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Original article

6-Substituted 9-fluoroquino[3,2-b]benzo[1,4]thiazines display strong antiproliferative and antitumor properties☆

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ABSTRACT

6-Substituted 9-fluoroquino[3,2-b]benzo[1,4]thiazines – a new type of tetracyclic azaphenothiazines –were obtained from of 6*H*-9-fluoroquinobenzothiazine by the introduction of appropriate substituents to the thiazine nitrogen atom (alkyl, aminoalkyl, amidoalkyl, sulfonamidoalkyl and nitrogen half-mustard groups). The compounds displayed differential cytotoxic as well as antiproliferative actions against human peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin A (PHA). In addition, they suppressed lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNF-α) production by whole blood human cell cultures. Two compounds (**4** and **15**, with the propargyl and methanesulfonamidopropyl groups) were selected for further experiments because of lack of cytotoxicity and strong antiproliferative actions. Compound **4** showed strong suppressive actions on growth of L1210, SW948, A-431 and CX-1 tumor cell lines which were close to those of cisplatin, the reference drug (e.g. GI_{50} of 2.28 μg/mL *vs.* 1.86 μg/mL for L1210 cells). Further, the compound appeared to be equally effective as cyclosporine A (CsA) in the inhibition of human two-way mixed lymphocyte reaction (MLR). The compound did not significantly inhibit interleukin 2 (IL-2)-induced growth of CTLL-2 cell line. In addition, inhibition of prostaglandin (PG) synthesis by indomethacin or block of PG receptors did not interfere with the inhibitory effect of the compound on PHA-induced cell proliferation. Therefore, it is likely that the compound acts by inhibiting cell cycle as proposed for other phenothiazines. Further studies are required for the elucidation of the mechanism of action and therapeutic utility of these compounds in more advanced *in vivo* models.

1. Introduction

Classical tricyclic phenothiazines with pharmacophoric aminoalkyl substituents at the nitrogen atom and simple additional electron-withdrawing substituents in position 2 are the source of valuable neuroleptic, antihistaminic, antitussive and antiemetic drugs [1].

N-substituted dibenzo-1,4-thiazine system provides a valuable molecular template for the development of new compounds having a wide variety of new types of activities. The structural modifications have been carried out by several approaches but the most promising one involves the introduction of a new substituent to the nitrogen atom and the substitution of one or two benzene rings with homoaromatic and heteroaromatic rings (mainly azine ones). The modified phenothiazine structures can contain not only the tricyclic but also tetracyclic, pentacyclic and even hexacyclic ring system and additional nitrogen atoms in the aromatic rings [2–5]. In the last decade, one can find a high number of reports regarding new activities of phenothiazines (both classical and modified), such as anticancer, antibacterial, antiviral and anti-inflammatory activities, multidrug resistance reverting potency as well as dealing with potential benefits in the treatment of Alzheimer's, Creutzfeldt-Jakob's and AIDS-associated diseases [2,3,5–12].

Our strategy for modification of the phenothiazine structure is based on the introduction of the pyridine and quinoline rings instead of the benzene ones to form a variety of azaphenothiazines, such as dipyridothiazines [13,14], diquinothiazines [15,16] and

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quinobenzothiazines [15,17,18]. Some these compounds exhibited promising immunosuppressive and anticancer activities against human tumor cell lines derived from colon, breast, kidney, lung, ovary, prostate, central nervous system, melanoma and leukemia [19–21]. Recently, we found that introduction of the additional substituent in the positions 8–10 of quino[3,2-b]benzo[1,4]thiazines influenced the antiproliferative and cytotoxic effects [21]. One of these compounds with the fluorine atom in position 9, and only hydrogen atom at the thiazine nitrogen atom (6*H*-9-fluoroquino [3,2-b]benzo[1,4]thiazine, **1**), caused a block in the PHA-induced proliferative response of PBMC already at 10 μg/mL although this action was also associated with high cytotoxicity [21].

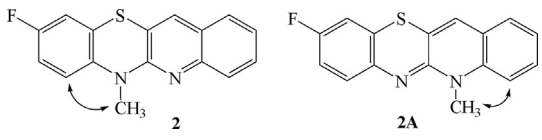
Based on our hitherto experience with this class of compounds regarding their potential therapeutic utility, we explored the possibility of decreasing their cytotoxicity by introducing various substituents to the nitrogen atom in position 6 of compound **1** to form 6-substituted 9-fluoroquinobenzothiazines. Compounds devoid of toxicity while at the same time exhibiting strong antiproliferative and/or anti-inflammatory properties would be attractive candidates for the treatment not only of neoplastic, autoimmune and allergic diseases but also for preventing graft rejection. In this work we evaluate their cytotoxicity and suppressive actions on mitogen-induced lymphocyte proliferation, tumor necrosis alpha production and tumor cell growth. In preliminary *in vitro* models we also try to investigate their possible mechanism of action. The synthesis, structures of the compounds, potential therapeutic applications and a presumable mechanism of action are discussed.

2. Results and discussion

2.1. Chemistry

The tetracyclic parent compound, 6*H*-9-fluoroquinobenzothiazine (**1**), was synthesized from the reaction of 2,2′-dichloro-3,3′-diquinolinyl disulfide with *p*-fluoroaniline in monomethyl ether of diethylene glycol or from the fusion reaction of 5,7-diaza-6,13-dithiapentacene with *p*-fluoroaniline hydrochloride as described in Ref. [16]. The transformation of reactant **1** into its N-substituted derivatives was achieved in one or three steps. As the substituents, the alkyl, non-cyclic and cyclic dialkylaminoalkyl, phthalimidoalkyl, acetylaminoalkyl, chloroethylureidoalkyl and methanesulfonylaminoalkyl groups were chosen.

As the very strongly electronegative fluorine atom in *para* position decreases the basicity of the thiazine nitrogen atom, it was crucially important to find which of two nitrogen atoms was alkylated. There are some examples of the alkylation of the azine nitrogen atoms in azaphenothiazines [22–26]. The benzene and quinoline ring proton signals in the quinobenzothiazine system differed in shapes and chemical shifts (6.78–7.20 and 7.22–7.93 ppm, respectively). To find out which nitrogen atom was alkylated, the mononuclear NOESY and ROESY experiments for *N*-methylated product was carried out to study the spatial proximity of the protons in the molecule. It appeared that the methyl group protons at 3.60 ppm correlated with the benzene signal at 6.85 ppm assigned to the proton H-7. This spatial correlation indicated that the structure of compound **2** is 6-substituted (in alternative 5-substituted structure **2A**, the signal would be at 7.79 ppm) (Scheme 1). Those experiments showed also the spatial proximity of the benzene (H-7/H-8) and quinoline (H-1/H-2, H-2/H-3 and H-3/H-4) protons. The full assignment of the protons and carbon signals in the ^{1}H and ^{13}C NMR spectra of compound **2** was achieved with the use of two-dimensional HSQC and HMBC spectra (Supplementary data). Whereas the HSQC spectrum showed which proton was bonded to the carbon atom ($^{1}J_{C,H}$ connectivity), the HMBC spectrum indicated C–H relationship through three (predominantly) and two bonds ($^{3}J_{C,H}$ and $^{2}J_{C,H}$ connectivities).



