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A novel arylethynyltriazole acyclonucleoside inhibits proliferation of drug-resistant pancreatic cancer cells

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ABSTRACT

Novel arylethynyltriazole acyclonucleosides were synthesized and assessed for their anticancer activity on drug-resistant pancreatic cancer MiaPaCa-2 cells. One lead compound was found to have much more potent apoptosis-related antiproliferative effects than gemcitabine, the current first-line treatment for pancreatic cancer. Further investigations showed that this active compound did not inhibit DNA synthesis, which means that it does not resemble gemcitabine and may involve a different mechanism of action.

Pancreatic cancer is one of the most devastating forms of human cancer: it is associated with a 5-year survival rate of only 3%.[1] The current first-line treatment is based on gemcitabine (Fig. 1), a nucleoside drug which acts mainly by inhibiting DNA synthesis along with other possible modes of action.[2] However, gemcitabine is only moderately effective, since the response rate in humans is only 12%. In addition, drug resistance develops very fast, resulting in a median survival period of only five months.[1,2] Consequently, there is an urgent need to develop new and more efficacious drug candidates for combating pancreatic cancer, especially the drug-resistant form.[3]

We recently discovered several novel aryltriazole ribonucleosides with promising inhibitory effects on the proliferation of drug-resistant pancreatic cancer cells (**1**–**4** in Fig. 1).[4–6] With the special triazole heterocycle as nucleobase, the corresponding nucleosides may be endowed with novel mechanisms of action, different from the traditional nucleoside analogues with natural nucleobases.[7] Ribavirin, the first synthetic triazole nucleoside antiviral drug discovered 40 years ago,[8a] constitutes one of the best examples in this regard, because it exerts its antiviral activities by several modes of action including the inhibition of viral RNA polymerases and viral capping enzymes, the lethal mutagenesis of viral RNA genomes, the interference of host inosine monophosphate dehydrogenase (IMPDH) and the modulation of the host immune responses.[8] Recently, there has been renewed interest in ribavirin and its apoptosis-related anticancer effect.[9a,b] The anticancer mechanisms of ribavirin are also complex and pleiotropic: ribavirin may inhibit the activity of an oncogene, the eukaryotic translation initiation factor eIF4E, or modulate the immune system through eIF4E and/or IMPDH.[9b] Ribavirin treatment led to significant clinical improvement in patients with poor-prognosis acute myeloid leukemia (AML),[9c] with its clinical benefit likely being far beyond AML.[9d] Furthermore, the aromatic moieties appended on the triazole ring of these ribonucleosides are expected to yield enlarged aromatic systems serving as nucleobases.[7] These triazole nucleoside analogs may bind to the corresponding biological targets via greater and stronger interactions and thus provide novel mechanisms of action for combating cancer. It was established for example that triazole ribonucleoside **2** (Fig. 1) has potent anticancer effects involving entirely new mechanisms,[4,7] namely down-regulation of the heat shock protein Hsp27, a small molecular chaperone which contributes importantly to the drug resistance observed in many forms of cancer and provides a novel target for developing new means of combating drug resistance in pancreatic cancer.[10]

In our ongoing efforts to develop novel triazole nucleosides[11–18] capable of anticancer activity, we have been focusing on acyclic aryltriazole nucleoside analogs. Replacing ribose sugar with an acyclic sugar component is known to yield nucleoside analogs with further interesting biological properties. Acyclovir (Fig. 1) is one of the most successful examples of acyclic nucleoside drugs showing antiviral activity.[19] Although acyclic ribavirin analogues were first

