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# Novel 2′-alkoxymethyl substituted klavuzon derivatives as inhibitors of Topo I and CRM1

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ABSTRACT

In this work, 2′-alkoxymethyl substituted klavuzon derivatives were prepared starting from 2-methyl-1-naphthoic acid in eight steps. Anticancer potencies of the synthesized compounds were evaluated by performing MTT cell viability test over cancerous and healthy pancreatic cell lines, along with CRM1 inhibitory properties in HeLa cells by immunostaining and Topo I inhibition properties by supercoiled DNA relaxation assay. Their cytotoxic activities were also presented in hepatocellular carcinoma cells (HuH-7) derived 3D spheroids. Among the tested klavuzon derivatives, isobutoxymethyl substituted klavuzon showed the highest selectivity of cytotoxic activity against pancreatic cancer cell line. They showed potent Topo I inhibition while their CRM1 inhibitory properties somehow diminished compared to 4′-alkylsubstituted klavuzons. The most cytotoxic 2′-methoxymethyl derivative inhibited the growth of the spheroids derived from HuH-7 cell lines and PI staining exhibited time and concentration dependent cell death in 3D spheroids.

## 1. Introduction

Chemotherapy has a central role in the treatment of cancer. Despite the recent advances in targeted therapies, discovery of novel anticancer agents that selectively inhibit the growth of cancer cells is one of the strategy to develop novel anticancer drug candidates. To this end, we present the development of novel klavuzon derivatives that possess selective cytotoxic activity in pancreatic cancer cell line (MIA PaCa-2) compared to healthy one (HPDEC).

Pancreatic cancer and hepatocellular carcinoma are two of the most deadly diseases and there is still need to develop novel anticancer agents for the treatment or stop the progress of these illnesses. As it is a known fact that molecules isolated from the natural sources inspire the scientist to develop novel drug molecules. In recent years, we especially interested in to develop the conformationally constrained analogues of goniothalamin (GTN) [1] as a member of Styryllactone family [2].

Goniothalamin (GTN) is a natural product and its *R*-enantiomer (**(R)-1**) was first extracted from the bark of *Cryptocarya caloneura* [3] and its selective cytotoxic activity over cancer cell lines was first reported in 2005 by Chatchai and coworkers. They showed that (*R*)-goniothalamin is more cytotoxic in COR-L23 (large cell lung carcinoma), LS174T (human colon adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cell lines while it is less cytotoxic in ST3 (mouse skin fibroblast) and HF (human fibroblast) healthy cell lines. [4] Later Al-Qubaisi and coworkers presented its selective cytotoxic activity over Hepatoblastoma HepG2 cells in 2011 [5].

In the recent decades, many studies were conducted to assess anticancer properties of goniothalamin. Cytotoxic activitiy of (*R*)- and (*S*)-goniothalamin derivatives have been reported in these studies [5–12]. Similar therapeutical benefits of (*R*)-goniothalamin were also shown in *in vivo* studies. When healthy rats are treated with (*R*)-goniothalamin (300 μg, 14 days), it causes no changes in body weight, hematological and biochemical parameters [13]. When anticancer agent **1** was applied to Sprague-Dawley rats having cancer developed by 7,12-dimethylbenzanthracene (DMBA) it stopped the cancer growth and caused the accumulation of p53 protein [14]. In another *in vivo* study, racemic and enantiomerically pure forms of goniothalamin stops the growth of Ehrlich solid tumor model in vivo [10]. Among these studies Chromosome Maintenance 1 (CRM1) and Topoisomerase I (Topo I) proteins have been found as the intramolecular target for GTN so far [9,15].

Klavuzons have similar pharmacophore unit with GTN. Syntheses and anticancer properties of compounds 4′-methylklavuzon and 2′-methylklavuzon (**(*R*)-2** and **(*R*)-3**) were first described by our group in

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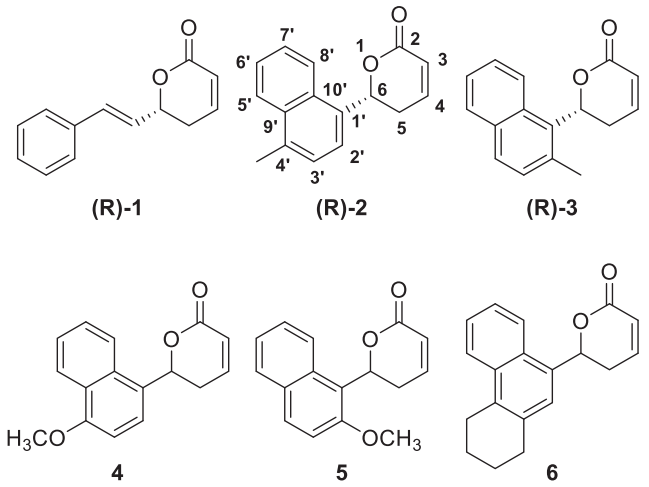



**Fig. 1.** Structures of (*R*)-goniothalamin ((*R*)-**1**) and klavuzon derivatives ((*R*)-**2**, (*R*)-**3**, and **4**–**6**).

