in DMF (10 mL) at 100 °C under nitrogen. [b] Isolated yields.

was noticed when an inorganic base *e.g.* K_2CO_3 was used in place of Et_3N irrespective of the presence or absence of XPhos (entries 3 and 4, Table 1). A change of solvent from DMF to 1,4-dioxane decreased the yield significantly in spite of the presence of XPhos (entry 5, Table 1). The use of other Pd-catalysts along with XPhos was also studied. Compound **9a** was obtained in poor yield when $Pd(PPh_3)_4$ was used in the presence of Et_3N or K_2CO_3 in DMF (entries 6 and 7, Table 1) and no product formation was noticed in 1,4-dioxane even after 6 h (entry 8, Table 1). The use of 10% Pd/C in the presence of Et_3N or K_2CO_3 in DMF was also found to be unsuitable (entries 9 and 10, Table 1). Overall, the best result was obtained with $Pd(OAc)_2$ as a catalyst, K_2CO_3 as a base and DMF as a solvent in the presence of XPhos. However, since the omission of ligand gave an almost similar yield of **9a**, further reactions were performed under the conditions of entry 4 of Table 1.

The scope and generality of the present reaction was then studied using a variety of amines *e.g.* **7a–h** and **8a–g** under the optimised reaction conditions (Table 2). Both *N*-aryl and *N*-alkyl substituted 3-chloroquinoxaline-2-amines participated well affording the 1,3-disubstituted pyrrolo[2,3-*b*]quinoxalines **9a–h** and **10a–g**. The formation of **9** or **10** was indicated by the appearance of a singlet near 7.4–7.9 δ due to the C-2 proton (though this singlet was merged with other aromatic protons in some cases) and disappearance of the CH_2 protons of **7** or **8** near 4.1–4.7 δ in their corresponding ^{1}HNMR spectra (see ESI†). A Heteronuclear Multiple Bond Correlation (HMBC) study performed by using compound **10b** indicated two 3-bond correlations for the ring junction carbon A [with H_h (7.91 δ, s) and H_e (7.52 δ, dd, J = 8.4, 1.2 Hz) separately] and

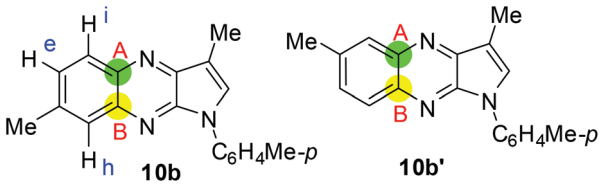
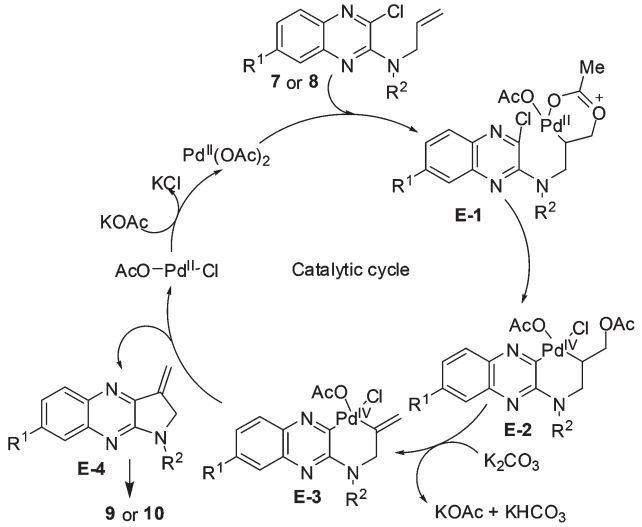


Fig. 3 Structure of compound **10b** and its regioisomer **10b′**.