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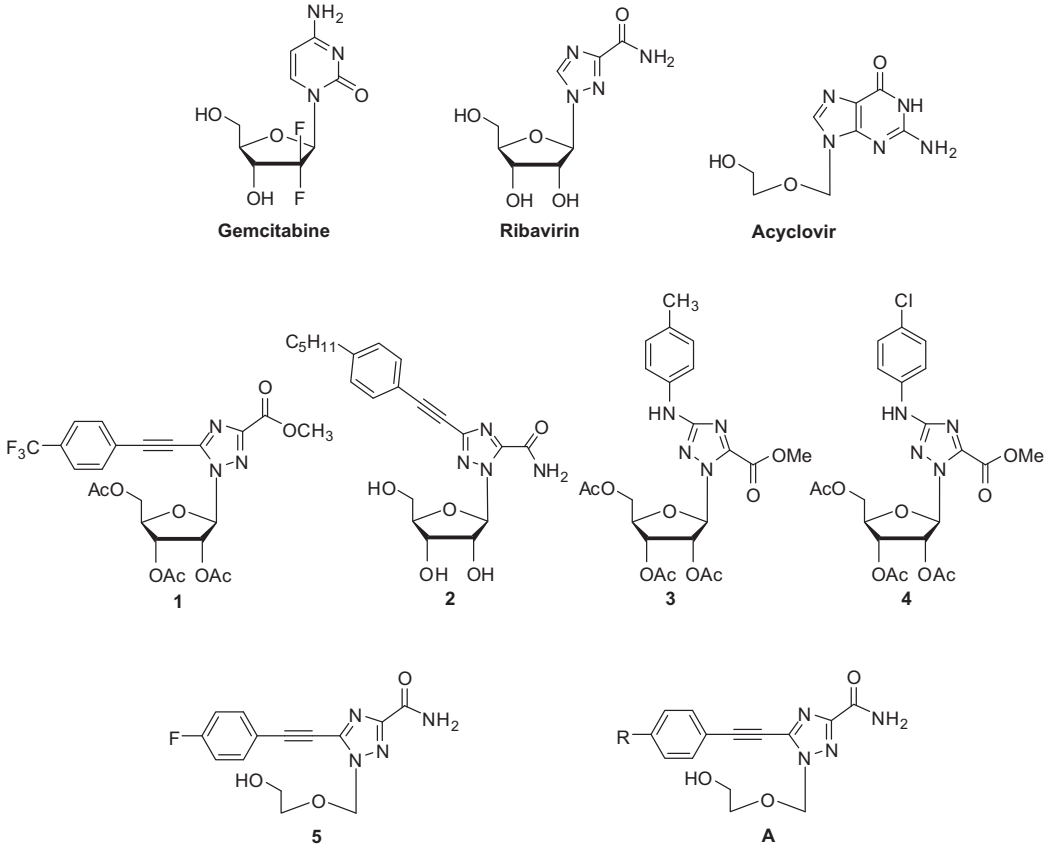


Figure 1. Gemcitabine, ribavirin, acyclovir, the identified anticancer triazole ribonucleoside leads **1**–**4** and antiviral triazole acyclonucleoside **5**, as well as the proposed arylethynyltriazole acyclonucleosides (**A**).

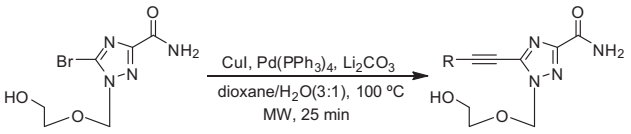
synthesized in 1985 by Beauchamp et al., none of them showed interesting biological activity.[20] We have recently identified an arylethynyl acyclonucleoside **5** (Fig. 1),[13] which has significant antiviral effects on the hepatitis C virus (HCV). All these findings led us to study more closely the presence of the acyclic sugar component in aryltriazole nucleoside analogs, with a view to designing new nucleoside analogs with antitumoral properties. Here we report on our recent studies, in which novel arylethynyltriazole acyclonucleoside analogs were synthesized by performing Sonogashira cross-coupling reactions and the anticancer effects were assessed on cancer cells. One of these novel acyclonucleoside analogs was found to have particularly noteworthy antiproliferative effects on drug-resistant pancreatic cancer MiaPaCa-2 cells, presumably via caspase-dependent apoptosis induction processes. In addition, the potency of this analog is greater than that of gemcitabine, the reference drug to treat pancreatic cancer.