Scheme 1.

The alkylations of compound **1** with alkyl (methyl, allyl, propargyl) and dialkylaminoalkyl (2-diethylaminoethyl, 3-dimethylaminopropyl, 3-dimethylamino-2-methylpropyl, 1-pyrrolidinoethyl, 1-piperidinoethyl, 1-methyl-2-piperidinoethyl) halides were carried out in DMF in the presence of sodium hydride or potassium *tert*-butoxide and in dioxane in the presence of sodium hydroxide (see Experimental) to give derivatives **2**–**10** in good yields (Scheme 2).

Compound **1** was also transformed into compounds possessing the aminoalkyl derivative substituents. Reactions of compound **1** with phthalimidopropyl and phthalimidobutyl bromides in toluene in the presence of sodium hydride gave the phthalimidoalkyl derivatives **11** and **16**. The hydrolysis of those products with hydrazine in ethanol led to aminoalkyl derivatives **12** and **17**. As those compounds were unstable, they were quickly transformed into the acetamidoalkyl, chloroethylureidoalkyl and methanesulfonamidoalkyl derivatives **13**–**15** and **18**–**20** through the reactions with acetic anhydride, 2-chloroethyl isocyanate and methanesulfonyl chloride (Scheme 3).

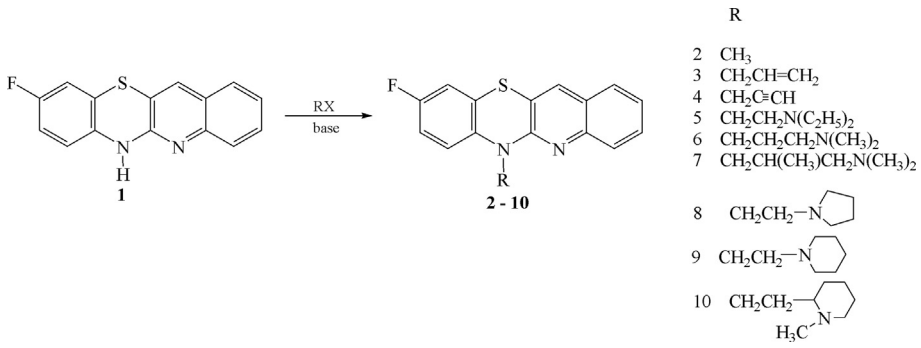
2.2. Biological activities

The compounds were tested for their cytotoxic effects against human PBMC (Table 1) at the concentrations of 100, 10 and 1 μg/mL. The cytotoxic properties were differential among the compounds depending on their structures. Compounds with the alkyl (methyl, allyl and propargyl, **2**–**4**), phthalimidoalkyl (**11**, **16**), acetylaminopropyl (**13**) and sulfonamidoalkyl (**15**, **20**) substituents were nontoxic. Compounds with the dialkylaminoalkyl (**5**–**10**), chloroethylureidoalkyl (**14**, **19**) substituents were strongly cytotoxic at the concentration of 100 μg/mL, most of them also at 10 μg/mL.

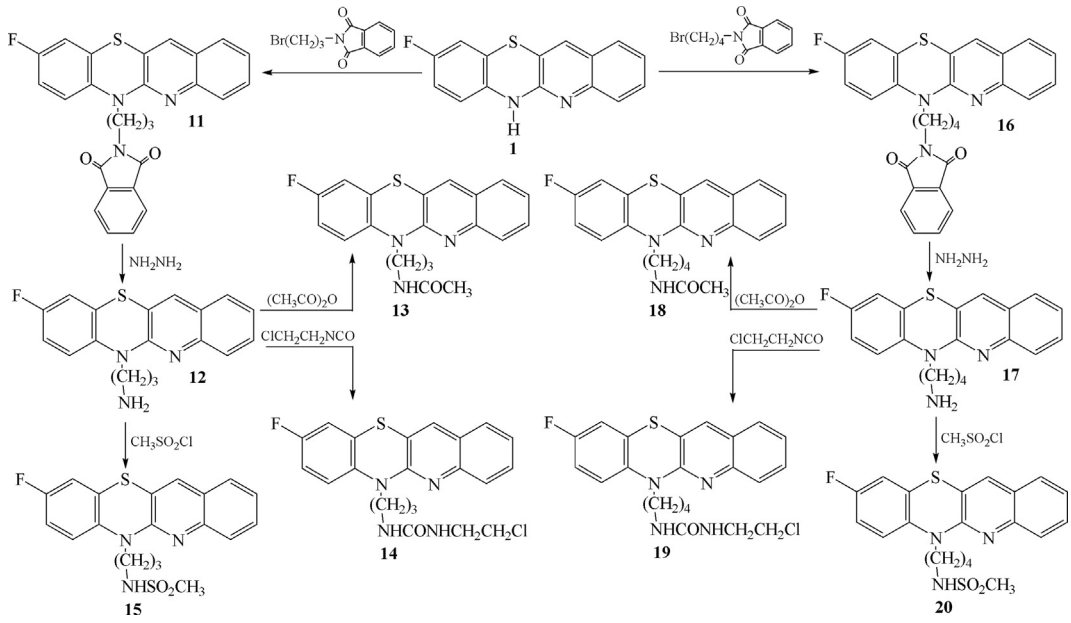
The compounds displayed very strong inhibitory actions in the proliferative response of PBMC to PHA already at low concentration (1 μg/mL) and LPS-induced TNF-α production in whole blood cultures at 5 μg/mL (Table 1). Higher concentrations of the compounds deepened the inhibitory effects in the proliferation assay as well as in induction of TNF-α (not shown).

For further investigations we selected compounds showing no (**15**) or very low (**4**) cytotoxicity and strong antiproliferative properties were tested for growth inhibition of L1210, SW948, A-341 and CX-1 tumor cell lines (Table 2). These cell lines were chosen because of their different origin and location in the organism (L1210 lymphoma, A-431 epidermal tumor, SW948 and CX-1 colon tumors). Differential suppressive action of the compounds on growth of these cells could be expected and thus indicate their preferential therapeutic use. Cisplatin [27,28], a commonly used antitumor agent served as a reference drug. In nearly all concentrations in those models compound **4** was more potent in inhibiting cell line growth than compound **15** and its activity was only slightly lower as that for cisplatin except SW948 cells. The growth inhibition $(GI)_{50}$ values of compound **4** for L1210, A431 and CX-1 cells, in comparison to cisplatin, were as follows: 2.28, 2.84 and 10.83 μg/mL *vs.* 1.86, 1.28 and 13.16 μg/mL, respectively.

