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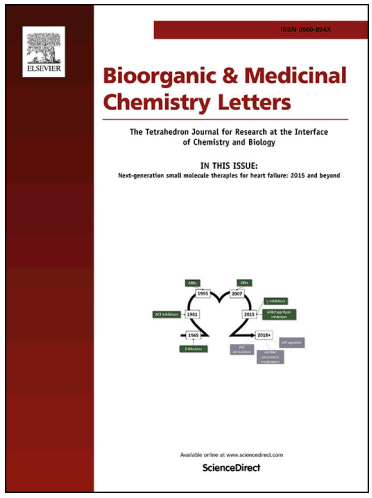

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l*H*-Pyrazolo[3,4-*b*]quinolin-3-amine derivatives inhibit growth of colon cancer cells *via* apoptosis and sub G1 cell cycle arrest.

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Running title*: Anticancer activity of lH-Pyrazolo[3,4-b]quinolin-3-amines*

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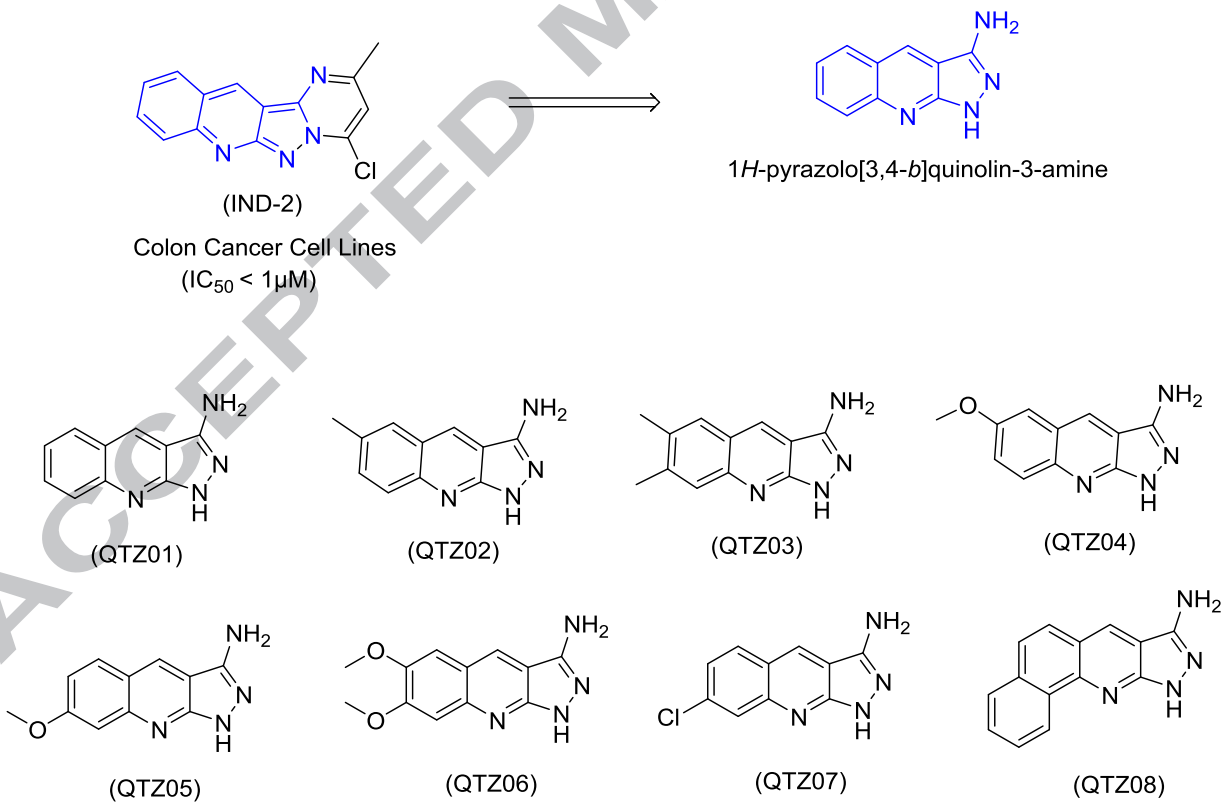
Abstract

A series of l*H*-Pyrazolo[3,4-b]quinolin-3-amine derivatives were synthesized and evaluated for anticancer efficacy in a panel of ten cancer cell lines, including breast (MDAMB-231 and MCF-7), colon (HCT-116, HCT-15, HT-29 and LOVO), prostate (DU-145 and PC3), brain (LN-229), ovarian (A2780), and human embryonic kidney (HEK293) cells, a non-cancerous cell line. Among the eight derivatives screened, compound **QTZ05** had most potent and selective antitumor activity in the four colon cancer cell lines with IC_{50} values ranging from 2.3 to 10.2 µM. Furthermore, **QTZ05** inhibited colony formation in HCT-116 cells in a concentration-dependent manner. Cell cycle analysis data indicated that **QTZ05** caused an arrest in the sub G1 cell cycle in HCT-116 cells. **QTZ05** induced apoptosis in HCT-116 cells in a concentration-dependent manner that was characterized by chromatin condensation and a reduction in mitochondrial membrane potential. The findings our study suggest that **QTZ05** may be a valuable prototype for the development of chemotherapeutics targeting apoptotic pathways in colorectal cancer cells.