Arylethynyltriazole acyclonucleoside **A** was synthesized in a single step, using our previously developed Pd-catalyzed Sonogashira cross-coupling method in aqueous media under microwave irradiation (Table 1).[13,21] In line with our previous findings, good to excellent yields were obtained with both alkylacetylene (Table 1, entry 1) and heterocyclic arylacetylenes (Table 1, entries 7 and 8) in comparison with the reference *n*-butylphenylacetylene (Table 1, entry 2). Electron-withdrawing groups such as the Cl, Br, and CN groups located at the *para*-position of the phenyl ring in phenylacetylene resulted in lower yields (Table 1, entries 3–5), possibly because the reduced nucleophilicity and increased electrophilicity of the alkyne induced by the electron-withdrawing group impede the transmetallation process and, in addition, induce undesirable Michael additions.[22] It is worth mentioning that the yield was much lower in the case of **A4,** and that considerable amounts of side products could be observed on the TLC plate. This may be mainly due to the presence of the Br atom in **A4**, which may further react with the remaining excess alkyne. For reasons so far not fully understand, no reaction occurred with 4-ethynylaniline, and the starting materials were recovered with a yield of up to 80%.

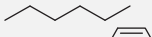
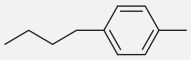
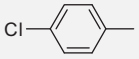
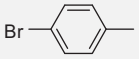
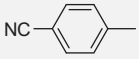
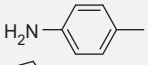
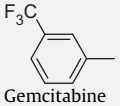
We then assessed the anticancer activity of the above synthesized compounds and that of the previously synthesized arylethynyltriazole acyclonucleosides[13] by performing MTT assays to determine the inhibition on the proliferation of drug-resistant human pancreatic cancer MiaPaCa-2 cells.[23] Among all the acyclic nucleosides tested, **A4** turned out to be the most promising lead, since it was found to significantly inhibit the growth of MiaPaCa-2 cells in a dose-dependent manner (Fig. 2). In addition, it is much more potent than the control reference, gemcitabine, which is currently the first-line drug used to treat pancreatic cancer. In our previous study on aryltriazole nucleosides, only nucleoside analogs bearing a ribose sugar component showed effective anticancer activity (**1**–**4** in Fig. 1).[4–7] The results obtained in the present study on the active compound **A4** show that the acyclic analog **A4** may also constitute a promising new structural lead with potent anticancer effects. To our knowledge, **A4** is the first acyclic aryltriazole nucleoside analog reported to inhibit the proliferation of drug-resistant pancreatic cancer MiaPaCa-2 cells more efficiently than gemcitabine.

We were particularly interested in the role of the acyclic sugar component relating to the anticancer activity of **A4**. We therefore synthesized two analogues of **A4**, **6** and **7**,[24,25] which have different sugar components from **A4**. Compound **6** is a ribose analog of **A4**, whereas **7** has the acyclic sugar part being protected by acetyl group (Fig. 3). No anticancer activity was observed with **6**, neither did **7** elicit any significant activity (data not shown). Therefore, the

Table 1
Synthesis of arylethynyltriazole acyclonucleosides **A** via the Sonogashira cross-coupling in aqueous media using microwave irradiation, and their inhibition on the cell growth of drug-resistant human pancreatic cancer MiaPaCa-2 cells



A

Entry	R	Products	Yields[a] (%)	Inhibition (%)
1		A1	80	NA
2		A2	90	21
3		A3	67	22
4		A4	48 [b]	69
5		A5	53	19
6		A6	0(80)[c]	—
7		A7	75	NA
8		A8	70	NA
9		A9	72	13
10		A10	72	21
11	Gemcitabine			30

NA: no activity.
[a] 0.05 equiv $Pd(PPh_3)_4$, 0.05 equiv CuI, 2.0 equiv Li_2CO_3, dioxane/H_2O (3:1), 100 °C, microwave irradiation, 25 min.
[b] By-products were observed by TLC.
[c] No product and about 80% of starting material was recovered.