2008 and it was found that they are more cytotoxic in cancer cells compared to GTN. Especially 4′-methyl substituted klavuzon had remarkable cytotoxic activity (Fig. 1) [1]. Later structure activity relationship studies indicated that exchange of methyl groups (**4** and **5**) with methoxide decreases the activity of the klavuzons, while compound **6** showed a remarkable cytotoxic activity in pancreatic cancer cell line [15,16]. Structure activity relationship of a list of 4′-alkyl substituted klavuzon derivatives revealed that the size of the substituent is inversely related with potency of the klavuzon derivatives. On the other hand, selectivity of these derivatives was not so promising for pancreatic cancerous and healthy cell lines (MIA PaCa-2 and HPDEC) [16].

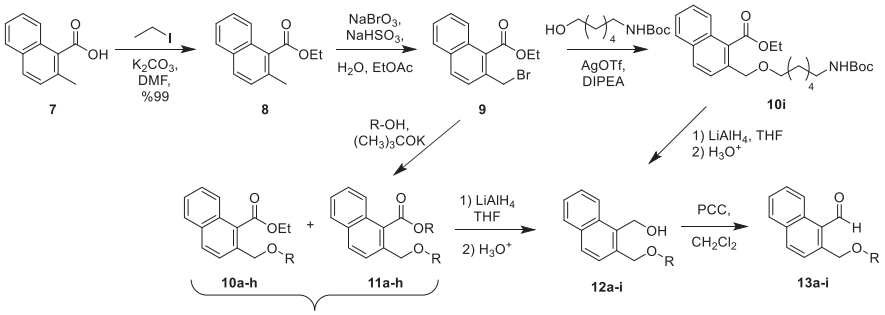
Similar to GTN, klavuzon derivatives also inhibits CRM1 and Topo I activities [15,16]. Both proteins are valuable intracellular targets to develop novel anticancer agents [17–19]. In the current study, a list of racemic 2′-alkoxymethyl substituted klavuzon derivatives have been synthesized and their anticancer activity potentials are presented. Among the tested compounds 2′-isobutoxymethyl substituted klavuzon has been found as the most selective klavuzon derivative in the literature.

## 2. Results and discussion

The route for these racemic syntheses of novel 2′-alkoxymethyl substituted klavuzon derivatives (**16a-i**) have been summarized in Tables 1 and 2. Transformation of 2-methyl-1-naphthoic acid (**7**) to ethyl 1-naphthoate ester (**8**) was the starting point for these syntheses. This conversion has been performed quantitatively by the treatment of 2-methyl-1-naphthoic acid by ethyl iodide in the presence of potassium carbonate. This ester (**8**) was also prepared by refluxing the compound **7** in ethanol in the presence of catalytic amount of sulfuric acid but this procedure gave only 48% yield.

In the next step, various reaction conditions have been tried to perform the benzylic bromination of ethyl 2-methyl-1-naphtoate (**8**). Treatment of compound **8** with benzoyl peroxide and bromotrichloromethane in refluxing benzene [20] or treatment with NBS and benzoylperoxide in heated acetonitrile had failed [21]. Afterwards, a method developed by Ishii and coworkers was used for benzylic bromination reaction by using $NaBrO_3/NaHSO_3$ in EtOAc-water mixture [22]. Although this procedure is quite sensitive to both temperature and volume of the ethyl acetate used in the reaction, the yield of the

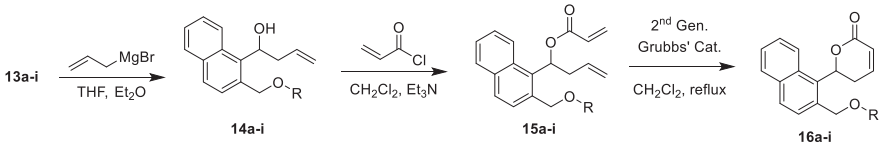
**Table 1**
Conversion of 2-methyl-1-naphthoic acid (**7**) into 2-alkoxymethyl-1-naphthaldehyde derivatives (**13a-i**).

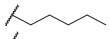
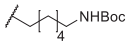



| Entry | R | Yield % | | | |
|---|---|---|---|---|---|
| | | 10a-i | 11a-i | 12a-i | 13a-i |
| **a** | | 66[a] | -[x] | 83 | 77 |
| **b** | | 60[a] | -[x] | 85 | 78 |
| **c** | | -[x] | 52[a] | 85 | 74 |
| **d** | | -[x] | -[x] | 12[b] | 76 |
| **e** | | 44[a] | -[x] | 81 | 86 |
| **f** | | -[x] | -[x] | -[x] | 23[c] |
| **g** | | 42[a] | -[x] | 97 | 84 |
| **h** | Ph | 80[a], 28[a] | -[x], 30[a] | 90 | 86 |
| **i** | NHBoc (4) | 14[a] | -[x] | 65 | 68 |

[a,b,c] Yields for the last 2, 3 and 4 steps, respectively.
[x] Compounds couldn't be purified.