Keywords: *lH-Pyrazolo[3,4-b]quinolin-3-amines; Cytotoxicity; colorectal cancer; Apoptosis*

Cancer still remains the leading cause of human deaths worldwide, with 8.2 million reported deaths (around 13% of all deaths) in 2012[1]. Despite an increasing understanding of the molecular biology of cancer and the consequent increase in the development of anticancer compounds, most of the chemotherapeutic drugs currently used for treating many types of cancer are often rendered ineffective due to the development of drug resistance during treatment [2]. Furthermore, treatment may be discontinued as a result of drug-related toxicities[2]. Therefore, there is a continuous need for the development of novel chemotherapeutic drugs for cancer therapy that can surmount these aforementioned limitations.

Figure 1: Design Strategy and Eight 1*H*-pyrazolo[3,4-*b*]quinolin-3-amines reported in this study.

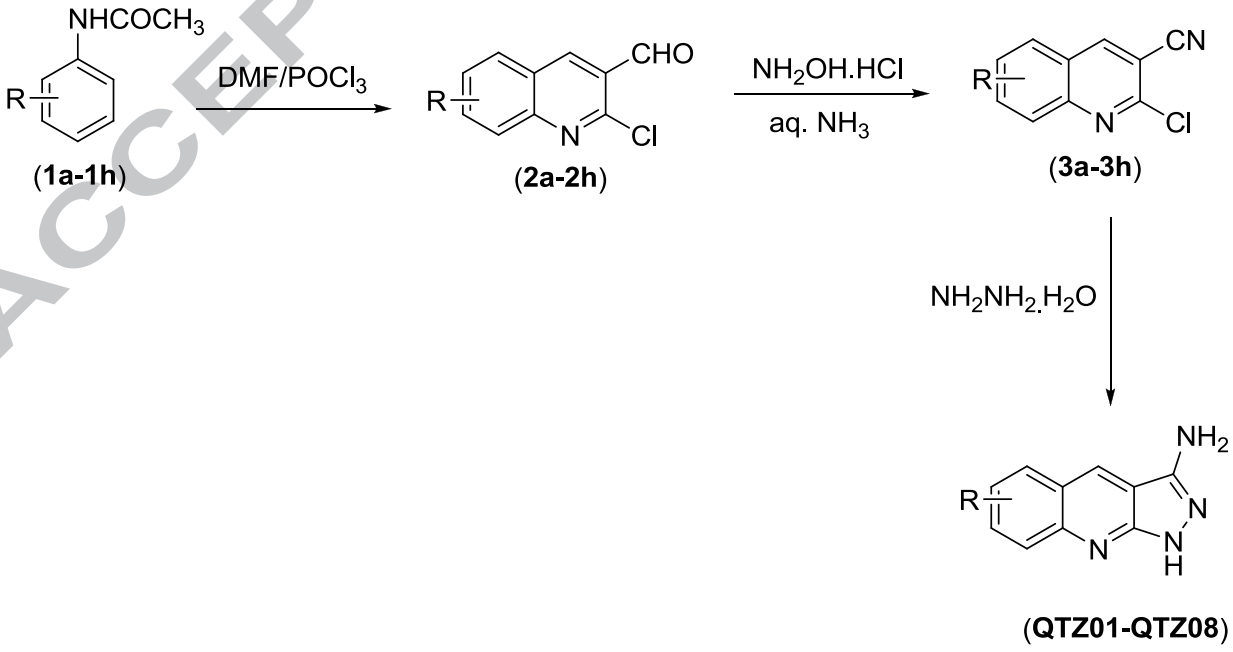


Functionalized quinolines and their hetero-fused analogues represent an important class of organic molecules and they are present in numerous natural products [3, 4]. Pyrazoloquinolines,

which have quinoline and pyrazole moieties in their molecular framework, is an excellent example of this class of compounds that have abroad spectrum pharmacological efficacies,[5, 6], [7],[8], [9], [10-13]. Notably, we are interested in the antitumor efficacy of pyrazoloquinolines, which is elicited through inhibition of topoisomerase II[7], CHK1 kinase[14], DNA-dependent protein kinase[15], Oncogenic *Ras* protein[16] and by induction of apoptosis[17].

During the course of our investigations on polycyclic quinolines with antitumor efficacy, we reported the discovery of IND-2, a pyrimido[1",2":1,5]-pyrazolo[3,4-*b*]quinoline (**Figure 1**) with potent cytotoxic and apoptosis-inducing properties in colon cancer cells[18]. Thus, in this study, we chose to evaluate 1*H*-pyrazolo[3,4-*b*]quinolin-3-amines, the major substructural unit and synthetic precursor of IND-2, for their anticancer efficacy (**Figure 1**). Eight pyrazolo[3,4-*b*]quinolin-3-amine derivatives (**Figure 1**), bearing different substituents on the fused benzo ring, were synthesized and their anticancer efficacy determined in a panel of 10 cancer cell lines.

Scheme 1: Synthesis of l*H*-Pyrazolo[3,4-*b*]quinolin-3-amine derivatives.