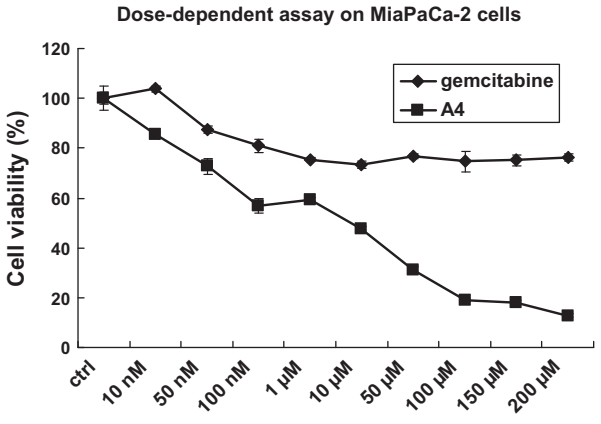
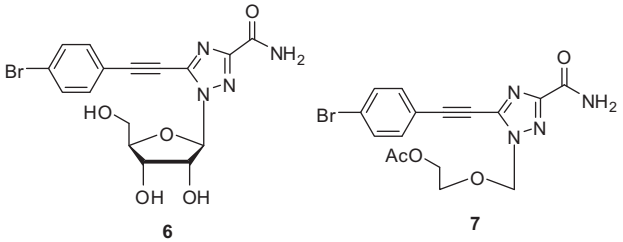


Figure 2. Inhibitory effect of **A4**, in comparison with gemcitabine, on the drug-resistant pancreatic cancer MiaPaCa-2 cell growth, assessed by MTT test.

deprotected acyclic sugar component appears to be essential and contributed crucially to the observed anticancer activity of **A4**.

We further studied the apoptosis-inducing anticancer effects of **A4** on MiaPaCa-2 cells, using fluorescence-activated cell sorting (FACS) flow cytometry,[26] in comparison with non-treated control



6 7

Figure 3. Structural analogs of **A4**.

cells. After 48 h of treatment, the cells were labeled with propidium iodide and immediately analyzed. An increase in the fraction of cells undergoing apoptosis (sub-G1/G0 fraction) was observed in cells treated with **A4** in comparison with non-treated control cells (Fig. 4A), which indicates that **A4** acts mainly by inducing apoptosis. To further confirm this point, caspase-3/7 activity was determined by performing a colorimetric assay and measuring the cleavage of the luminogenic substrate containing the tetrapeptide sequence DEVD.[27] The results obtained showed the occurrence of a twofold increase in caspase-3/7 activation in cells treated with **A4** as compared with non-treated control cells (Fig. 4B). Collectively, these findings suggest that a caspase-3/7-dependent process of apoptosis induction was involved.

We then examined whether **A4** exerted its antiproliferative effects by inhibiting DNA synthesis, because the anticancer nucleoside drug gemcitabine is known to inhibit DNA replication and subsequently induce apoptosis, resulting in effective anticancer effects.[28] The action of **A4** on the metabolism of nucleic acids was assessed in MiaPaCa-2 cells by measuring the incorporation of [^{3}H]-thymidine into the DNA.[29] As shown in Figure 5, no significant inhibitory effects on DNA synthesis were observed with **A4**, whereas more than 80% DNA inhibition was achieved with gemcitabine under the same conditions. This finding suggests that **A4** may have a different mechanism of action from that of

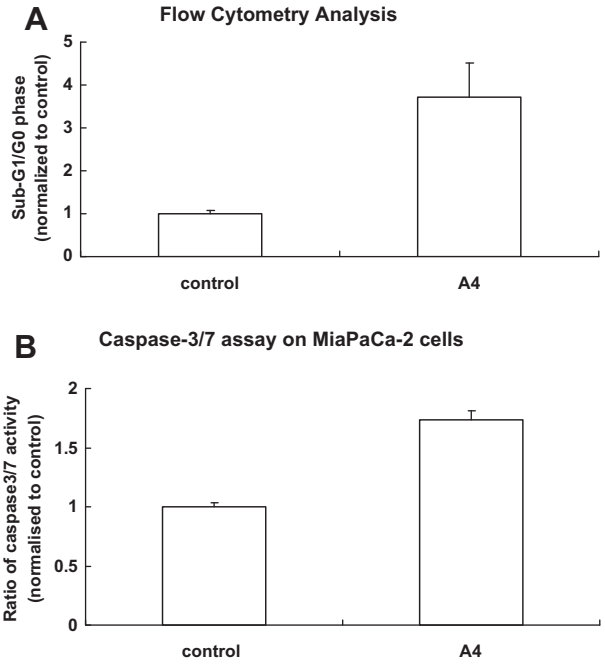


Figure 4. Apoptosis-induction in MiaPaCa-2 cells after treatment with **A4**, using non treatment as control. (A) Flow cytometry was used to quantify the percentage of cells undergoing apoptosis (cells in sub-G1/G0);[26] (B) Caspase-3/7 activity was measured using Caspase-Glo luminescent assay.[27]

