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# Investigation of the spectrum of applicability of quinolineamides

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**ABSTRACT**

*Antiproliferative activity of the synthesized compounds was tested by the MTS assay against the wild type of human colon adenocarcinoma cell line (HCT116) and mutants with disabled TP53 gene (HCT116 p53-/-). The compounds were also tested for their cytotoxicity against murinemelanoma cell line (B16-F10) and normal human dermal fibroblasts(NHDF). One of the studied compounds (**3g**) was found active inhibiting the proliferation of all tumor cell lines.Moreover, spectroscopic characterization for the quinolines was performed. The cellular localization of two compounds with the highest fluorescence intensity(**3e** and**3h**) was also determined. These results suggest the quinoline derivatives accumulate in mitochondria but not penetrate the nuclei of cells. In summary, agroup of quinoline analogues showing good potential as candidates for imaging agents. The most active compound being superior to standarddoxorubicine is interesting leading structure for further modification.*

**Keywords**: quinoline derivatives, antiproliferative activity, anticancer, fluorescent dyes

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## INTRODUCTION

Compounds with quinoline moiety are well known due to their broad biological activities such as antifungal, antibacterial, antiprotozoic [1-4], antiretroviral [5] and antituberculotic agent [5,6]. Some quinoline compounds also have antineoplastic, antiasthmatic and antiplatelet activity [7-12] and HIV-1 replication inhibitors [13-16].Recently, some quinoline derivatives have been synthesized and reported as potent anticancer agents [17-24]. Serda and co-workers reported thatquinoline–based thiosemicarbazones showed antitumor efficiency involving iron chelation mechanism [25].

On the other hand fluorescent compounds have attracted attention due to their broad applications[26-28]. There are several reports on the synthesis and applications of styryl dyes build onquinoline. Qian Li et al. synthesized a group of styryl-based neutral compounds for potential use as in vivo imaging agents for β-amyloid plaques. They found that a class of styrylquinoline compounds displays specific binding to β-amyloid fibrils [29]. Staderini and co-workers synthesized 6-methyl-4'-amino-2-styrylquinolineas a small molecule, potentially useful to diagnose, deliver targeted therapy and monitor response to therapy in protein misfolding diseases [30]. In this application the compounds should be relatively safe for the cells at least within the concentration scope of the good fluorescence level. Another important feature is he Stockes shift which is the difference between absorption and emission peaks. Last but not least from the point of view of applications it is favorable if dyes have preference to accumulate in specific part of the staining material eg. organelle.

With these in mind we focused our attention on biological activity quinoline analogues and fluorescent compounds capable of staining cancer cells. The quinoline amides series we were studying are lipophilic compounds that potentially easily enter the cell membranes. While their antiproliferative activities render them interesting as leading structures for anticancer agents or (in case of non-active compounds)as dying agents.

## MATERIALS AND METHODS

**Synthesis**

All reagents were purchased from Sigma-Aldrich. TLC experiments were performed on alumina- backed silica gel 40 F254 plates (Merck, Darmstadt, Germany). The plates were illuminated under UV (254 nm) and evaluated in iodine vapor or UV. The melting points were determined on Boetius PHMK 05 (VEB Kombinat Nagema, Radebeul, Germany) and are uncorrected. The purity of the final compounds was checked by the HPLC separation module Waters Alliance 2695 XE (Waters Corp., Milford, MA, U.S.A.). All NMR spectra were recorded on a Bruker AM-500 (499.95 MHz for $^1$H) instrument (Bruker BioSpin Corp., Germany). Chemicals shifts are reported in ppm (δ) to internal$Si(CH_3)_4$, when diffused - easily exchangeable signals are omitted.

**8-Hydroxyquinaldine-7-carboxylic acid (2).** Obtained as yellow crystalline solid with 40% yield. Mp 229-230 $^o$C [15].

**8-Hydroxy-2-methylquinoline-7-carboxylic acid benzylamide (3a).** Obtained as white crystalline solid with 86% yield. Mp215-218$^o$C [31].