The inhibition of the proliferative response of lymphocytes prompted us to check the ability of the compounds to inhibit two-



Scheme 2.



Scheme 3.

way mixed lymphocyte reaction of human PBMC (Fig. 1A–C). This is a relevant laboratory model to study potential therapeutic utility of compounds in prevention of allogeneic graft rejection. Three combinations of PBMC populations (three different donors) and the most active compounds (**4** and **15**) were used in the experiments. Cyclosporine A, a classical drug, applied for decades for prevention of allograft rejection and originally found to inhibit mixed lymphocyte reaction [29] served as a reference drug. The results showed that at concentration of 10 μg/mL compound **4** inhibited the proliferative response of lymphocytes to a similar degree as CsA. It is encouraging that compound **4**, devoid of toxicity, exhibits potent antitumor and antiproliferative properties, comparable to those of cisplatin and CsA, the reference drugs of established mechanism of action but high toxicity [27–29]. It is highly plausible that the compounds act by blocking cell cycle in the early stage of cell activation as proposed for phenothiazines [12,30]. Such a mechanism could explain no effect of the compounds on survival of PBMC in 24 h culture, measured by the results of colorimetric MTT assay which merely reflect activity of respiratory enzymes in mitochondria [31], presumably not affected by the compounds.

In additional experiments, we applied two *in vitro* models to confirm or exclude two possible mechanisms of the inhibitory action of the compounds. One possibility was an interference with IL-2 dependent cell growth typical for rapamycin [32]. Fig. 2 shows that compound **4** inhibited CTLL-2 cell growth to a limited degree and only at 10 μg/mL concentration. The inhibition by cisplatin was much stronger but most likely caused by a direct cytotoxic effect.

An interference of the compound in the prostanoid (PG) synthesis and action was also likely since phenotiazines were shown to inhibit phosphodiesterase and prostaglandin dehydrogenase [33]. Therefore, we applied indomethacin, a PG synthesis inhibitor and antagonists of PG receptors: L-798106 for EP3 and AH23848 for EP2/EP4 in the model of PHA-induced PBMC proliferation. It appeared, however, that none of the applied compounds affected the inhibition of cell proliferation by 10 μg/ml of compound **4** (Fig. 3).

In conclusion, compound **4**, showing similar activities when compared with cisplatin and cyclosporine A in respective models, could be potentially applied in prevention of tumor growth or graft rejection, alone or in combination with other immune suppressors. The property of inhibition of TNF-α by this compound could be advantageous in both cases. For example, TNF-α despite its established cytotoxic role against tumors may also contribute to tumor progression [34]. There is, however, no doubt that TNF-α is involved in graft rejection [35]. Although the mechanism of the antiproliferative action of compound **4** is far of being established we did

Table 1
The degree of cytotoxicity against PBMC of the selected compounds, effects on PHA-induced proliferative response of PBMC and LPS-induced TNF-α production by whole blood cell cultures. The toxicity of the compounds was tested in 24 h culture of PBMC and the antiproliferative effects of the compounds in a 4-day test on PBMC stimulated with PHA. The suppressive effects of the compound on TNF-α production were tested in LPS-stimulated 24 h whole blood cultures. The toxicity of the compounds was tested at 100, 10 and 1 μg/mL. Effects of the compounds on PHA-induced proliferation at 1 μg/mL, as well as at 5 μg/mL for determination of TNF-α, were shown. The results, originally calculated as OD values (toxicity and proliferation) or pg/mL (TNF-α assay), are shown in percentage inhibition as compared with appropriate DMSO controls. NT – nontoxic.

No	Cytotoxicity			Antiproliferative activity	TNF-α inhibition
	μg/ml				
	100	10	1	1	5
2	NT	NT	0.84	27.75	62.65
3	NT	2.97	12.80	34.38	57.65
4	16.39	11.57	5.27	88.76	61.60
5	87.39	88.56	16.77	93.00	80.25
6	88.22	87.63	10.48	94.18	83.54
7	90.87	92.34	NT	95.76	82.93
8	89.88	91.69	11.80	95.42	79.65
9	89.54	44.92	13.84	88.35	71.17
10	89.82	90.71	3.24	84.21	83.62
11	NT	NT	NT	31.28	64.24
13	25.18	NT	NT	54.04	72.78
14	92.37	46.90	NT	94.41	79.61
15	NT	NT	NT	80.30	65.33
16	NT	13.88	5.33	66.50	66.83
18	76.59	NT	NT	71.28	69.57
19	90.71	89.54	7.97	94.95	87.66
20	NT	NT	NT	46.97	79.76

Table 2
Anticancer activity (GI_{50}) of selected compounds **4** and **15** and cisplatin as a reference drug against cancer lines L1210, SW948, A-431 and CX-1. The test was performed in 96-well flat-bottom culture plates. L-1210 cells were incubated at 1.5×10^4 cells/100 μL/well while SW948, A-431 and CX-1 at 2.5×10^4 cells/100 μL/well. The preparations were added to the cultures at the concentration range 50–0.39 μg/mL. After 72 h incubation in a cell culture incubator the cell viability was determined using MTT colorimetric method and originally calculated as OD values. The control cultures contained DMSO dilutions present in respective compound doses. The results are presented as GI_{50} as calculated from comparisons with DMSO controls.

Compound	GI_{50} μg/mL			
	L1210	SW948	A-431	CX-1
4	2.28	44.50	2.84	10.83
15	20.47	21.61	9.65	10.67
Cisplatin	1.86	7.75	1.28	13.16

not demonstrate possible involvement of the compound in the prostaglandin metabolism. Also, the interference of the compound with IL-2-dependent CTLL-2 growth was very moderate and could rather result from metabolic disturbances in CTLL-2 cells by compound **4**. It is more likely that the mechanism of action of the studied compounds resembles those proposed for other members of phenothiazine family [12] namely inhibition of cell cycle by interference in some signaling pathways.