**Table 2**
Preparation of 2′-alkoxymethyl substituted klavuzon derivatives (**16a-i**) starting from 2-alkoxymethyl-1-naphthaldehydes (**13a-i**).




| Entry | R | Yield % 14a-i | 15a-i | 16a-i |
|---|---|---|---|---|
| **a** |  | 94 | 88 | 78 |
| **b** |  | 94 | 97 | 85 |
| **c** |  | 94 | 89 | 85 |
| **d** |  | 98 | 91 | 67 |
| **e** |  | 77 | 76 | 73 |
| **f** |  | 96 | 79 | 68 |
| **g** |  | 89 | 75 | 88 |
| **h** |  Ph | 72 | 95 | 74 |
| **i** |  NHBoc, 4 | 98 | 44 | 99 |

compound **9** was almost quantitative under optimized conditions. Repeatability of the quantitative yield was not so high and in some trials the reaction gave an inseparable mixtures of compounds **8** and **9**. Then, this mixture of product (**9**) and starting material (**8**) was used in the next step without purification (Table 1).

In the next step, various primary and secondary alcohols treated with potassium *tert*-butoxide were reacted with ethyl 2-bromomethyl-1-naphthoate (**9**) to yield corresponding ethyl 2-alkoxymethyl-1-naphtoate derivatives (**10a-h**) besides their transesterification products (**11a-i**). Because of their similar polarities, purification of the ethyl esters from transesterification products was not always successful. Yields of the purified esters were summarized in Table 1. Methoxy, ethoxy, 1-pentoxy, isobutoxy and benzyloxy substituted ethyl esters (**10a**, **10b**, **10e**, and **10 g-h**) were isolated successfully. Among the transesterification products, 1-propoxy and benzyloxy substituted ones (**11c** and **11h**) were isolated. On the other hand, the products for 1-butoxy and isopropoxy substituted esters (**10d**, **10f**, **11d** and **11f**) could not be purified. Hence, mixtures of esters were used in the next step for these cases.

**Table 3**
Antiproliferative activity of 2′-alkoxymethyl substituted klavuzon derivatives (**16a-i**) over cancerous (MIA PaCa-2) and immortalized healthy (HPDEC) pancreatic cell lines after 48 h. of incubation.

| Comp. | $IC_{50}$ (in μM) MIA PaCa-2 | HPDEC | SI |
|---|---|---|---|
| **CPT** | 0.18 ± 0.01 | 0.21 ± 0.01 | 1.17 |
| **GTN** | 8.53 ± 0.88 | 17.75 ± 0.68 | 2.08 |
| **3** | 1.86 ± 0.17 | 0.53 ± 0.05 | 0.28 |
| **16a** | 0.61 ± 0.09 | 0.54 ± 0.10 | 0.89 |
| **16b** | 0.72 ± 0.11 | 0.70 ± 0.05 | 0.97 |
| **16c** | 2.46 ± 0.42 | 1.98 ± 0.03 | 0.80 |
| **16d** | 3.63 ± 0.14 | 3.21 ± 0.23 | 0.88 |
| **16e** | 3.25 ± 0.39 | 3.61 ± 0.16 | 1.11 |
| **16f** | 2.87 ± 0.51 | 1.23 ± 0.08 | 0.43 |
| **16g** | 3.02 ± 0.02 | 5.53 ± 0.22 | 1.83 |
| **16h** | 3.21 ± 0.19 | 1.60 ± 0.02 | 0.50 |
| **16i** | 0.70 ± 0.05 | 0.42 ± 0.02 | 0.60 |

SI: Selectivity Index = $IC_{50}$ value calculated for HPDEC / $IC_{50}$ value calculated for MIA PaCa-2, where $IC_{50}$ is the concentration that inhibit the cell growth by 50%. CPT: camptothecin.

Formation of 6-(Boc-amino)-1-hexanoxy substituted ethyl ester (**10i**) could not be achieved by this procedure, however it was successfully produced by addition of alcohol in the presence of silver triflate and DIPEA. Reduction of the esters (**10** and **11**) with lithium aluminium hydride followed by hydrolysis gave the corresponding alcohols **12a-i**. In the next step, oxidation of these primary alcohols (**12a-i**) with PCC yielded 2-alkoxymethyl substituted 1-naphthaldehyde derivatives (Table 1).

At this point, addition of allymagnesium bromide to 2-alkoxymethyl substituted 1-naphthaldehydes (**13a-i**) yielded homoallylic alcohols (**14a-i**) in 72–98% yields. Surprisingly, $^1$H NMR spectra of 1-butoxymethyl substituted homoallylic alcohol (**14d**) in $CDCl_3$ implies the formation of atropisomers. The absence of the atropisomerism in $^1$H NMR spectra of compound **14d**, acquired in $CD_3OD$, strongly suggest that atropisomers should be formed through an intramolecular hydrogen bonding between the hydroxyl group alcohol and oxygen of ether. After conversion of homoallylic alcohols (**14a-i**) to acrylate esters (**15a-i**) by addition of acryloyl chloride, ring-closing metathesis gave the novel klavuzon derivatives in good yields (Table 2).