(**1a-1h**) (**2a-2h**) (**3a-3h**) (**QTZ01-QTZ08**)

The synthesis of 1*H*-pyrazolo [3,4-*b*]quinolin-3-amines was accomplished, using a three-step protocol, as shown in scheme 1. The first two steps involved the synthesis of the starting material, 2-chloroquinoline-3-carbaldehydes (**2a-2h**), from suitable acetanilides (**1a-1h**). This was followed by subsequent conversion to the corresponding 2-chloroquinoline-3-carbonitriles (**3a-3h**), using well-established procedures as described previously[18-20]. The obtained 2-chloroquinoline-3-carbonitriles on addition with hydrazine hydrate in under reflux conditions produced 1*H*-pyrazolo[3,4-b]quinolin-3-amines in good yields (**QTZ01-QTZ08**)[8].

The IR spectra of the synthesized 1*H*-pyrazolo[3,4-*b*]quinolin-3-amines displayed two characteristic peaks for the NH and NH_2 groups in the region 3424-3325 cm^{-1} and 3300-3256 cm^{-1}, respectively. In the ^1H NMR spectra, the signal for NH and NH_2 appeared as singlets at δ 11.24-12.5 ppm (D_2O exchangeable) and δ 5.52-5.96 ppm (D_2O exchangeable), respectively. The ^1H NMR spectra of compounds also showed a singlet in the region δ 8.28-8.72 ppm due to the presence of the C13 proton of the 1*H*-pyrazolo[3,4-*b*]quinoline ring. Further, the presence of singlet, doublet, triplet and multiplets at around δ 6.77-8.03 ppm in the compounds indicated aromatic protons in the benzo ring of the pyrazolo[3,4-*b*]quinolinyl moiety. The structures assigned for the compounds were also supported by the elemental analysis and mass spectral data.

The cytotoxicity of synthesized compounds was determined in a panel of ten cancer cell lines, including breast (MDAMB-231 and MCF-7), colon (HCT-116, HCT-15, HT-29 and LOVO), prostate (DU-145 and PC3), brain (LN-229), ovarian (A2780), and a non-cancerous cell line, human embryonic kidney (HEK293) **(Table 1).** The cytotoxicity was determined using the MTT assay with a series of different concentrations for each compound was used (0, 0.1, 03, 1, 3, 10, 30, and 100 μM). The results are expressed as IC_{50} and are summarized in **Table 1**.

Table 1: Activity of 1H-pyrazolo[3,4-b]quinolin-3-amine derivatives against various cell lines (cancerous and non-cancerous).

IC_{50} ± SD, µM						
Comp. code	**Breast**		**Prostate**		**Brain**	**Ovarian**
	MDAMB-231	**MCF-7**	**DU-145**	**PC3**	**LN-229**	**A2780**
QTZ-1	>100 ± NA	81.5 ± 28.4	>100 ± NA	>100 ± NA	7.8 ± 5.5	83.9 ± 6.5
QTZ-2	>100 ± NA	>100 ± NA	>100 ± NA	>100 ± NA	61.2 ± 12.4	98.3 ± 13.7
QTZ-3	>100 ± NA	78.8 ± 13.0	>100 ± NA	>100 ± NA	38.9 ± 17.0	>100 ± NA
QTZ-4	62.3 ± 5.9	49.5 ± 8.4	76.5 ± 12.6	>100 ± NA	93.7 ± 25.3	35.8 ± 10.6
QTZ-5	50.6 ± 8.9	59.2 ± 11.9	53.4 ± 11.5	87.1 ± 25.1	7.8 ± 2.3	56.6 ± 12.6
QTZ-6	77.9 ± 5.6	72.1 ± 7.7	73.4 ± 16.7	65.2 ± 15.5	26.0 ± 5.3	19.6 ± 25.1
QTZ-7	89.9 ± 19.6	>100 ± NA	>100 ± NA	>100 ± NA	58.6 ± 4.9	59.9 ± 10.5
QTZ-8	>100 ± NA	73.6 ± 12.6	>100 ± NA	59.7 ± 16.9	54.3 ± 8.2	13.5 ± 8.5

IC_{50} ± SD, µM					
Comp. code	**Colon**				**Normal**
	HCT-116	**HCT-15**	**HT-29**	**LOVO**	**HEK 293/pcDNA.3.1**
QTZ-1	67.7 ± 7.4	58.6 ± 6.2	95.7 ± 22.4	88.6 ± 16.1	>100 ± NA
QTZ-2	>100 ± NA	>100 ± NA	32.9 ± 5.9	89.6 ± 15.4	>100 ± NA
QTZ-3	>100 ± NA	>100 ± NA	>100 ± NA	>100 ± NA	>100 ± NA
QTZ-4	57.0 ± 5.8	69.3 ± 8.6	85.2 ± 13.6	55.6 ± 8.6	>100 ± NA
QTZ-5	2.3 ± 0.9	9.6 ± 3.6	3.9 ± 0.5	10.2 ± 11.0	45.8 ± 12.2
QTZ-6	40.8 ± 14.6	12.8 ± 3.9	19.4 ± 8.3	33.4 ± 12.3	81.1 ± 15.4
QTZ-7	56.6 ± 4.6	44.8 ± 8.6	42.3 ± 10.8	77.5 ± 20.3	36.6 ± 4.8
QTZ-8	76.1 ± 21.4	68.6 ± 13.3	98.7 ± 14.9	80.6 ± 23.5	>100 ± NA