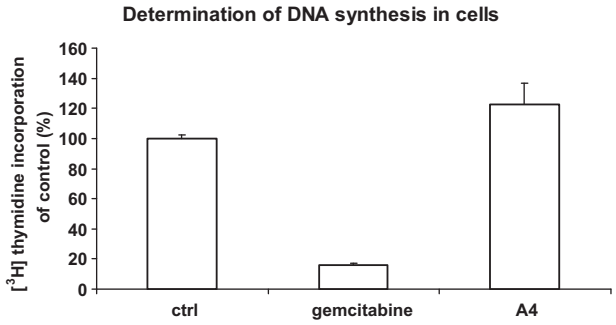


Figure 5. Inhibition of **A4** on DNA synthesis by measuring the incorporation of [^{3}H]-dTMP in MiaPaCa-2 cells, in comparison with gemcitabine.[29]

gemcitabine. Whether **A4** resembles ribavirin in anticancer mechanisms is an open question and requires further insightful investigation, which is not in the scope of the present work. As the modes for ribavirin action in cancer therapy are complex, pleiotropic, and not yet fully understood, a comparison of **A4** with ribavirin may be helpful to underpin their anticancer mechanisms. This will constitute the research program of our future investigation.

In conclusion, a series of novel arylethynyltriazole acyclonucleoside analogs was synthesized by performing Pd-catalyzed Sonogashira cross-coupling reactions using a previously described simple, efficient one-step procedure. The antiproliferative effects of these analogs were assessed on drug-resistant human pancreatic cancer MiaPaCa-2 cells. One lead compound was found to have superior antiproliferative effects to and different mechanism from gemcitabine, the current reference treatment for pancreatic cancer. This finding suggests that this acyclonucleoside could be a promising candidate in the search for new anticancer drugs with novel mechanisms of action. We are currently engaged on detailed study on its mode of action and further structure/activity relationship analyses, the results of which are essential to our ongoing search for potent novel anticancer drug candidates.

Acknowledgments

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Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra and MS analysis of all new compounds are included) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.093.

References and notes

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21. *General procedure for preparing* A: The terminal alkynes (0.24 mmol), tetrakis(triphenylphosphine)palladium(0) (11.6 mg, 0.01 mmol), CuI (1.9 mg, 0.01 mmol), Li_2CO_3 (29.6 mg, 0.4 mmol) and 5-bromo-1-[(2-hydroxyethoxy)methyl]-1,2,4-triazole-3-carboxamide (0.2 mmol) were suspended in 2.8 mL of dioxane/H_2O (3:1) under argon. The vessel was sealed and irradiated at 100 °C for 25 min, and then cooled to room temperature. The reaction mixture was concentrated under reduced pressure and the crude residue was purified by flash chromatography on silica gel (CH_2Cl_2/CH_3OH, 20:1). The purified material was dried in vacuo to afford the corresponding products.
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23. *In vitro cell growth inhibition assay*: Pancreatic cancer chemo-resistant MiaPaCa-2 cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS). Cells were seeded at a density of 15,000 cells per well in 96 well View Plate (Packard) in 250 μL of medium containing the same components as described above. Cells were allowed to attach overnight and then culture medium was removed and replace with fresh media alone as control or containing different compounds. Plates were further incubated at 37 °C and 5% CO_2 for 48 h. The number of viable cells remaining after the appropriate treatment was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) colorimetric assay.
24. Compound **6** was synthesized following the protocol described in Ref. 5: ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.06 (br s, 1H, –C(O)N*H*), 7.79 (br s, 1H, –C(O)N*H*), 7.76 (d, 2H, J = 8.8 Hz, phenyl-H), 7.68 (d, 2H, J = 8.5 Hz, phenyl-H), 6.01 (d, 1H, J = 4.0 Hz, H-1′), 5.65 (br s, 1H, –OH), 5.33 (br s, 1H, –OH), 4.82 (t, 1H, J = 5.6 Hz, –OH), 4.53–4.50 (m, 1H, H-2′), 4.26–4.23 (m, 1H, H-3′), 4.02–3.96 (m, 1H, H-4′), 3.63–3.41 (m, 2H, H-5′); ^{13}C NMR (62.5 MHz, DMSO-d_6): δ 159.6, 157.1, 139.7, 133.9, 132.2, 124.6, 118.4, 96.0, 90.5, 86.2, 75.6, 74.1, 70.5, 62.0; ESI-HRMS: calcd. for $C_{16}H_{16}BrN_4O_5^+$ 423.0299, found 423.0299; IR: 2231 cm^{-1} (–C≡C–).
25. *Preparation of* **7**: 40.0 mg (0.11 mmol) of **A4** was dissolved in 6 mL acetic anhydride and stirred under the catalyst 4-DMAP (0.01 mmol, 1.2 mg) until TLC indicated the complete consumption of **A4**. Then the solvent was removed and the residue purified by flash chromatography on silica gel (CH_2Cl_2/MeOH,