As it was mentioned above, CRM1 and Topo I proteins are two reported intracellular targets for both GTN and klavuzon derivatives [9,16,23]. Before testing their effect over the activity of these proteins, their antiproliferative activities on cancerous (MIA PaCa-2) and healthy (HPDEC) pancreatic cell lines were determined by MTT cell proliferation assay (Table 3). Because, camptothecin (CPT) is one of the well-known Topo I poison, and goniothalamin (GTN) inhibits the same intramolecular targets with klavuzons both compounds were used as positive control. Synthesis and biological activities of compound **3** has been reported by our research group in a previous study [1] and it was also used as a third control in order to draw a structure activity relationship. As it can be seen from the table, CPT was the most cytotoxic compounds on both cell lines without any selectivity. Contrarily, GTN was found to be the least cytotoxic compound with the highest selectivity index.

In terms of cytotoxic potencies, all klavuzon derivatives were more cytotoxic in both cell lines compared to GTN. It seems that the activity of the klavuzon derivatives inversely related with the size of the substituent at 2′- position of klavuzon with one exception compound **16i**. Selectivity of the klavuzon derivatives are sensitive to the size of the substituent. A number of the klavuzon derivatives (**3**, **16f** and **16 h-i**)

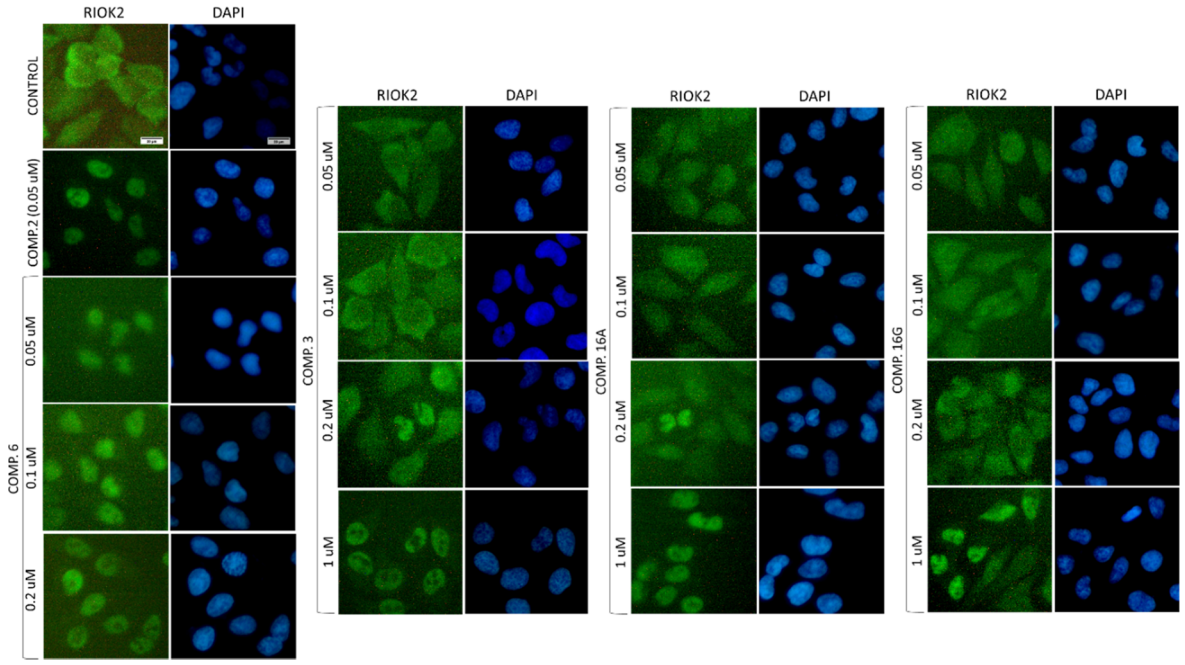



**Fig. 2.** Concentration dependent CRM1 inhibition in HeLa cells by using compounds **3**, **6**, **16a** and **16g** after 6 h. of incubation. Green channel shows RIOK2 protein which is a Serine/threonine-protein kinase. Blue channel shows nucleus staining with DAPI. RIOK2 is observed as localized in nucleus with increasing concentrations. (Scale bar = 20 μm, control group only 1% DMSO). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed selective toxicity over healthy (HPDEC) cell line. Another group of klavuzon derivatives (**16a-e**) had comparable selectivity profile with that of CPT. Interestingly, 2′-isobutoxymethyl substituted klavuzon (**16g**) was significantly selective on pancreatic cancer (MIA PaCa-2) cell line. Although the selective cytotoxic activity of GTN and compound **16g** was quite similar, klavuzon derivative (**16g**) is 2.8 times more cytotoxic in cancer (MIA PaCa-2) cell line and 3.2 fold more cytotoxic in healthy (HPDEC) cell line (Table 3).