**8-Hydroxy-2-methylquinoline-7-carboxylic acid 4- fluorobenzylamide (3b).** White solid, 26% yield. Mp 190-196 $^o$C [31].

**8-Hydroxy-2-methylquinoline-7-carboxylic acid 4- methylbenzylamide (3c).** White solid 36% yield. Mp 192-200 $^o$C [31].

**8-Hydroxy-2-methylquinoline-7-carboxylic acid 4- methoxybenzylamide (3d).** White solid 34 % yield. Mp 180-190 $^o$C [31].

**8-Hydroxy-2-methylquinoline-7-carboxylic acid (3- phenylbuthyl)-amide (3e).** White crystalline solid, 73 % yield. Mp196-201 $^o$C [31].

**8-Hydroxy-2-methylquinoline-7-carboxylic acid phenethylamide (3f).** White crystalline solid, 73 % yield. Mp193-201$^o$C [31].

**2-Hydroxybenzoic acid N'-(8-hydroxy-2-methylquinoline-7-carbonyl)-hydrazide (3g).** Yellow crystalline solid, 38% yield. Mp 147-150$^o$C [31].

**8-Hydroxy-2-methylquinoline-7-carboxylic acid phenethylamide (3h).** White crystallinesolid, 62 % yield. Mp 228-235 $^o$C [31].

**8-Hydroxy-2-methylquinoline-7-carboxylic acid (2-(4- fluorophenyl)-ethyl)-amide (3i).** Obtained as white solid with 24% yield. Mp 221-224 $^o$C.
**$^1$H NMR** (400 MHz, DMSO-*d6*): *δ*2.62 (s, 3H), 4.12 (s, 2H), 6.96 (d, *J* = 8.3 Hz, 1H), 7.28 (dd, *J* = 13.0 Hz, 6.2 Hz, 2H), 7.33 (d, *J* = 8.3 Hz, 1H), 7.45 (dd, *J* = 13.5 Hz, 7.4 Hz, 1H), 7.56 (t, *J* = 7.3 Hz, 1H), 7.76 (d, *J* = 8.4 Hz, 1H), 8.04 (d, *J* = 8.2 Hz, 1H); **$^{13}$C NMR** (100 MHz, DMSO-*d6*): *δ*24.5, 35.9 (d, $^3$*JC-F* = 4.4 Hz), 112.4, 115.5 (d, $^2$*JC-F* = 21.2 Hz), 115.6, 121.1, 121.3, 122.5, 124.7 (d, $^4$*JC-F* = 3.6 Hz), 126.7, 129.1, 130.9 (d, $^3$*JC-F* = 8.3 Hz), 131.2 (d, $^3$*JC-F* = 3.4 Hz), 135.9, 140.0, 155.4, 160.3 (d, $^1$*JC-F* = 246.5 Hz), 171.8; **AE** calc for $C_{18}H_{15}FN_2O_2$: 69.67 C, 4.87% H, found: 69.81 C, 5.01% H

**5-Nitro-8-hydroxy-2-methylquinoline-7-carboxylic acid 2-fluorobenzylamide (5a).**Obtained asyellow solid with 84 % yield. Mp 231-233 $^o$C.
**$^1$H NMR** (400 MHz, DMSO-*d6*): *δ*2.58 (s, 3H), 4.13 (s, 2H), 7.22-7.30 (m, 2H), 7.40-7.49 (m, 1H), 7.53 (d, *J* = 8.7 Hz, 1H), 7.53-7.57 (m, 1H), 8.39 (bs, 2H), 9.08 (d, *J* = 8.7 Hz, 1H), 9.14 (s, 1H); **$^{13}$C NMR** (100 MHz, DMSO-*d6*): *δ*23.9, 35.9 (d, $^3$*JC-F* = 4.4 Hz), 112.4, 115.5 (d, $^2$*JC-F* = 21.2 Hz), 120.9, 121.0, 122.6, 123.8 124.6 (d, $^4$*JC-F* = 3.6 Hz), 125.4, 128.2, 131.0 (d, $^3$*JC-F* = 8.3 Hz), 131.2 (d, $^3$*JC-F* = 3.4 Hz), 132.6, 146.3, 155.3, 160.3 (d, $^1$*JC-F* = 246.6 Hz), 168.3; **AE** calc for $C_{18}H_{14}FN_3O_4$ 60.84 C, 3.97% H, found: 60.95 C, 4.12% H