3. Conclusion

Selected 6-substituted 9-fluoroquinobenzothiazines from the studied series are attractive as potential antitumor, antiproliferative and anti-inflammatory therapeutics. Their value is further enhanced by lack of cytotoxicity. The introduction of the substituents at the thiazine nitrogen atom of the 9-fluoroquinobenzothiazine system decreased most often the compound cytotoxicity in comparison with the hydrogen atom. The most promising is compound **4** with the propargyl substituent at the thiazine nitrogen atom. It showed very strong antiproliferative action and comparable suppressive actions with cisplatin on growth of L1210, SW948, A-431 and CX-1 tumor cell lines. Further, it appeared to be equally effective as CsA in inhibition of two-way MLR. It is possible that compound **4** inhibits cell cycle as proposed for phenothiazines which will be a subject of our next study. Further studies will be also aimed at evaluation of therapeutic utility of these compounds in several mouse models *in vivo*.

4. Experimental

Melting points were determined in open capillary tubes on a Boetius melting point apparatus and were uncorrected. The NMR spectra were recorded on Bruker Fourier 300 and Bruker DRX spectrometers (^1H at 300 and 500 MHz, ^{13}C at 75 MHz) in $CDCl_3$. Two-dimensional NOESY, ROESY, HSQC and HMBC spectra of compound **2** were recorded on a Bruker Avance spectrometer at 600 MHz. Fast Atom Bombardment (FAB MS) mass spectra were run on a Finnigan MAT 95 spectrometer at 70 eV.

4.1. Synthesis

6*H*-9-fluoroquinobenzothiazine (**1**) was obtained in the reaction of 2,2′-dichloro-3,3′-diquinolinyl disulfide with *p*-fluoroaniline in MEDG or in the fusion reaction of 5,7-diaza-6,13-dithiapentacene with *p*-fluoroaniline hydrochloride as described in Ref. [16].

4.1.1. Synthesis of 9-fluoro-6-methylquinobenzothiazine (**2**) and 6-allyl-9-fluoroquinobenzothiazine (**3**)

To a solution of 6*H*-9-fluoroquinobenzothiazine (**1**) (134 mg, 0.5 mmol) in dry DMF (5 mL) NaH (120 mg, 5 mmol, 60% NaH in mineral oil was washed out with hexane) was added. The reaction mixture was stirred at room temperature for 1 h, alkyl halide (methyl iodide, allyl bromide, 1.5 mmol) was added and the stirring was continued for 24 h. The reaction mixture was poured into water (25 mL). The resulting solid was filtered off, washed with water and purified by column chromatography (aluminum oxide, $CHCl_3$) to give 6-substituted compounds **2** and **3**:

1. 9-Fluoro-6-methylquinobenzothiazine (**2**) (125 mg, 89%), mp 98–99 °C. ^1H NMR ($CDCl_3$) δ: 3.60 (s, 1H, CH_3), 6.85 (m, 1H, H-7), 6.91 (m, 2H, H-8, H-10), 7.30 (t, 1H, H-2), 7.54 (t, 1H, H-3), 7.55 (d, 1H, H-1), 7.67 (s, 1H, H-12), 7.79 (d, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 33.98 (CH_3), 113.76 (d, $J_{C\text{-}F} = 22.5$ Hz, C-10), 113.92 (d, $J_{C\text{-}F} = 21.0$ Hz, C-8), 115.97 (d, $J_{C\text{-}F} = 7.5$ Hz, C-7), 117.91 (C-11a), 122.37 (d, $J_{C\text{-}F} = 6.3$ Hz, C-10a), 124.26 (C-2), 125.83 (C-12a), 126.33 (C-1), 127.34 (C-4), 129.31 (C-3), 132.18 (C-12), 139.28 (d, $J_{C\text{-}F} = 2.5$ Hz, C-6a), 145.90 (C-4a), 153.21 (C-5a), 158.60 (d, $J_{C\text{-}F} = 202.5$ Hz, C-9). EIMS: *m*/*z*: 282 (M, 100), 281 (M−1, 38), 267 (M−CH_3, 20). Anal. calcd. for $C_{16}H_{11}FN_2S$: C 68.07, H 3.93, N 9.92. Found: C 67.85, H 3.95, N 9.79.

2. 6-Allyl-9-fluoroquinobenzothiazine (**3**) (131 mg, 85%), mp 77–78 °C. ^1H NMR ($CDCl_3$) δ: 4.88 (d, 2H, CH_2), 5.31 (m, 2H, CH_2), 6.12 (m, 1H, CH), 6.81 (m, H-7, H-8), 6.91 (m, 1H, H-10), 7.26 (t, 1H, H-2), 7.50 (t, 1H, H-3), 7.52 (d, 1H, H-1), 7.58 (s, 1H, H-12), 7.73 (d, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 48.57 (NCH_2), 113.44 (d, $J_{C\text{-}F} = 24.0$ Hz, C-10), 113.74 (d, $J_{C\text{-}F} = 21.0$ Hz, C-8), 116.95 (CH_2=), 117.15 (d, $J_{C\text{-}F} = 7.5$ Hz, C-7), 117.42 (C-11a), 122.06 (d, $J_{C\text{-}F} = 8.3$ Hz, C-10a), 124.24 (C-2), 125.92 (C-12a), 126.18 (C-1), 127.39 (C-4), 129.25 (C-3), 131.90 (C-12), 134.18 (CH =), 137.81 (d, $J_{C\text{-}F} = 3.0$ Hz, C-6a), 145.74 (C-4a), 152.01 (C-5a), 158.42 (d, $J_{C\text{-}F} = 241.5$ Hz, C-9). FAB MS: *m*/*z*: 309 (M + 1, 40), 293 (M−CH_3, 85), 268 (M + 1−C_3H_5, 100), 267 (M−C_3H_5, 50). Anal. calcd. for $C_{18}H_{13}FN_2S$: C 70.11, H 4.25, N 9.08. Found: C 69.90, H 4.22, N 9.83.