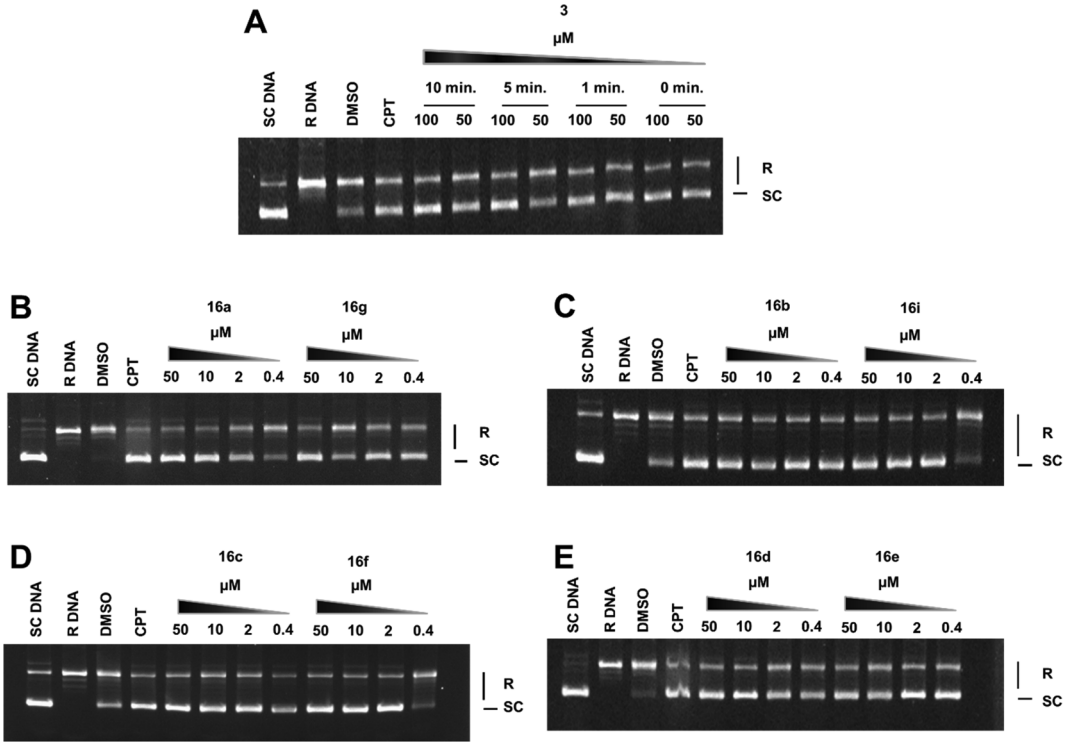



**Fig. 3.** Panel for time and concentration dependent relaxation of pUC19 (SC DNA: Supercoiled DNA and R is the relaxed form) by Topo I in the presence of klavuzon derivatives. All reactions were carried out for 30 min. CPT (camptothecin) was used as positive control. A) Time dependent inhibition by 2′-methylklavuzon (**3**). B) Concentration dependent inhibition of Topo I, preincubated for 10 min. with 2′-methoxymethylklavuzon (**16a**) and 2′-isobutoxymethylklavuzon (**16 g**). C) Concentration dependent inhibition of Topo I, preincubated for 10 min. with 2′-ethoxymethyklavuzon (**16b**) and compound **16i**. D) Concentration dependent inhibition of Topo I, preincubated for 10 min. with 2′-propoxymethylklavuzon (**16c**) or 2′-isopropoxymethylklavuzon (**16f**). E) Concentration dependent inhibition of Topo I, preincubated for 10 min. with 2′-butoxymethylklavuzon (**16d**) or 2′-pentyloxymethylklavuzon (**16e**).

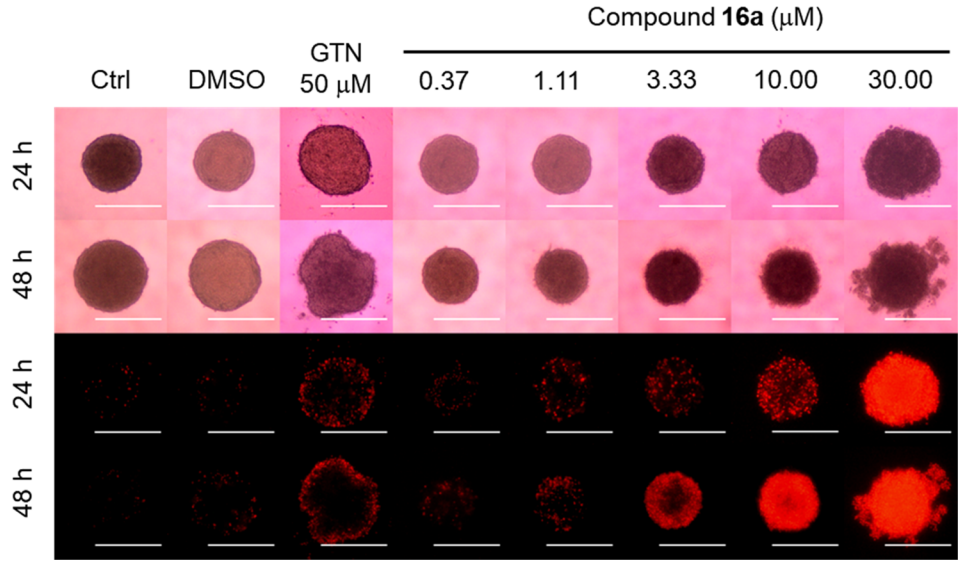



**Fig. 4.** Time and concentration dependent cytotoxic activity of 2′-methoxymethylklavuzon (**16a**) in comparison with 1% DMSO and 50 μM GTN (goniothalamin) in 3D spheroids of HuH-7 cells. Scale bar = 500 μm.