**5-Nitro-8-hydroxy-2-methyl-7-carboxylic acid benzylamide (5b).**Yellow crystalline solid 61% yield. Mp219-228 $^o$C [31].

**8-Hydroxy-2-methyl-5-nitroquinoline-7-carboxylic acid 4-methylbenzylamide (5c).** Yellow crystalline solid, 78% yield. Mp 228-232 $^o$C [31].

**8-Hydroxy-2-methyl-5-nitroquinoline-7-carboxylic acid 4-methoxybenzylamide (5d).** Yellow crystalline solid, 63 % yield. Mp 211-214 $^{o}$C [31].

**Cell culture**

The human colon adenocarcinoma cells (HCT116), murine melanoma cell line (B16-F10) and normal human dermal fibroblast (NHDF) cells were obtained from the American Type Culture Collection,HCT116 p53-/- were obtained from Professor Marek Rusin from Center of Oncology in Gliwice. Cells were grown as monolayer cultures in 75cm$^2$ flasks (Nunc) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12). The medium was supplemented with 12% heat inactivated fetal bovine serum (PAA) and for NHDF with 15% fetal bovine serum (Gibco) and 100 µg/mL of gentamycin (Gibco). The cell lines were maintained at 37˚C in a 5% $CO_2$incubator.

**Cytotoxicity assay**

Exponentially growing cells were harvested by trypsinization of sub-confluent cultures. Cells were seeded at concentrations 5.0 x 10$^3$ cells per well into 96-well cell culture microtiter plates (Nunc) and cultured for 18 h. After this time, the growth medium was exchanged for medium containing of the compounds in the concentrations range 0.5 µM – 50 µM. Stock solution of the investigate œmpounds were prepared in sterileDMSO. The final concentration of DMSO in medium was 0.2 %. After 72 h incubations with the investigated compounds under standard cell culture conditions, medium was replaced with 100 µL of DMEM without phenol red. Metabolic activity of viable cells was determined by adding 20 µL of The CellTiter 96®AQ$_{ueous}$One Solutions – MTS (Promega) to each well followed by incubating for 1 h. The MTS assay is a colorimetric method for determining the number of viable cells. A standard solution containing 100 µL of DMEM without phenol red and 20 µL of MTS solution was used to determine ”blank” absorbance. The absorbance was measured at 490 nm using Synergy$^{TM}$4microplate reader (BioTek). The inhibitory concentration ($IC_{50}$) was defined as the compound concentration necessary to reduce the cells proliferation to 50% of the untreated control cells and expressed as means ± standard deviation (SD) in Graph Pad Prism 5 software. Each individual compound was tested in triplicate in a single experiment, with each experiment being repeated 3-5 times.

**Absorption and fluorescence measurements**

In the photophysical characterization of the quinoline compounds DMSO was used as a solvent. The ground-state absorptionspectra were recorded at room temperature with a Hitachi U-2900 spectrophotometer over the wavelength range of 200 – 800 nm. A typical concentration of 1.0 x 10$^{-5}$ M was used for the measurement. Fluorescence spectra were obtained with a Hitachi F-7000fluorescence spectrometer in a rage of wavelength 250 – 800 nm. A typical concentration of range 1.0 x 10$^{-5}$ M - 1.0 x 10$^{-7}$M was used for the emission measurements.