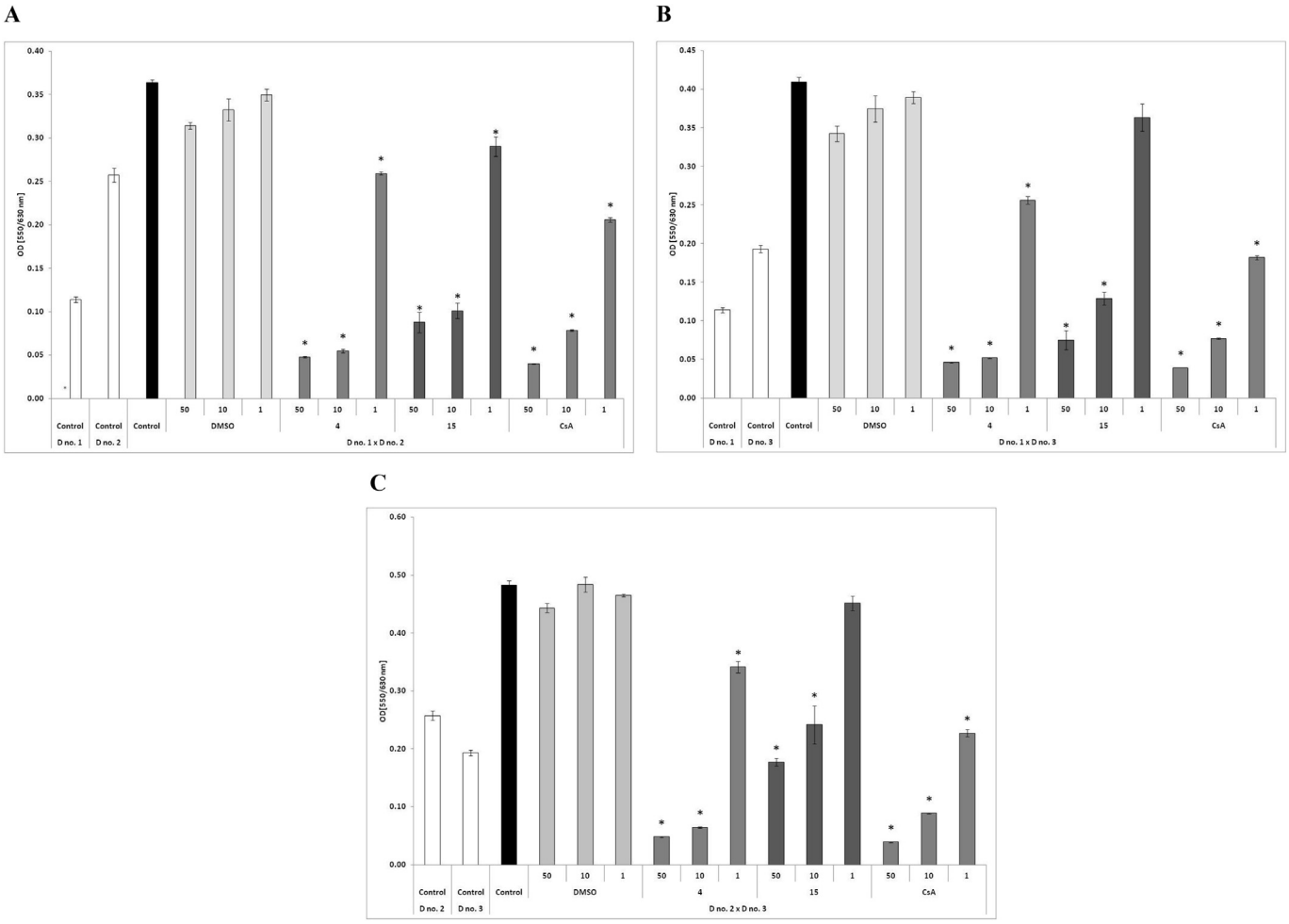


Fig. 1. Effects of compound **4** and **15** on two-way mixed lymphocyte reaction of human PBMC. Three combinations (A, B, C) of blood donors (D) were used. For the assay combinations of untreated PBMC from two individuals were used. 2×10^5 cells from each individual (total volume of 100 μL) were combined in each well of 96-well flat bottom culture plates. The compounds were used at a final concentrations of: 50, 10 and 1 μg/mL. CsA was used as a reference drug. Appropriate DMSO dilutions in the culture medium, corresponding to the compound's concentrations (0.5%, 0.1% and 0.01%) served as control cultures. After 120 h incubation the cell proliferation was determined by MTT colorimetric method and the results presented as mean OD values ±SE. (*, $P < 0.001$) when compared with DMSO cultures.

*4.1.2. Synthesis of 9-fluoro-6-propargylquinobenzothiazine (**4**)*

To a solution of 6*H*-9-fluoroquinobenzothiazine (**1**) (134 mg, 0.5 mmol) in dry DMF (5 mL) potassium *tert*-butoxide (80 mg, 0.72 mmol) was added. The mixture was stirred at room temperature for 1 h. Then 80% solution of propargyl bromide in toluene (0.15 mL, 1.35 mmol) was added dropwise. The solution was stirred at room temperature for 24 h and poured into water (20 mL), extracted with methylene chloride (20 mL), dried with Na_2SO_4 and evaporated to the brown oil. The residue was purified by column chromatography (silica gel, $CHCl_3$) to give compound **4** (121 mg, 78%), mp 124–125 °C. ^{1}H NMR ($CDCl_3$) δ: 2.31 (s, 1H, CH), 4.95 (s, 2H, CH_2), 6.91 (m, 2H, H-7, H-8) 7.20 (m, 1H, H-10), 7.30 (t, 1H, H-3) 7.50 (t, 1H, H-3), 7.54 (d, 1H, H-1) 7.68 (s, 1H, H-12), 7.82 (d, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 35.78 (CH), 72.24 (C), 79.75 (NCH_2), 113.72 (d, $J_{C\text{-}F} = 25.5$ Hz, C-10), 113.97 (d, $J_{C\text{-}F} = 22.5$ Hz, C-8), 116.74 (d, $J_{C\text{-}F} = 8.3$ Hz, C-7), 117.21 (C-11a), 122.20 (d, $J_{C\text{-}F} = 8.3$ Hz, C-10a), 124.60 (C-2), 126.02 (C-12a), 126.25 (C-1), 127.66 (C-4), 129.38 (C-3), 132.25 (C-12), 137.12 (d, $J_{C\text{-}F} = 2.3$ Hz, C-6a), 145.60 (C-4a), 151.48 (C-5a), 158.75 (d, $J_{C\text{-}F} = 242.3$ Hz, C-9). FAB MS: *m/z*: 307 (M + 1, 100), 281 (M−C_2H_2, 30), 268 (M−C_3H_3, 70). Anal. calcd. for $C_{18}H_{11}FN_2S$: C 70.57, H 3.62, N 9.14. Found: C 70.38, H 3.60, N 8.92.