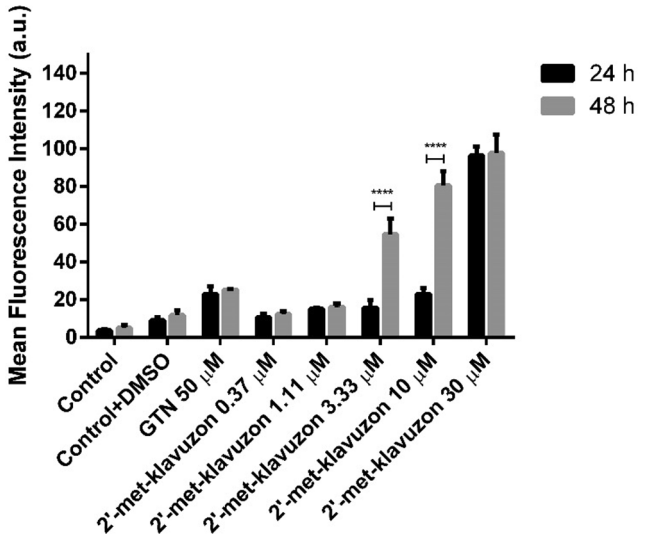



**Fig. 5.** Mean Fluorescence Intensity analysis for spheroids which are treated with PI after 24 h and 48 h incubation. Two-way ANOVA with Sidak's multiple comparison test: ****$P$ value is smaller than 0.0001 ($P < 0.0001$).

One of the well-known covalent inhibitor of CRM1 is leptomycin B which reacts with Cys528 residue present at NES-binding groove of CRM1 protein by Michael addition reaction [24]. Later, this residue was targeted with many different thiol reactive compounds such as anguinomycin, [25] selinexor, [26] valtrate, [27] acetoxychavicol acetate, [28] piperlongumine, [29] curcumin, [30] and goniothalamin [9]. Among these leptomycin, anguinomycin and selinexor inhibit CRM1 at low nanomolar concentrations and only selinexor has been approved by FDA for treatment of multiple myeloma so far.

CRM1 inhibitory properties of two novel klavuzon derivatives (the least and the most selective compounds: **16a** and **16g**) were studied along with previously reported klavuzon derivatives (**2**, **3** and **6**). Compounds **2** and **6** are the most active klavuzon derivatives reported in literature and as it can be seen Fig. 2, they completely inhibit CRM1 at 0.05 μM concentration. However, 2′-methylklavuzon (**3**) started to inhibit CRM1 at 0.2 μM concentration but still it could not inhibit CRM1 fully at this concentration. Somehow, displacement of methyl substituent from position 4′- to 2′- made a dramatic effect in the CRM1 inhibitory properties. However the CRM1 inhibitory activities of compounds **2**, **3**, and **6** are still comparable with the activity of selinexor. Similar to 2′-methylklavuzon (**3**), two novel 2′-alkoxymethyl substituted klavuzon derivatives (**16a** and **16g**) are not effective as much as 4′-methylklavuzon (**2**) in terms of CRM1 inhibition. Hence, the selective toxicity of compound **16g** may not be related with CRM1 inhibition (Fig. 2). RIOK2 protein was used to demonstrate effects of CRM1 protein that facilitates the process of intracellular translocation of RIOK2 from nucleus to cytoplasm under normal conditions.

Previous studies showed that Topo I's vicinal cysteine residues 504 and 505 should be the target for thiol reactive Topo I inhibitors such as *N*-ethylmaleimide. In the same study, authors also mentioned that Cys505 plays a critical role during the poisoning of the Topo I by derivatives of CPT [31]. Afterwards, Topo I inhibitory properties of other thiol reactive species such as derivatives of naphthoquinone, [32] cyclometalated gold III, [33] 1-ethylkuquinone, [34] CY13II, [35] and oxindolimine-metal complexes [36] were reported. Since 2′-methylklavuzon (**3**) is a thiol reactive compound it was preincubated with Topo I enzyme for 0, 1, 5 and 10 min at two different concentrations before the supercoiled DNA relaxation assay (Fig. 3A). Although compound **3** inhibited Topo I at 50 and 100 μM concentrations in all preincubation times, 10 min of preincubation time was chosen for further experiments.