Cell survival assay was determined by the MTT assay. IC_{50} values are represented as means of three independent experiments performed in triplicate. A mean IC_{50} value of 100 µM was the cut off. The compounds were screened on breast (MDAMB-231 and MCF-7), colon (HCT-116, HCT-15, HT-29, and LOVO), prostate (DU145), brain (LN-229), and ovarian (A2780) cancer cell lines. Human embryonic kidney cells (HEK293/pcDNA 3.1) is a non-cancerous cell line.

According to the cytotoxicity data in **Table 1**, the compounds **QTZ01**, **QTZ05**, **QTZ06** and **QTZ08** displayed variable efficacy on the inhibition of the growth of the human tumor cell lines HCT-116, HCT-15, HT-29, LOVO, LN-229 and A2780. Compound **QTZ05,** with a 7-OCH_3 substituent in the benzo ring of the 1*H*-pyrazolo[3,4-i]quinolin-3-amine moiety, was the most potent in the series. **QTZ05** had broad spectrum cytotoxic efficacy in colon cancer cells, with IC_{50} values ranging from 2.3 to 10.2 µM. **QTZ05** also displayed excellent selectivity for colon cancer cells compared to non-cancerous HEK293 cells, with 20, 5, 11 and 4-fold greater selectivity for HCT-116, HCT-15, HT-29 and LOVO cells, respectively (**Table 1)**.

Figure 2: Morphological analysis of the cytotoxic effects of **QTZ05** (1, 10 and 30 µM) or vehicle on the colon cancer cells, HCT-116, HCT-15, and LOVO and human embryonic kidney cells HEK293, incubated for 72 h. The cells were photographed for each triplicate incubation using an inverted microscope (Olympus, BX53F) with digital cameras. A representative image is shown for each treatment. The data were acquired and analyzed with CellSens software.

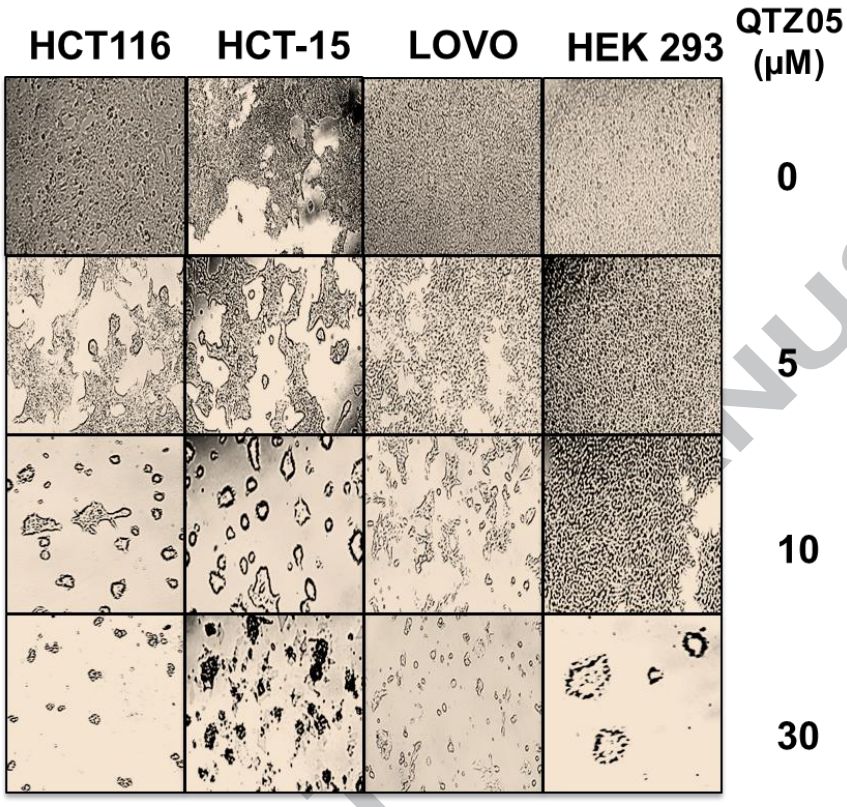
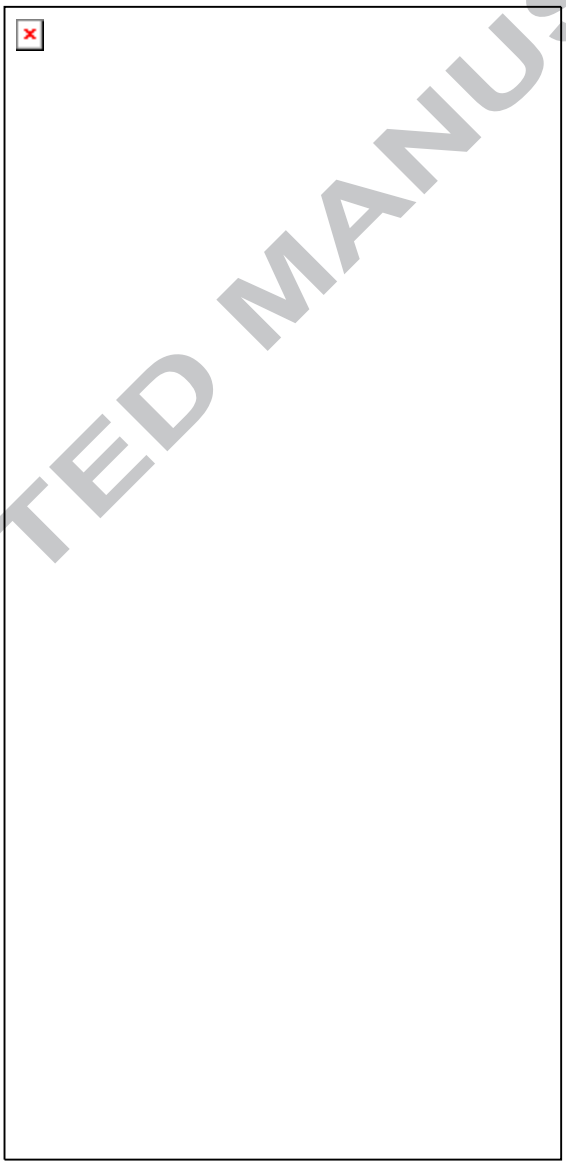