*4.1.3. Synthesis of 6-dialkylaminoalkyl-9-fluoroquinobenzothiazines (**5**–**10**)*

A mixture of 6*H*-9-fluoroquinobenzothiazine (**1**) (134 mg, 0.5 mmol) sodium hydroxide (300 mg, 7.5 mmol) and hydrochloride of dialkylaminoalkyl chloride (1.5 mmol, 2-diethylaminoethyl − 260 mg, 3-dimethylaminopropyl − 240 mg, 3-dimethylamino-2-methylpropyl − 260 mg, 2-(1-pyrrolidyl)ethyl − 260 mg, 2-(1-piperidyl)ethyl − 280 mg, 2-(1-methyl-2-piperydinyl)ethyl − 300 mg) in dry dioxane (5 mL) was refluxed for 3 h. After cooling the reaction mixture was poured into water (25 mL) and extracted with chloroform (3 × 10 mL). The combined extracts were washed with water to pH = 7 and dried over Na_2SO_4. Chloroform was evaporated and the residue was purified by column chromatography (Al_2O_3, $CHCl_3$) to give compounds **5**–**10**:

*4.1.3.1. 6-(2-Diethylaminoethyl)-9-fluoroquinobenzothiazine (**5**).* (140 mg, 76%), an oil. ^{1}H NMR ($CDCl_3$) δ: 1.16 (t, 6H, $2CH_3$), 2.73 (m, 4H, $2CH_2$), 2.94 (m, 2H, CH_2), 4.32 (m, 2H, NCH_2), 6.82 (dd, 1H, H-7), 6.87 (dd, 1H, H-10), 7.01 (t, 1H, H-8), 7.25 (t, 1H, H-2), 7.49 (t, 1H, H-3), 7.51 (d, 1H, H-1), 7.58 (s, 1H, H-12), 7.66 (d, 1H H-4). FAB MS *m/z*: 368 (M + 1, 90), 295 (M−$NH(C_2H_5)_2$, 100), 268 (M-$C_2H_4N(C_2H_5)_2$, 35). Anal. calcd. for $C_{21}H_{22}FN_3S$: C 68.64, H 6.03, N 11.43. Found: C 68.49, H 6.00, N 11.21.

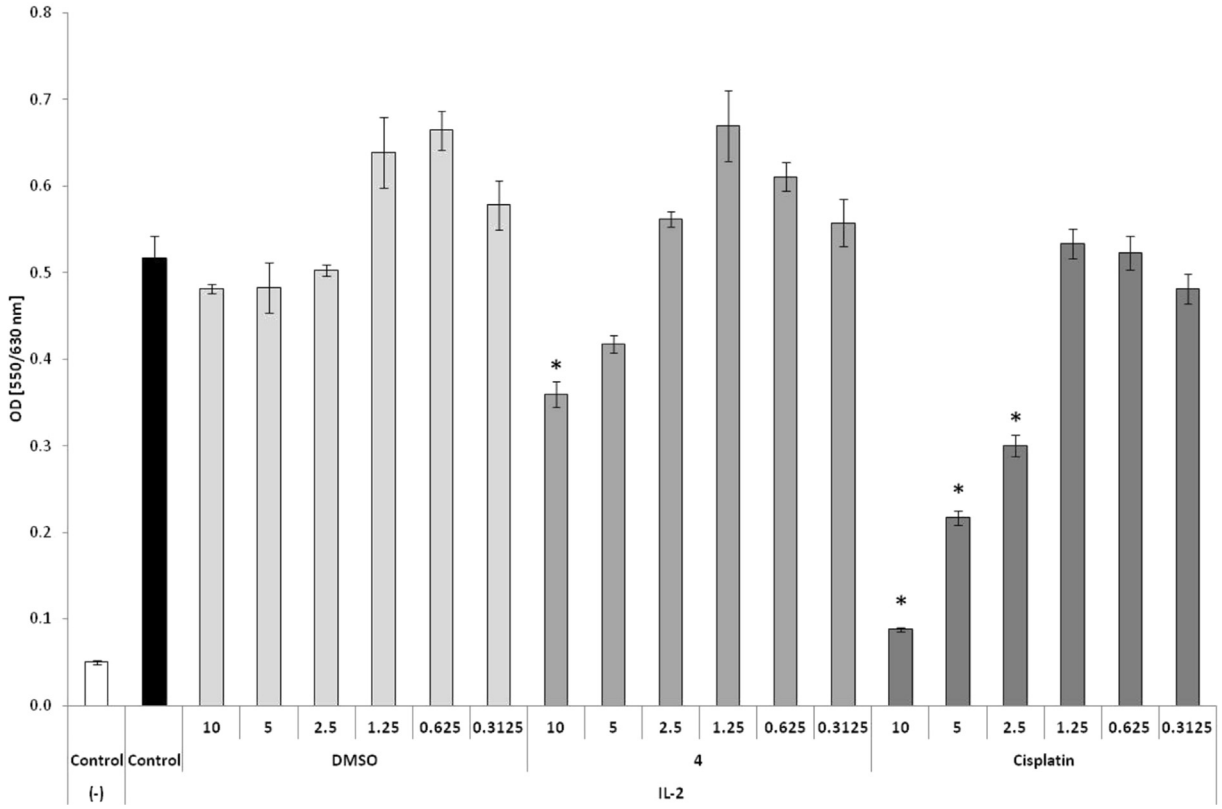


Fig. 2. Effects of the compound on IL-2 supported growth of CTLL-2 cell line (*, $P < 0.001$) when compared with control or DMSO-containing cultures (0.1–0.003125%). CTLL-2 cells (2×10^3/well/100 μL) were distributed in flat bottom 96-well plates. IL-2, was added at a dose of 8 ng/mL and compound **4** was used at 10–0.3 μg/mL concentration range. After 24 h incubation the cell viability was determined by MTT colorimetric method and the results presented as mean OD values ± SE.

*4.1.3.2. 6-(3-Dimethylaminopropyl)-9-fluoroquinobenzothiazine (**6**).* (140 mg, 79%), m. p. an oil. ^{1}H NMR ($CDCl_3$) δ: 2.06 (m, 2H, CH_2), 2.31 (s, 6H, $2CH_3$), 2.50 (m, 2H, CH_2), 4.25 (t, 2H, NCH_2), 6.87 (m, 3H, H-7, H-8, H-10), 7.25 (t, 1H, H-2), 7.49 (t, 1H, H-3), 7.52 (d, 1H, H-1), 7.59 (s, 1H, H-12), 7.71 (d, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 24.45 (CCH_2), 44.10 (NCH_2), 45.60 (NCH_3), 57.40 (NCH_2), 113.71 (d, $J_{C\text{-}F}$ = 24.8 Hz, C-10), 113.94 (d, $J_{C\text{-}F}$ = 21.8 Hz, C-8), 116.43 (d, $J_{C\text{-}F}$ = 8.3 Hz, C-7), 117.77 (C-11a), 122.29 (d, $J_{C\text{-}F}$ = 8.3 Hz, C-10a), 124.11 (C-2), 125.87 (C-12a), 126.19 (C-1), 127.32 (C-4), 129.18 (C-3), 131.82 (C-12), 137.85 (d, $J_{C\text{-}F}$ = 2.3 Hz, C-6a), 145.86 (C-4a), 152.27 (C-5a), 158.33 (d, $J_{C\text{-}F}$ = 241.5 Hz, C-9). FAB MS *m*/*z*: 354 (M + 1, 80), 309 (M + 1−NH$(CH_3)_2$, 100), 282 (M + 1−C_2H_4NH$(CH_3)_2$, 85), 268 (M-C_3H_6NH$(CH_3)_2$, 55). Anal. calcd. for $C_{20}H_{20}FN_3S$: C 67.96, H 5.70, N 11.89. Found: C 67.89, H 5.65, N 11.73.