Concentration dependent inhibitions of Topo I preincubated with 50, 10, 2 and 0.4 μM of novel klavuzon derivatives (**16a-g** and **16i**) were also investigated (Fig. 3B-3E). As a summary, most of the klavuzons showed Topo I inhibition at all tested concentrations except compounds **16a**, **16i** and **16f**. These three klavuzon derivatives showed minimal or no Topo I inhibition at 0.4 μM concentrations which was the lowest tested concentration (Fig. 3B, Lane 8; Fig. 3C, Lane 12; Fig. 3D, Lane 12). More interestingly the same derivatives were the ones that show selective toxicity in healthy (HPDEC) pancreatic cell line (Table 3).

It is quite difficult to draw a clear conclusion, however it was expected to have a similar $IC_{50}$ values in both cell lines. Although it is expected that drug candidates should have limited cytotoxic activity in healthy cell line, strong cytotoxic activity in HPDEC cell line can only be explained by mechanism of actions of klavuzon derivatives. Duplication times of MIA PaCa-2 and HPDEC cell lines are quite similar (~40 h. and ~ 34 h. respectively) and Topo I plays an important role during cell division process. Inhibition of Topo I by klavuzons may halt the cell division in both cell lines.

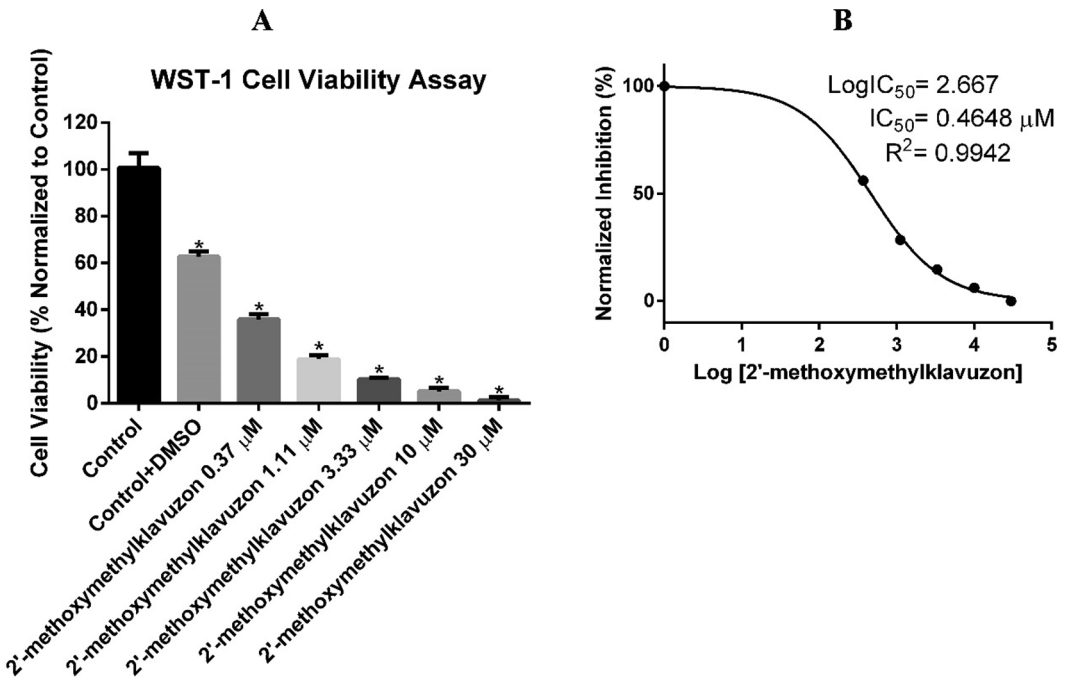



**Fig. 6.** Concentration dependent cytotoxic activity of 2′-methoxymethylklavuzon over HuH-7 derived 3D spheroids. Two-way ANOVA with Dunnett's multiple comparison test: *$P$ value is smaller than 0.05 ($P < 0.05$).

Another important point is that, HPDEC cells are Human Pancreatic Duct Epithelial Cells immortalized by HPV16 E6/E7 genes. Both E6 and E7 proteins inhibits the tumor suppressor proteins p53 and Rb (retinoblastoma) respectively. CRM1 is the cargo protein that transport p53 from nuclei to cytoplasm. Hence stability of the p53 is also depends on the activity of CRM1 cargo protein. Inhibition of CRM1 increases the stability of p53 in healthy HPDEC cell lines and causes cell death by evading immortality.

According to the literature time dependent Topo I inhibitory properties of *N*-ethylmaleimide can be seen clearly at 600 μM concentration [31]. Naphthoquinone derivative causes Topo I inhibition at 100–500 μM concentrations. Similar time dependent CRM1 inhibition was also observed for cyclometalated gold III, 1-ethylkuquinone, CY13II, and oxindolimine-metal complexes at 6.5, 5, 25, and 25 μM concentrations respectively. It seems that most of the klavuzon derivatives reported in this work show superior Topo I inhibition property compared to other thiol reactive inhibitors present in the literature.