Figure 2 illustrates the cytotoxicity of **QTZ05** in colon cancer cells compared to normal cells. Furthermore, **QTZ05,** at 7.6 µM, also significantly inhibited the growth of the brain cancer cell line. **QTZ01**, the unsubstituted derivative, also inhibited LN-229 cells at concentration similar to that of **QTZ05**. However, neither **QTZ05** nor **QTZ01** had significant cytotoxic efficacy in other cancer cell lines **(Table1)**. Compound **QTZ06,** with a 6,7-dimethoxy substitution, had moderate cytotoxic efficacy against the colon cancer cell lines (HCT-15 and HT-29 (IC_{50} < 20 µM) and A2780 ovarian cancer cells (IC_{50} = 19.6 µM) (**Table 1**). In contrast, compound **QTZ08**, a benzo fused derivative, had selective and moderate growth inhibitory efficacy in A2780 cancer cells (IC_{50} = 13.6 µM) **(Table 1)**.

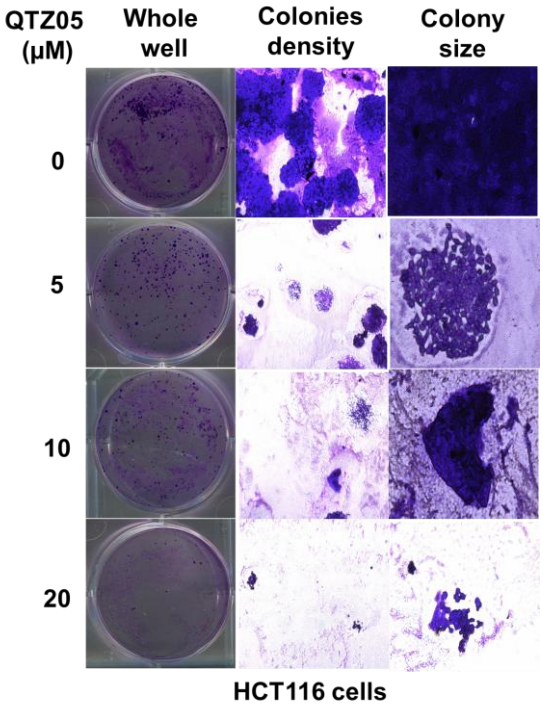
Overall, the results of the MTT assay establishes compound **QTZ05** to be the most interesting candidate for further mechanistic investigations in terms of its potent cytotoxicity on colon cancer cell lines.

Figure 3: The cytotoxicity of **QTZ05** on HCT116 cells over time. The cells were incubated with, 10, 20 and 30 µM of **QTZ05** or vehicle for 72 h. The pictures were taken every 15 min from the same spot by a CytoSMART™ Lux 10X system for live cell imaging. The confluence at each time point were used to generate confluence curves over time for each compound or vehicle.