*4.1.3.3. 6-(3-Dimethylamino-2-methylpropyl)-9-fluoroquinobenzothiazine (**7**).* (130 mg, 71%), an oil. ^{1}H NMR ($CDCl_3$) δ: 0.98 (t, 3H, CH_3), 2.26 (m, 9H, $2CH_3$, CH_2, CH), 4.32 (m, 2H, NCH_2), 6.89 (m, 2H, H-7, H-10), 7.01 (d, 1H, H-8), 7.28 (t, 1H, H-2), 7.53 (t, 1H, H-3), 7.54 (d, 1H, H-1), 7.68 (s, 1H, H-12), 7.74 (d, 1H, H-4). FAB MS *m*/*z*: 368 (M + 1, 85), 323 (M + 1−NH$(CH_3)_2$, 100), 309 (M + 1−CH_2N$(CH_3)_2$, 10), 281 (M + 1−C_2H_4N$(CH_3)_2$, 55), 268 (M + 1−C_4H_8N$(CH_3)_2$, 35). Anal. calcd. for $C_{21}H_{22}FN_3S$: C 68.64, H 6.03, N 11.43. Found: C 68.50, H 5.99, N 11.18.

*4.1.3.4. 9-Fluoro-6-(1-pyrrolidylethyl)quinobenzothiazine (**8**).* (130 mg, 71%), an oil. ^{1}H NMR ($CDCl_3$) δ: 1.86 (m, 4H, $2CH_2$), 2.73 (m, 4H, $2CH_2$), 2.98 (t, 2H, CH_2), 4.40 (t, 2H, NCH_2), 6.83 (m, 2H, H-7, H-10), 6.96 (d, 1H, H-8), 7.25 (t, 1H, H-2), 7.49 (d, 1H, H-1), 7.52 (t, 1H, H-3), 7.53 (s, 1H, H-12), 7.70 (m, 1H, H-4). FAB MS *m*/*z*: 366 (M + 1, 30), 295 (M + 1−H$(NCH_2)_4$, 100), 268 (M + 1−$C_2H_4NC_4H_8$, 30). Anal. calcd. for $C_{21}H_{20}FN_3S$: C 69.02, H 5.52, N 11.50. Found: C 68.88, H 5.49, N 11.37.

*4.1.3.5. 9-Fluoro-6-(1-piperidylethyl)quinobenzothiazine (**9**).* (150 mg, 79%), an oil. ^{1}H NMR ($CDCl_3$) δ: 1.53 (m, 2H, CH_2), 1.67 (m, 4H, $2CH_2$), 2.61 (m, 4H, $2CH_2$), 2.82 (t, 2H, CH_2), 4.38 (m, 2H, NCH_2), 6.84 (m, 2H, H-7, H-10), 7.02 (d, 1H, H-8), 7.26 (t, 1H, H-2), 7.49 (d, 1H, H-1), 7.50 (t, 1H, H-3), 7.58 (s, 1H, H-12), 7.71 (d, 1H, H-4). FAB MS *m*/*z*: 380 (M + 1, 80), 378 (M−1, 100), 295 (M-NC_5H_{10}, 40), 268 (M-$C_2H_4NCH_3$, 45), 250 (M + 1−$C_2H_4NC_5H_{10}$, 35). Anal. calcd. for $C_{22}H_{22}FN_3S$: C 69.63, H 5.84, N 11.06. Found: C 69.42, H 5.80, N 10.89.

*4.1.3.6. 9-Fluoro-6-(1-methyl-2-piperidylethyl)quinobenzothiazine (**10**).* (150 mg, 76%), an oil. ^{1}H NMR ($CDCl_3$) δ: 1.65 (m, 4H, $2CH_2$), 1.84 (m, 2H, CH_2), 2.09 (m, 4H, $2CH_2$), 2.45 (s, 3H, CH_3), 2.92 (m, 1H, CH), 4.28 (m, 2H, NCH_2), 6.83 (d, 1H, H-7), 6.87 (m, 2H, H-8, H-10), 7.26 (t, 1H, H-2), 7.50 (t, 1H, H-3), 7.54 (d, 1H, H-1), 7.60 (s, 1H, H-12), 7.68 (d, 1H, H-4). FAB MS *m*/*z*: 394 (M + 1, 80), 281 (M-$CH_2NC_6H_{12}$, 65), 268 (M-$C_2H_4NC_6H_{12}$, 40). Anal. calcd. for $C_{23}H_{24}FN_3S$: C 70.20, H 6.15, N 10.68. Found: C 70.01, H 6.11, N 10.42.

*4.1.4. Synthesis of 9-fluoro-6-phthalimidoalkylquinobenzothiazine (**11** and **16**)*

To a stirred solution of 6*H*-9-fluoroquinobenzothiazine (**1**) (268 mg, 1 mmol) in dry toluene (10 mL) NaH (240 mg, 10 mmol, washed out with hexane) was added. The mixture was refluxed for 30 min and a solution of *N*-(3-bromopropyl)phthalimide (600 mg, 2.2 mmol) or *N*-(4-bromobutyl)phthalimide (620 mg, 2.2 mmol) in dry toluene (5 mL) was added. The mixture was refluxed for 24 h.