Scheme 4 Proposed mechanism for the ligand/PTC-free intramolecular Heck cyclization of **7** or **8**.

one 3-bond correlation for the other carbon B [with H_i (8.15 δ, d, J = 8.4 Hz)] (Fig. 3, see also ESI†). An opposite HMBC result was expected for the regioisomer **10b′**, hence **10b** appeared to be the structure of the isolated product. Based on an earlier report[12] we propose a Pd(II)–Pd(IV) catalytic cycle instead of a classical Pd(0)–Pd(II) cycle for the present ligand free intramolecular Heck reaction (Scheme 4). Thus the reaction proceeds *via* generation of a Pd(II) complex **E-1** *in situ* which undergoes oxidative addition with the proximal C–Cl bond of the 3-chloroquinoxaline moiety generating the Pd(IV) species **E-2**. The elimination of acetate group in the presence of K_2CO_3 generates **E-3**. Subsequent reductive elimination of a Pd(II) species from **E-3** facilitates C–C bond formation in an intramolecular fashion leading to **E-4**. The exocyclic double bond of **E-4** undergoes a facile isomerisation to afford the thermodynamically more stable compound **9** or **10**. The Pd(II) species regenerates the $Pd(OAc)_2$ to complete the catalytic cycle.

Among all the synthesized compounds, nine were studied for PDE4B inhibition using an enzyme based assay[13] (Table 3). Most of them showed significant PDE4B inhibition (>60%), at 30 μM, except **9h**. The other compounds *i.e.* **9b**, **9c**, **9f** and **10b** showed solubility related issues in the medium used for the assay. The active compounds were also tested[7] for luciferase inhibition separately where none of them showed significant inhibition (Table 3) with **10g** showing the highest inhibition of ~14%. In a dose response study, **9a** and **9d** (which displayed

Table 3 Inhibition of PDE4B and luciferase by **9** and **10** at 30μM

Entry	Compound	% Luciferase inhibition	% PDE4B inhibition[b]	S.D.	Glide score[a]
1	**9a**	8.44	76.12	2.23	−8.02
2	**9b**	—	ND	—	
3	**9c**	—	ND	—	
4	**9d**	7.89	70.06	3.47	−8.03
5	**9f**	—	ND	—	
6	**9h**	4.86	46.88	2.49	—
7	**10a**	12.87	63.04	3.10	—
8	**10b**	—	ND	—	
9	**10g**	14.24	60.87	2.58	—

[a] For PDE4B. [b] S.D.: standard deviation. ND: not determined.

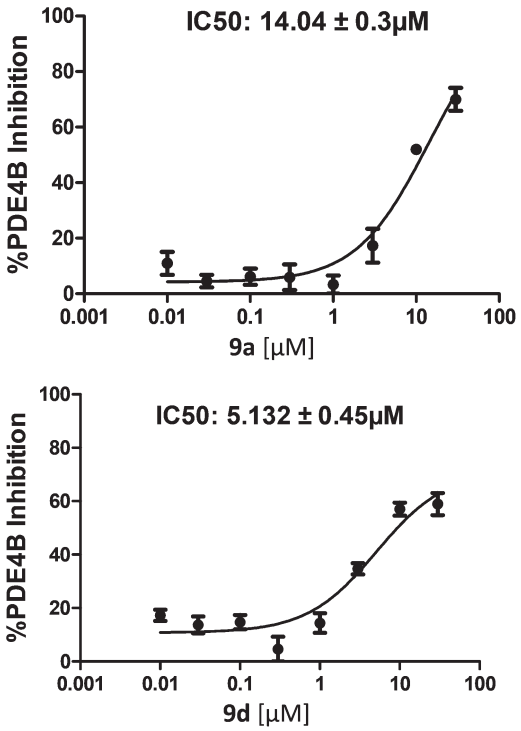


Fig. 4 Dose-dependent curve for **9a** and **9d**.