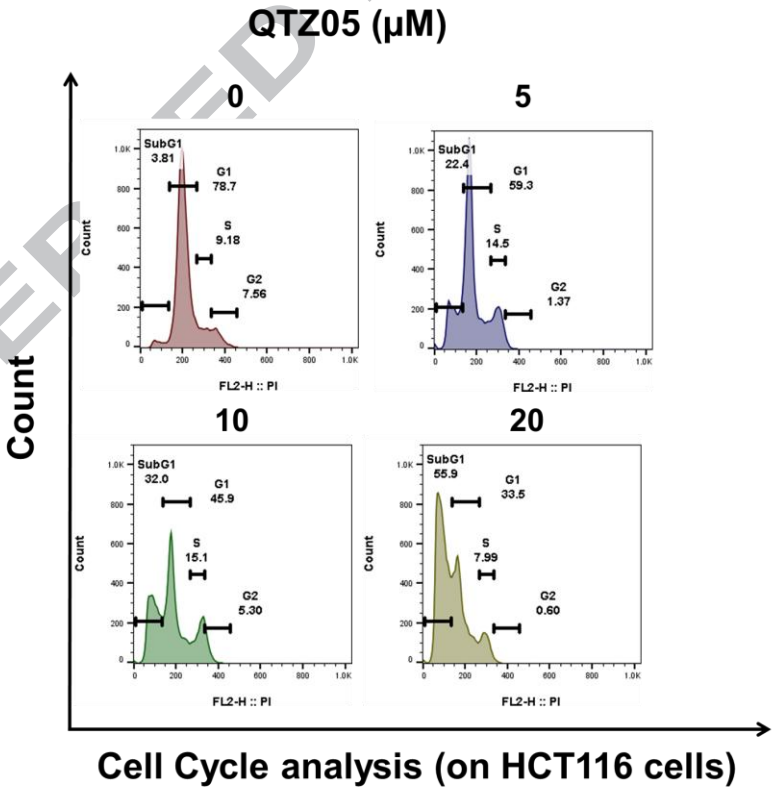
First, we investigated the cytotoxicity of **QTZ05** on HCT116 cells over time. The cytotoxicity of different concentrations of **QTZ05** (0, 10, 20, and 30 µM) at different times (every 15 minutes up to 72 h) was determined. **Figure 3** shows representative pictures of the cell confluence for the different concentrations (0, 10, 20, and 30 µM) at 0, 12, 24, 36, 48, 60, and 72 h. Clearly, control cells were able to grow and replicate over time, and they reached high confluence after 3 days of incubation (≈ 60%, **Figure 3**). However, the cells incubated with QTZ05 grew significantly slower over time and they only reached a very low confluence at the experiment (≈ or < that 20% confluence for all 3 concentrations of **QTZ05**, **Figure 3**). These results also indicated that **QTZ05** produces cytotoxicity at relatively early time points (≈20 h of incubation). A detailed graph(**Figure 3**) showing the results for each compound and each time point was generated to clarify the cytotoxicity over time.

Figure 4: QTZ05 inhibits the colony formation rate of HCT 116 cells. The cells were incubated with different concentrations **QTZ05** (5, 10 or 20 uM) or vehicle. The pictures show the effect of **QTZ05** colonies density and colony size. Each experiment was repeated independently three times.



Next, we investigated the efficacy of **QTZ05** (5, 10 or 20 µM) to inhibit growth of HCT116 cells colonies. **Figure 4** shows the number of colonies formed, as well as a representation of the colony sizes in the presence of **QTZ05** (0, 5, 10, and 20 µM). **QTZ05** significantly inhibits colony formation abilities in HCT116 cells (**Figure 4**). In addition, the HCT116 cells that survive incubation with QTZ05 are unable to replicate (**Figure 4).** The mean colony formation rate was decreased significantly in HCT116 cells incubated with 5, 10, and 20 µM, respectively, of compound **QTZ05**, compared to control cells ($p < 0.001$ for all concentrations, **Figure 4**).

Figure 5: QTZ05 induces sub G1 cell cycle arrest in HCT116 cells. HCT116 cells were incubated with different concentrations (5, 10, 20 µM) of **QTZ05** or vehicle for 24 h and subjected to cell cycle analysis by flow cytometry of PI (X axis)/cell counts (Y axis). The data represents means ± SD of three independent experiments performed in triplicate.



Further, the effect of **QTZ05** on the cell cycle was analyzed using flow cytometry cell cycle analysis with propidium iodide (PI)). **QTZ05** produced a significant concentration-dependent

increase in the number of cells in the subG1phase in cells treated with **QTZ05** compared to cells incubated with vehicle (control cells, **Figure 5).** The percentage of cells in the sub G1 phase increased from 3.8% in control cells, to 22.4%, 32.0%, and 55.9% in cells incubated with5, 10, or 20 µM of **QTZ05**, respectively. **QTZ05** also significantly shifted the cells from the G1 phase, where incubation with 5, 10 or 20 µM of **QTZ05** decreased the number of cells in the G1 phase to 59.3%, 45.9% and 33.5%, respectively, compared to 78.7% in cells incubated with vehicle (**Figure 5).** In contrast, **QTZ05** (5, 10 or 20 uM) did not significantly alter the number of cells in the S or G2 phases (**Figure 5**). Our results indicate that **QTZ05** produces a concentration-dependent increase in the number of cells in the sub G1 phase of the cell cycle.