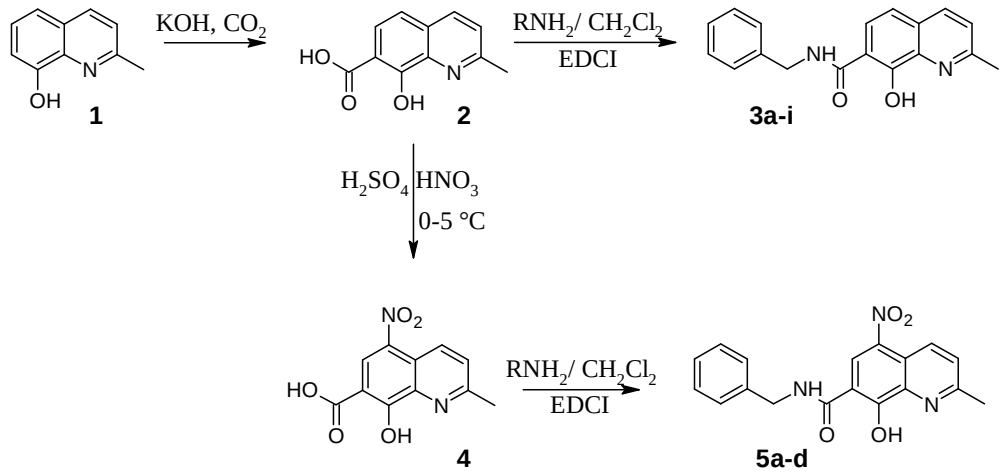
**The subcellular localization**

To visualize the compounds accumulation within the cells,20×10$^3$HCT116 cells in 100 µL growth medium were plated into 8-well LabTek chambered coverglass (Nunc) and incubated under standard conditions at 37°C, in a humidified atmosphere at 5% $CO_2$ for 24h.After this time, cells were treated with tested compounds (**3a - 3c**, **3e**, **3h, 3i**) at concentration of 25µM and incubated for further 5h. Then cells were rinsed with phosphate-buffered saline (PBS,pH 7.2) and serum-free medium containing MitoTracker® Orange CMTMRos(100 nM, Molecular Probes)was added for 30-minutes of incubation in the dark After incubation cells were washed three times with PBS and resuspended in DMEM without phenol red. Cells observation was carried out using inverted fluorescence microscope (IX81, Olympus)immediately after staining.

## RESULTS AND DISCUSSION

**Chemistry**

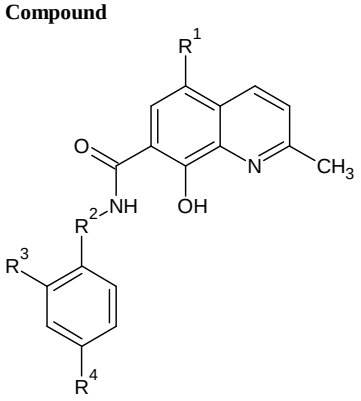
Compounds (**Table 1**) were obtained according to known multistep procedure consist of Kolbe-Schmidt carboxylation, then condensation with appropriate amine in presence of EDCI or DCC. Alternatively 7-carboxylic acid was nitrated in gentle condition and the product was subsequently reacted further as depicted in **Scheme 1**.




**Scheme1.Synthesis of quinoline analogues examined in this study**

**Table 1.Structure of synthesized compounds**

**Compound**

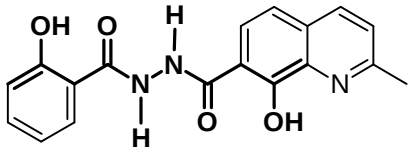


| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| **3a** | H | $CH_2$ | H | H |
| **3b** | H | $CH_2$ | H | F |
| **3c** | H | $CH_2$ | H | $CH_3$ |
| **3d** | H | $CH_2$ | H | $OCH_3$ |
| **3e** | H | $(CH_2)_4$ | H | H |
| **3f** | H | $(CH_2)_2$ | H | F |
| **3g** | H | NH-CO | OH | H |
| **3h** | H | $(CH_2)_2$ | H | H |
| **3i** | H | $CH_2$ | F | H |
| **5a** | $NO_2$ | $CH_2$ | F | H |
| **5b** | $NO_2$ | $CH_2$ | H | H |
| **5c** | $NO_2$ | $CH_2$ | H | $CH_3$ |
| **5d** | $NO_2$ | $CH_2$ | H | $OCH_3$ |