40:1). The purified material was dried in vacuo to afford 35.7 mg (80%) product **7** as a white solid: ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.56 (d, 2H, J = 8.5 Hz, phenyl-H), 7.47 (d, 2H, J = 8.5 Hz, phenyl-H), 7.09 (br s, 1H, –C(O)N*H*), 6.38 (br s,1H, –C(O)N*H*), 5.71 (s, 1H, –NCH_2O–), 4.20 (t, 2H, J = 4.6 Hz, –CH_2CH_2OAc), 3.89 (t, 2H, J = 4.6 Hz, –CH_2CH_2OAc), 2.03 (s, 3H, –$C(O)CH_3$); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 170.8, 160.3, 156.2, 140.8, 133.6, 132.2, 125.5, 118.7, 97.3, 78.3, 75.1, 68.3, 62.7, 20.9; ESI-HRMS: calcd for $C_{16}H_{16}N_4O_4Br^+$ 407.0349, found 407.0345.

26. *FACS flow cytometry*: Cells were seeded in 10 cm dishes at the density of 10^6 cells/dish and allowed to adhere and proliferate overnight. Culture medium was then removed and fresh media containing the proper concentration of compound was added. No treatment was done as negative controls. After 48 h of treatment, the cells were trypsinized and the collected cell pellet was washed with PBS and fixed in cold 70% ethanol overnight at 4 °C. After a wash with phosphate–citrate buffer, cells were treated with 200 μL RNase (500 μg/mL), labeled with 1 mL propidium iodide (50 μg/mL), and immediately analyzed by Fluorescence Activated Cell Sorting (FACS Calibur, Becton Dickinson, Le Pont-De-Claix, France). Cell death analysis was done on 10^6 cells, evaluating the sub-G1/G0 ratio. Each sample was performed in triplicate.
27. *Caspase-3/7 cleavage assay*: MiaPaCa-2 cells were initially seeded at 15,000 cells/well on 96-well plates. Twenty-four hours later, cells were treated with the test compound for 48 h. Next 100 μL of Caspase-Glo 3/7 Assay Reagent (Promega) was added to each well of a white 96-well plate containing 100 μL of blank, negative control cells or treated cells in culture medium. The caspase-3/7 activity was measured by the cleavage of the luminogenic substrate containing the tetrapeptide sequence DEVD according to the instructions of the manufacturer (Promega). The plate was incubated at room temperature for 1 h before measuring the luminescence of each well. Each experiment was performed in triplicate.
28. Huang, P.; Chubb, S.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. *Cancer Res.* **1991**, *51*, 6110.
29. *Determination of DNA synthesis*: MiaPaCa-2 Cells were seeded at a density of 15,000 cells per well in 96 well View Plate. Then the cells in exponential growth phase were treated with the compounds for 4 h and then labelled with [^{3}H]-thymidine (10 μCi/mL) for 4 h at 37 °C and 5% CO_2. Then the cells were harvested and DNA synthesis activity was determined according to the radioactivity by using liquid scintillation counting.