Recently we have shown that klavuzon derivatives also inhibit SIRT1 protein and that causes the accumulation of p53 protein inside the cell which may be the cause of sensitivity of the immortalized healthy cells toward klavuzon derivatives [37]. Additionally, inhibition of p53 activity in healthy cell lines was maintained by E6 protein which can bind directly to p53 and trigger proteasomal degradation via the ubiquitin pathway [38]. It is reported that acetylation of p53 can contribute the stability and activation of p53 protein. Acetylation of lysines in p53 inhibits proteasomal degradation by inhibiting ubiquitination, and acetylation of C-terminal region causes sequence-specific DNA binding activity by activating p53 [39]. Interestingly, acetylated p53 is one of the substrates of SIRT1 enzyme [40]. In the absence of SIRT1 activity cells became more sensitive to stress conditions apoptosis can be triggered by transcriptional activities of increased expression levels of acetylated p53 and its targets p21 and Bax proteins [39]. In this sense, SIRT1 inhibitory properties of klavuzons are also contributes to the stabilization p53 in the HPDEC cell lines. Hence cytotoxic activity is not unusual in immortalized healthy cell line (HPDEC) when CRM1 and SIRT1 proteins are inhibited by klavuzon. Inhibition of Topo I, CRM1 and SIRT1 may be the cause of lack of selective cytotoxic activity.

At last, compound **16a** was incubated with 3D spheroids that are assembled from HuH-7 (hepatocellular cancer) cell line in 96 well hanging drop plates at five different concentrations for 24 and 48 h. GTN (goniothalamin) was also used at 50 μM concentration as positive control to evaluate the relative toxicity of klavuzon derivative **16a**. At the end of the incubation time propidium iodide (PI) staining was performed for 3D spheroids in hanging drops. Visualization of the spheroids was performed with an inverted phase contrast - fluorescence microscope by using 4X objective just before and after the PI staining (Fig. 4). Image analysis of PI stained spheroids resulted the mean fluorescence intensities as shown in Fig. 5. In both control experiments (with or without 1% DMSO) the size of the spheroids increased over time and PI staining indicated minimal cell death. The sizes of the GTN treated spheroids were similar and the number of PI stained death cells were increased compared to control at the end of 24 and 48 h of incubation. On the other side, compound **16a** was much more effective and inhibits the growth of spheroids at the lowest tested concentration (0.37 μM), although there was not significant number of cell death. A clear dose and time dependent cell death in the spheroids were seen and spheroids started to crumble at the end of 48 h of incubation in the presence of 30 μM of compound **16a**.

Alternatively, WST-1 assay was also performed as a measure of cell viability in spheroids. As it shown in Fig. 6, 2′-methoxymethylklavuzon showed a clear dose dependent cytotoxic activity over the spheroids. One interesting point is the presence of significant amount of cell viability difference between the control and DMSO treated control spheroids. Such difference could not be seen in PI stained spheroids. Although WST-1 assay is mostly depends on the enzymatic activities of mitochondrial dehydrogenases, PI staining can only show late apoptotic or necrotic cells. Thus normalized dose dependent percent inhibition graph for compound **16a** was drawn and $IC_{50}$ value for the cytotoxic activity of compound **16a** in spheroids was calculated as 0.46 μM.

## 3. Conclusions

A number of 2′-alkoxymethyl substituted klavuzon derivatives have been reported as an effort to contribute multi-target drug discovery studies. Syntheses of these novel compounds were completed in eight steps. Previously 4′-alkyl substituted klavuzon derivatives were also

reported by our group for the same purpose. In terms of biological activities there were some similarities and differences between these two groups of klavuzon derivatives. One of the similarities is the potency and selectivity of the klavuzon derivatives in healthy and cancerous pancreatic cell lines. Substituents, that are larger in size, resulted a decrease in the cytotoxic activity of klavuzon, while larger substituents at 4′- and 2′-positions gave better selectivity index compared to that of 4′- and 2′-methyl substituted klavuzons. It is important to point out that none of the 4′-alkylklavuzons showed significant selective cytotoxic activity against cancer cells. On the other hand novel 2′-isobutoxymethylklavuzon (**16g**) was reported as the most selective klavuzon derivative among the reported klavuzons in the literature.

Both 2′- and 4′- substituted klavuzon derivatives shown to be the inhibitors of CRM1 and Topo I proteins. One interesting point in here was the potency of the CRM1 inhibition property was altered by the positions and the sizes of the substituents. It seems that a smaller substituents at 4′-position gave stronger CRM1 inhibition compared to those of 2′-substituted ones. This finding was especially important for us because it might be possible to tune klavuzons to inhibit a specific target by playing the sizes and the positions of the substituents.

3D cell cultures are one of the convenient ways of showing newly synthesized drugs on normal and cancerous cells. Because of the unique properties of 3D cell cultures, cytotoxic effects of the drugs can be evaluated as closer to in vivo conditions than *in vitro* conditions. In our case the compound **16a**, the most cytotoxic compound among the tested 2′-alkoxymethyl klavuzon derivatives, inhibited the increment in the size of HuH-7 derived 3D spheroids. Cytotoxic activity of compound **16a** in spheroids was visualized by PI staining and $IC_{50}$ value was calculated as 0.46 μM by WST-1 assay.

## Author contributions

All authors have approved the final version of the manuscript.

## Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.104162.

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