**Biological activity**

The antiproliferative activity of the synthesized compounds were assessed by the MTS assay. The results from the cytotoxicity assay are shown in **Table 2**. In general the tested compounds appeared to be inactive against all the cancer cell lines. An exception iscompound **3g**(**Figure 1**) which shownnoticeableantitumoractivity against all testedcell lines. It seems to be active, regardless of the p53 status, since the $IC_{50}$ values for HCT116 and HCT116 $p53^{-/-}$ are roughly equal. Similarly, its effectiveness against B16-F10 is observable on micromolar level ($IC_{50}$= 3.32 µM). It is noteworthy that compound **3g** has the chemical structure unlike the other tested compounds. Due to specific substitution pattern it mimics known diketo-acids inhibitors of HIV integrase. However its antiretroviral activity is rather poor [13]. Nevertheless the combined double salicylamide substructure could be valuable scaffold for further development as potential anticancer agent. Furthermore to the best of our knowledge antiproliferative activity of compounds built up on such scaffold was not reported. Compound **3g** was found to have similar or better activity than doxorubicin – clinically used agent.

**Figure 1.Structure of 3g**



All tested compounds were also examined for their cytotoxicity effects against the normal human dermal fibroblasts. All the tested compounds proved to be inactive with the exception of the compound **3g**.

**Table 2.Antiproliferative activity assay results of the tested quinoline compounds**

| Compound | Antiproliferative activity $IC_{50}$ (µM) | | | |
|---|---|---|---|---|
| | HCT116p53+/+ | HCT116p53-/- | B16F10 | NHDF |
| **3a** | >25 | >25 | >25 | >50 |
| **3b** | >25 | >25 | >25 | >50 |
| **3c** | >25 | >25 | >25 | >50 |
| **3d** | >25 | >25 | >25 | >50 |
| **3e** | >25 | >25 | >25 | >50 |
| **3f** | >25 | >25 | >25 | >50 |
| **3g** | 1.40±0.13 | 1.51±0.37 | 3.32±0.71 | 0,20±0,06 |
| **3h** | >25 | >25 | >25 | >50 |
| **3i** | >25 | >25 | >25 | >50 |
| **5a** | >25 | >25 | >25 | >50 |
| **5b** | >25 | >25 | >25 | >50 |
| **5c** | >25 | >25 | >25 | >50 |
| **5d** | >25 | >25 | >25 | >50 |
| **Doxorubicin** | 5.95±0,50 | 1.65±0,21 | - | - |

*Results are expressed as mean ± standard deviation from 3-5 experiments.*

Most of the tested compounds however remain inactive against both normal and cancer cells within relatively high concentrations range. As stressed above this may be useful in case of cellular imaging. Compound neutral to the cell with good fluorescent properties may appear useful for various applications. This prompted us to perform series of further measurements.

**Spectroscopic characterization**

The absorption and fluorescence spectra of tested quinoline compounds were summarized in **Table 3**. The fluorescence spectra of quinoline compounds have very similar profiles with a broad long wavelength band in the 400 – 550 nm region.