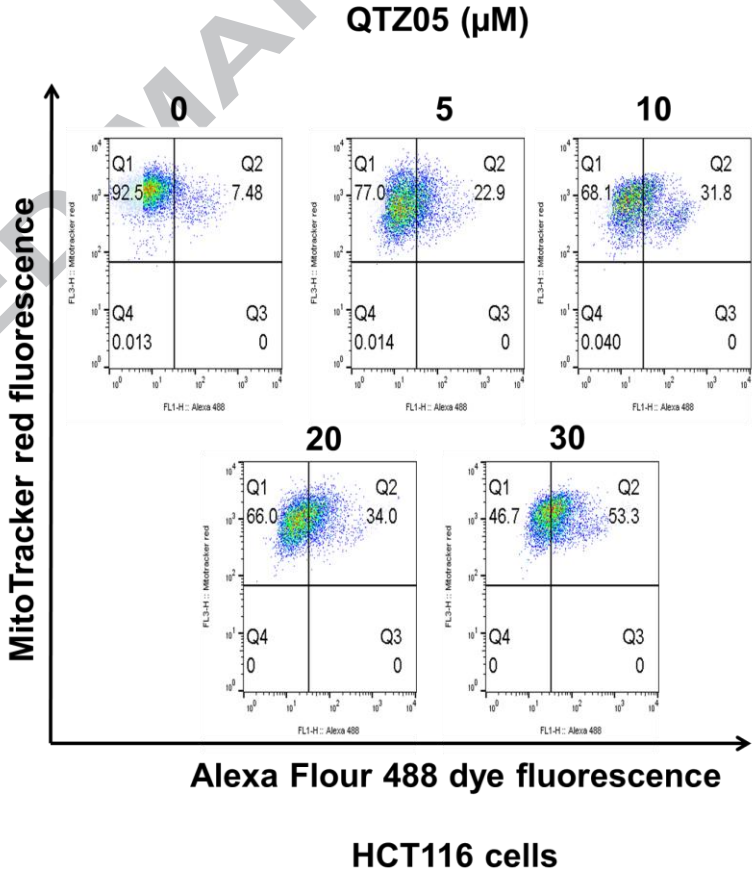


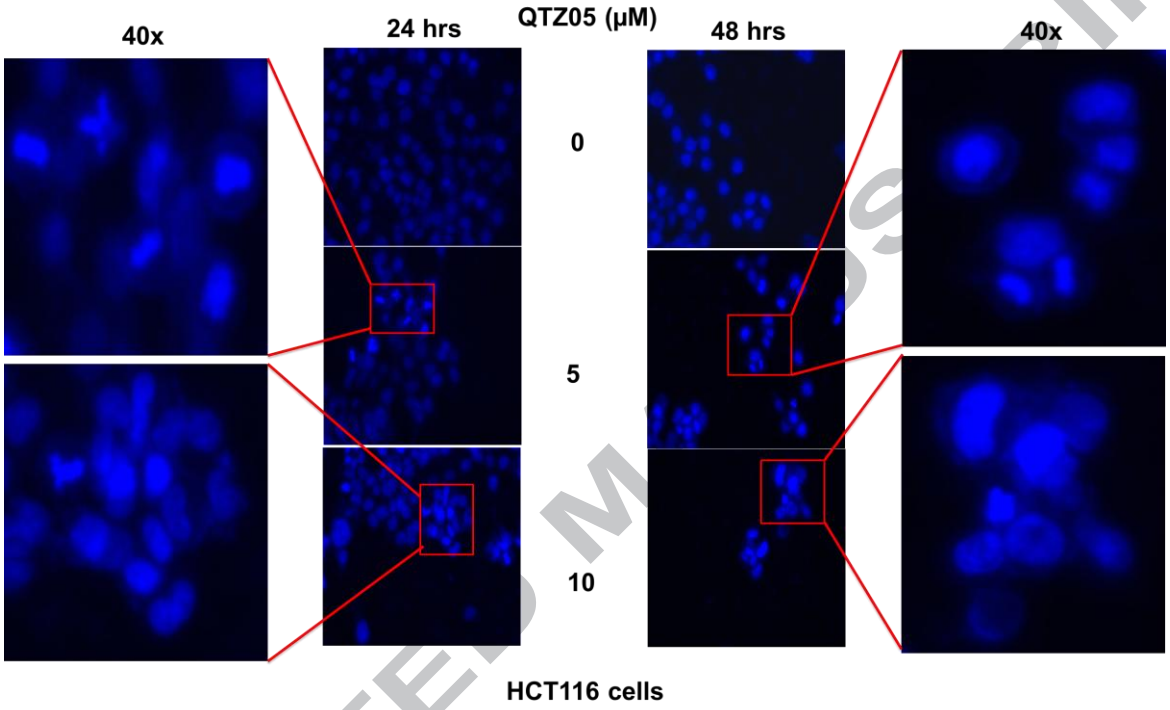
Figure 6: QTZ05 induces apoptosis in HCT116 cells. HCT116 cells in complete medium were incubated with **QTZ05** at 5, 10, 20, or 30 µM or vehicle for 24 h. Cells were then incubated with the reagents of the MitoTracker Red and Alexa Fluor 488 annexin V kits for flow cytometry. Representative results of HCT116 cells from at least two independent experiments, each performed in triplicate, are shown.