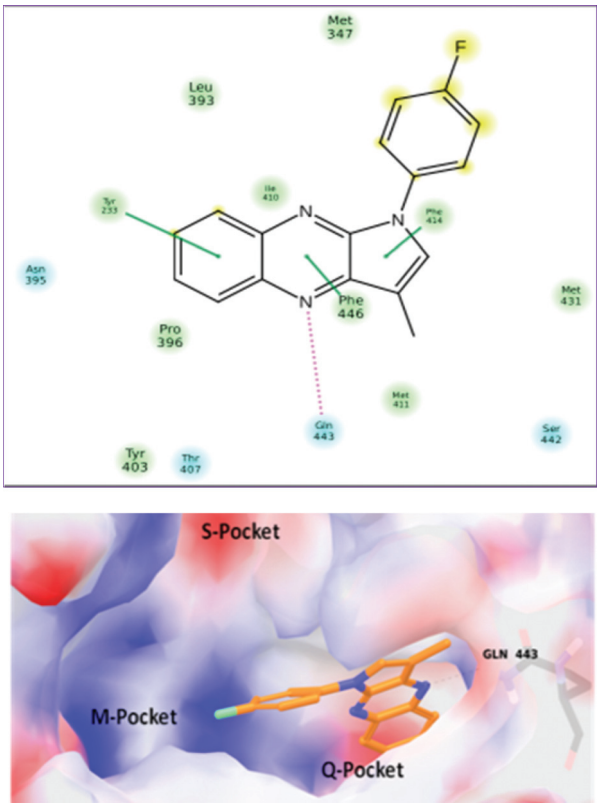


Fig. 5 Orientation of **9d** in the Q-pocket of the PDE4B enzyme.

>70% inhibition of PDE4B) showed IC_{50} = 14.0 and 5.1 μM, respectively (Fig. 4) comparable to rolipram's IC_{50} of ~1.0 μM.

Further, *in silico* studies of **9a** and **9d** were performed using PDE4B and luciferase. The three divisions or pockets of the PDE4B binding site include the M- or metal binding pocket, S- or solvent filled side pocket and Q- or core pocket *i.e.* the hydrophobic clamp. The docking studies indicated that both **9a** and **9d** are tightly bound in the Q-pocket of PDE4B. The nitrogen of the quinoxaline ring of **9a** was involved in an H-bond with the Gln 443 and two π–π stacking interactions with Phe 446 and Phe 414, whereas the pyrrole ring was involved in a π–π stacking interaction with Tyr 233 (see ESI, Fig. S1†). Notably, except for a few weak hydrophobic interactions **9a** did not show any strong interactions when docked into the luciferase (see ESI, Fig. S3†). Similarly, **9d** showed strong binding with PDE4B (two π–π stacking interactions with Phe 446 and Tyr 233 along with an H-bond with Gln 443; the pyrrole ring was involved in a π–π stacking interaction with Phe 414) (Fig. 5) and weak interactions with luciferase (see ESI, Fig. S4†). Thus the introduction of an aryl group on the pyrrole nitrogen increased selectivity for PDE4B over luciferase.

Some of the active pyrrolo[2,3-*b*]quinoxalines were tested against oral cancer cell lines *e.g.* CAL 27 *in vitro* at 10 μM using the sulphorhodamine B (SRB) assay[14] (gemcitabine[15] as a reference compound), where **9a**, **9d** and **10g** showed 23–25% growth inhibition after 72 h of treatment. However, none of them showed any effects on normal HEK 293 T cells [Human Embryonic Kidney 293 cells] when tested at 10 μM. Notably, gemcitabine[16] showed >50% growth inhibition of CAL 27 cells at 10 μM. To assess the safety profile of this class of heterocycle two representative compounds *i.e.* **9a** and **10a** were tested for toxicity in zebrafish embryos[17] (Fig. 6) in the range 1.0–30 μM. None of them showed toxic effects at these concentrations (Fig. 7) with No Observed Adverse Effect Level (NOAEL) ≥ 30 μM. Both the compounds showed no to mild hepatotoxicity when tested at 1–30 μM in the same model. Notably, none of these compounds was found to be positive compared to methotrexate[18] when tested for apoptotic activity (Fig. 8) indicating that they induce cancer cell death by non-apoptotic mechanisms such as necrosis, senescence, autophagy and mitotic catastrophe.[19]

In conclusion, we have described a unique strategy to develop PDE4 inhibitors devoid of luciferase inhibition *via* a