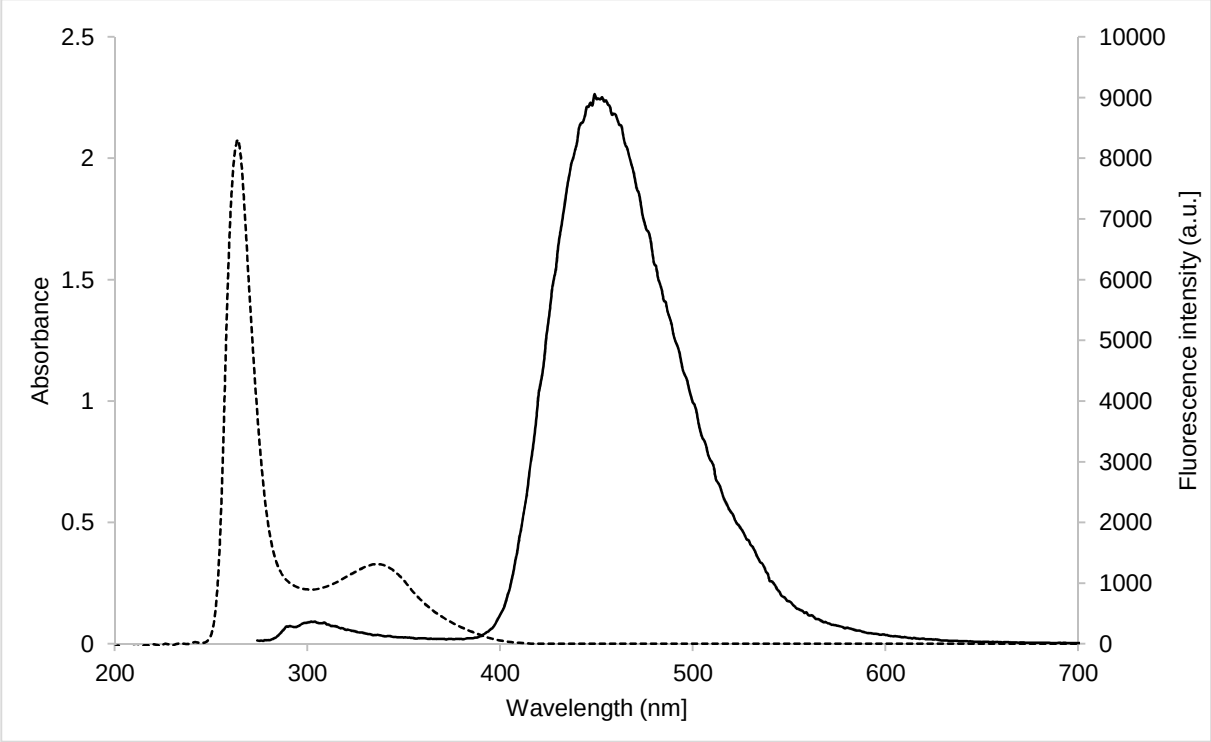
**Table 3.Absorption and fluorescence maxima and fluorescence intensity of quinoline compounds**

| Compound | $\lambda_{ex}$(nm) | $\lambda_{em}$(nm) | Fluorescence intensity[a] |
|---|---|---|---|
| **3a** | 260 | 452 | 4556 |
| **3b** | 260 | 449 | 4198 |
| **3c** | 260 | 452 | 4459 |
| **3d** | 264 | 452 | 1448 |
| **3e** | 264 | 449 | 9005 |
| **3f** | 264 | 453 | 3985 |
| **3g** | 260 | 441 | 312 |
| **3h** | 264 | 449 | 8853 |
| **3i** | 260 | 452 | 4213 |
| **5a** | 260 | 524 | 195 |
| **5b** | 260 | 523 | 4746 |
| **5c** | 260 | 524 | 213 |
| **5d** | 260 | 522 | 237 |

[a]*concentration solution $c=1x10^{-5}$ M*

Several quinolinederivatives were found to have interesting fluorescent properties. The highest fluorescence intensity was observed for compound**3e** (**Figure 2**) and **3h**. High fluorescence intensities wereobserved for compounds **3a**-**3c**, **3f**, **3i** and**5b**. Substitution of the main structure significantly affects the fluorescence properties of the tested compounds. In general compounds, unsubstituted aryl system or substituted $–CH_3$ –F showed the best fluorescence intensity. Substitution with $–OCH_3$ or -OH in the aryl ring has caused decrease of fluorescence. Theseproperties along with large Stokes shift value allow to easily observe the subcellular localization. Unfortunately the most active compound **3g** have very poor fluorescence, too weak for observations.