The induction of apoptosis is one of the main mechanisms by which many clinically approved anticancer drugs, despite differences in their structures and interaction with cellular

targets, produce cytotoxicity in cancer cells[21]. Therefore, we used the annexin mitotracker red kit to measure the mitochondrial membrane potential and the fluorescence of fluorochrome-conjugated annexin V as indicators for apoptosis levels. Annexin V can only bind to exposed phosphatidylserine (PS) that translocates from the inner to the outer side of the cell membrane during apoptosis[22]. As shown in **Figure 6**, about 92.5% of the control HCT116 cells are in quadrant I, indicating that most of the cells are viable and intact (**Figure 6**), whereas only a small portion of the cells express high annexin fluorescence, indicating apoptosis (7.48%) in quadrant II (**Figure 6**). However, more cells were shifted towards quadrant II after incubation with 5, 10, 20, or 30 μM of **QTZ05** (**Figure 6**). **QTZ-05** at 5, 10, 20 or 30 μM, produced a non-significant reduction (77%, 68.1%, 66%, and 46.7% respectively), in the percent of viable cells located in quadrant I (**Figure 6**). Furthermore, **QTZ05** produced a significant accumulation of cells in quadrant II in a concentration-depended manner (5 μM: 22.9%, 10 μM:31.8%, 20 μM: 34%, and 30 μM:53.3 % respectively, **Figure 6),** indicating that **QTZ05** induces apoptosis.

Nuclear condensation is one of the hallmarks of cellular apoptosis[23]. Therefore, we used DAPI staining to visualize nuclear changes after incubating HCT116 cells with different concentrations of **QTZ05** (0, 5 or 10 μM) for 24 and 48 h. As shown in **Figure 7**, control cells had typical oval nuclei (indicative of viable cells) with non-condensed, low bright - blue staining. Overall, HCT116 cells incubated with vehicle did not undergo a significant magnitude of apoptosis as virtually all of the cells had no nuclear condensation. However, the incubation of cells with 5 or 10 μM of **QTZ05** for 24 h significantly increased the number of cells with condensed, fragmented nuclei, (**Figure 7**). Furthermore, the incubation of cell with 5 or 10 μM of **QTZ05** for 48 h resulted in a higher chromatin condensation and brighter staining compared to cells incubated for 24 h (**Figure 7**). Overall, the above results indicate that QTZ05-induced cytotoxicity in HCT116 cells is due to apoptosis.

Figure 7: The effect of **QTZ05** on the nuclear morphology of HCT116 cells. The changes in the nuclear morphology of HCT116 cells upon incubation with different concentrations (5, or 10 µM) of **QTZ05** or vehicle for 24 or 48 h, respectively, was detected using DAPI staining. The cells were fixed and stained with the DAPI. Condensed and fragmented nuclei were observed under an EVOS fluorescent microscope at 40X.



In conclusion, a series of eight l*H*-Pyrazolo[3,4-*b*]quinolin-3-amineswere synthesized and their cytotoxic efficacy was determined in ten cancer cell lines. The compound **QTZ05,** with a 7-OCH_3 substituent in the benzo ring of 1*H*-pyrazolo[3,4-*b*]quinolin-3-amine moiety, had significant and relatively selective cytotoxic efficacy in four colon cancer cell lines, with IC_{50} values ranging from 2.3 to 10.2 µM. This cytotoxicity of **QTZ05** on colon cancer cells is not strong as our previously reported compound (IND-2), but the cytotoxicity on the other cells lines is also lowered. **QTZ05**'s cytotoxic efficacy in colon cancer cells is mediated by cell cycle arrest at the sub G1 phase and induction of apoptosis, as indicated by chromatin condensation and translocation of PS detected by Annexin V in HCT-116 cells. Overall, the findings our study suggest that l*H*-Pyrazolo[3,4-*b*]quinolin-3-amines,in particular, **QTZ05,**

may be an important prototype for the development of compounds targeting apoptotic pathways in colorectal cancer cells.

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Conflict of Interest: Authors declared no conflict of interest.

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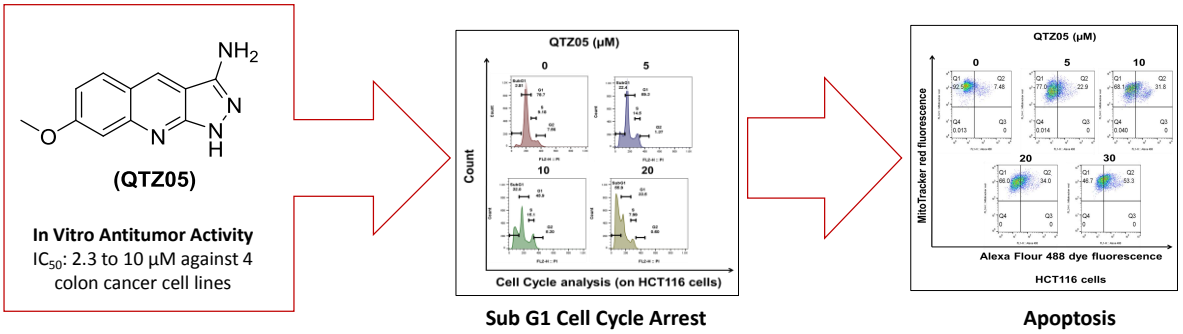

Highlights:

- Eight l*H*-Pyrazolo[3,4-*b*]quinolin-3-amine (QTZ) derivatives were synthesized
- The QTZ05 is the most potent compounds against colon cancer cells
- The QTZ05 acts *via* apoptosis and sub G1 cell cycle arrest.