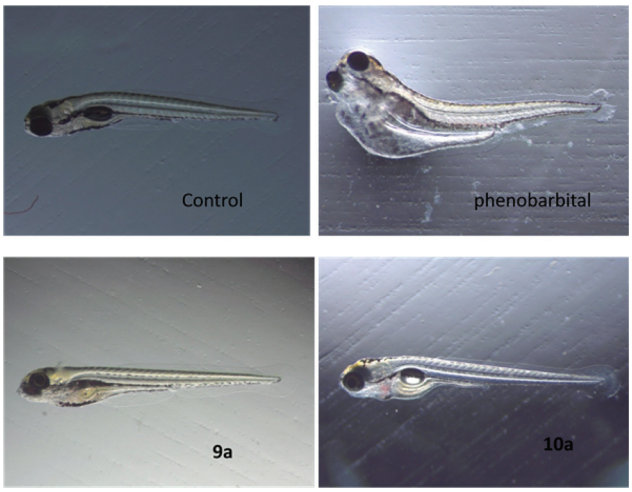


Fig. 6 Control embryo showing normal body; embryo treated with phenobarbital (positive control) at 3 mM showing severe abnormalities, body bent; compound **9a** and **10a** showing no and slight abnormalities respectively at 30 μM.

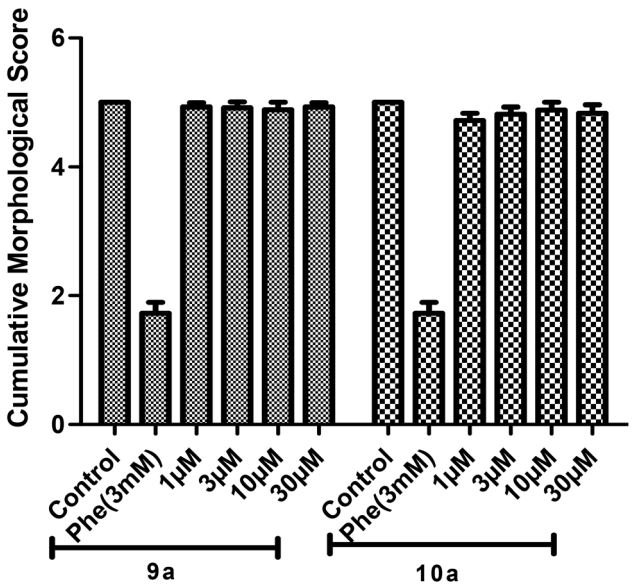


Fig. 7 Teratogenicity assay: each embryo was scored based on their level of toxicity from 5 being non-toxic and 0.5 being highly toxic. Mean (±S.D.) lesion score of all parameters different treatment groups. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Statistical significance was analyzed as control group *vs.* all groups.

rational drug design approach. A series of 1,3-disubstituted pyrrolo[2,3-*b*]quinoxalines designed for the potential inhibition of PDE4 were conveniently prepared by using a ligand and phase transfer catalyst (PTC) free intramolecular Heck reaction in good to excellent yields. Some of them showed significant inhibition of PDE4B but not luciferase along with growth inhibition of oral cancer cells when tested *in vitro*. They also showed an acceptable safety profile but no apoptosis in zebrafish embryos indicating that the present pyrrolo[2,3-*b*]-

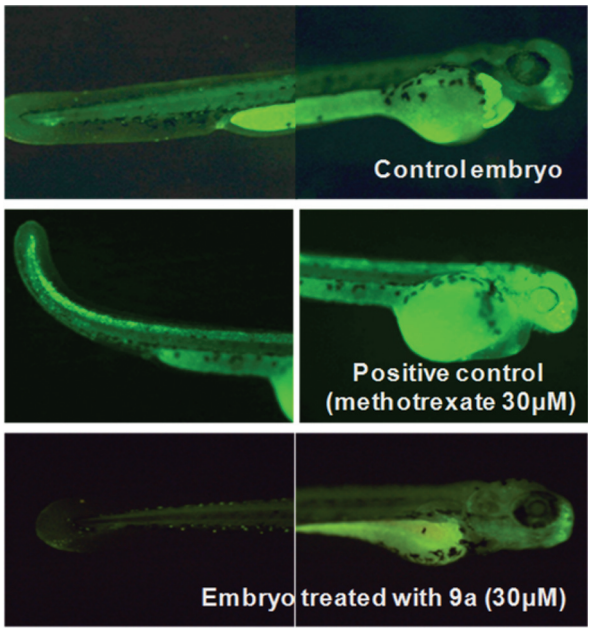


Fig. 8 Images of the embryos treated with **9a** and methotrexate for apoptosis.

quinoxaline based PDE4 inhibitors may be of further interest as potential agents against oral cancer.

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