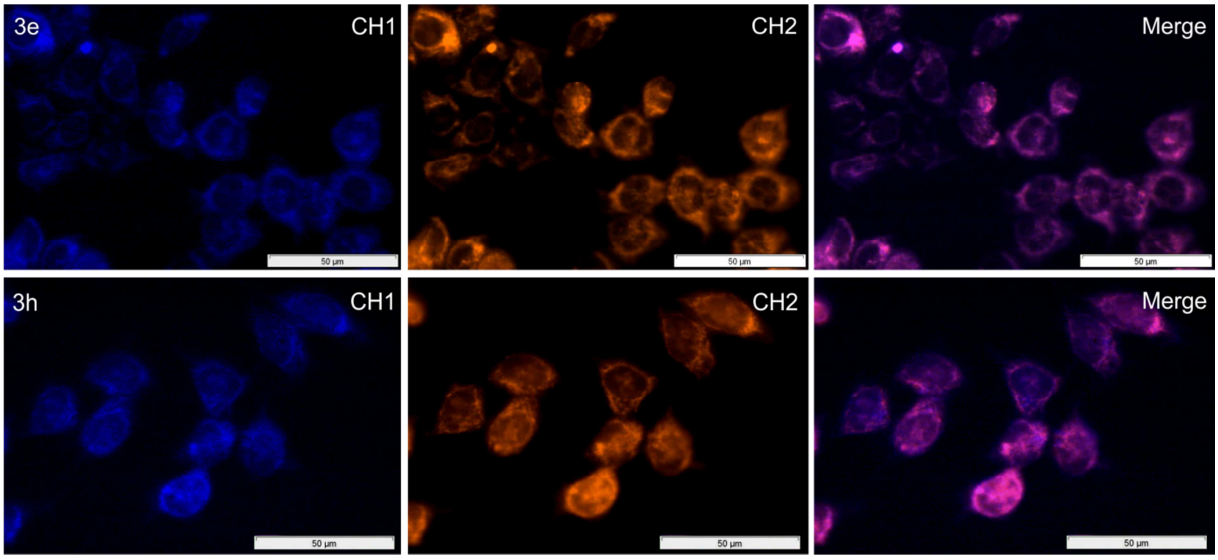
**Figure 2.Absorption and fluorescence spectra of compound 3e**




**Thesubcellular localization**

Subcellular localization of **3e** and **3h**compounds into human colon carcinoma cells (HCT116) is presented on **Figure 3**. Accumulation of compounds were determined following staining the organelle with specificfluorochrome. Both compounds penetrate the cell membranes after 5-hours incubation as strong blue signal could be observed. On the basis on staining with MitoTracker® dye we can assume that tested compounds were accumulated in mitochondria.

**Figure 3.The subcellular localization of 3e and 3h compound in HCT116 cells. Staining with 3e, 3h compounds (CH1) and MitoTracker®OrangeCMTMRos dye (CH2). Bars = 50µm**




## CONCLUSION

A series of quinolone derivatives with benzylamide substitution were synthesized. All compounds were used for biological assays. The *in vitro* tests for cytotoxicity activity were performed against colon cancer HCT116 and murine melanoma cancer B16-F10 with doxorubicin as standard. The compounds were also tested on normal

fibroblast NHDF. Compound **3g**shown very good inhibition of the cancer cell proliferation. However, further studies are necessary for confirmation of its highest activity as leading structure.

Additionally we investigated fluorescence properties of the compounds. Several of them can be successfully used as fluorescent dyes for visualization of mitochondria. High fluorescence intensity, large Stokes shift are strong advantage of the presented compounds.

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