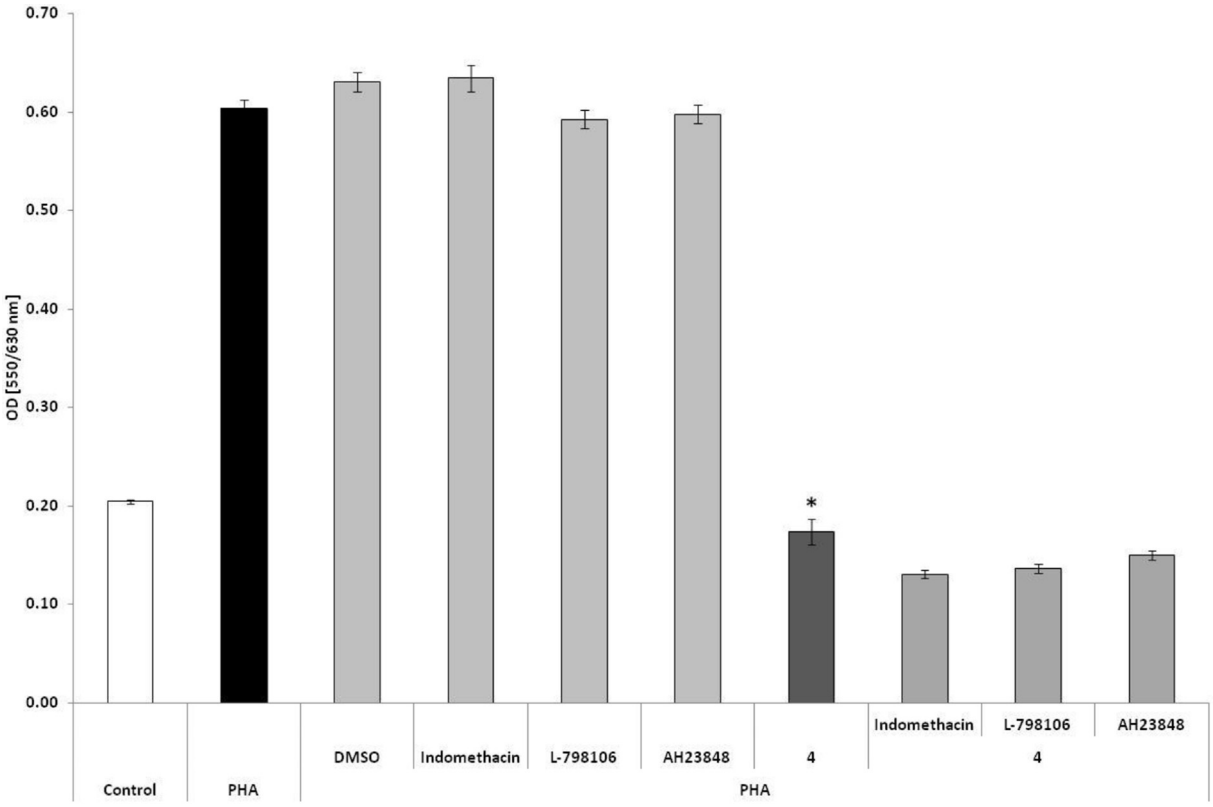


Fig. 3. Inhibition of prostaglandin synthesis and block of prostaglandin receptors do not interfere with the inhibitory action of compound **4**. (*, $P < 0.001$) when compared with 0.1% DMSO-containing cultures. PBMC were preincubated with indomethacin or prostaglandin receptor antagonists for 15 min, then for 30 min with compound **4** (10 μg/mL), followed by addition of PHA. After 4-day incubation the degree of cell proliferation was determined by MTT colorimetric method. The results are shown as mean OD values ± SE. For the sake of figure clarity additional DMSO controls were not shown since in these cases the OD values were almost identical with main control cultures.

Next toluene was evaporated *in vacuo* and the residue was extracted with $CHCl_3$ (2 × 5 mL). The extract was concentrated and purified by column chromatography (silica gel, $CHCl_3$) to give compounds **11** and **16**:

*4.1.4.1. 9-Fluoro-6-phthalimidopropylquinobenzothiazine (**11**).* (380 mg, 83%), mp 133–134 °C. ^{1}H NMR ($CDCl_3$) δ: 2.33 (m, 2H, CH_2), 3.91 (m, 2H, CH_2), 4.31 (m, 2H, NCH_2), 6.83 (d, 1H, H-7), 6.85 (m, 2H, H-8, H-10), 7.25 (t, 1H, H-2), 7.45 (t, 1H, H-3), 7.49 (d, 1H, H-1), 7.60 (s, 1H, H-12), 7.67 (m, 3H, H-4, $2H_{phthal.}$), 7.76 (m, 2H, $2H_{phthal.}$). ^{13}C NMR ($CDCl_3$) δ: 25.43 (CCH_2), 35.97 (NCH_2), 43.02 (NCH_2), 113.88 (d, $J_{C\text{-}F}$ = 24.8 Hz, C-10), 114.02 (d, $J_{C\text{-}F}$ = 22.5 Hz, C-8), 116.34 (d, $J_{C\text{-}F}$ = 8.3 Hz, C-7), 118.00 (C-11a), 122.74 (d, $J_{C\text{-}F}$ = 8.3 Hz, C-10a), 123.13 ($C_{phthal.}$), 124.26 (C-2), 125.82 (C-12a), 126.14 (C-1), 127.20 (C-4), 129.27 (C-3), 132.06 (C-12, $C_{phthal.}$), 133.86 ($C_{phthal.}$), 137.55 (C-6a), 145.50 (C-4a), 152.19 (C-5a), 158.45 (d, $J_{C\text{-}F}$ = 243 Hz, C-9). FAB MS *m/z*: 456 (M + 1, 100), 394 (M-C_3H_6F, 19), 268 (M-$C_{11}H_{10}O_2N$, 10). Anal. calcd. for $C_{26}H_{18}FN_3O_2S$: C 68.56, H 3.98, N 9.22. Found: C 68.37, H 3.95, N 8.99.

*4.1.4.2. 9-Fluoro-6-phthalimidobutylquinobenzothiazine (**16**).* (352 mg, 75%) 121–122 °C. ^{1}H NMR ($CDCl_3$) δ: 1.90 (m, 4H, $2CH_2$), 3.77 (m, 2H, NCH_2), 4.24 (m, 2H, NCH_2), 6.78 (d, 1H, H-7), 6.81 (m, 2H, H-8, H-10), 7.22 (t, 1H, H-2), 7.45 (d, 1H, H-3), 7.46 (d, 1H, H-1), 7.54 (s, 1H, H-12), 7.61 (d, 1H, H-4), 7.68 (m, 2H, $2H_{phthal.}$), 7.81 (m, 2H, $2H_{phthal.}$). FAB MS *m/z*: 470 (M + 1, 100), 295 (M-$CH_2C_8H_4NO_2$, 30), 281 (M-$C_2H_4C_8H_4NO_2$, 45), 268 (M-$C_3H_6C_8H_4NO_2$, 55). Anal. calcd. for $C_{27}H_{20}FN_3O_2S$: C 69.07, H 4.29, N 8.95. Found: C 68.88, H 4.22, N 8.79.

*4.1.5. Synthesis of 6-aminoalkyl-9-fluoroquinobenzothiazines (**12** and **17**)*

To a boiling solution of 6-phthalimidopropyl **11** (445 mg, 1 mmol) or 6-phthalimidobutyl derivative **16** (470 mg, 1 mmol) in ethanol (25 mL) 80% aqueous solution of hydrazine (0.2 mL, 5 mmol) was added. The mixture was refluxed for 2 h. After cooling the reaction mixture was acidified to pH = 2 with conc. hydrochloric acid and evaporated. Water (10 mL) was added to the residue, the resulting solid was filtered off and washed with 10% hydrochloric acid. Combined filtrates were alkalized to pH = 10 and the resulted solid was filtered off, washed with water, dried and purified by column chromatography (SiO_2, $CHCl_3$–EtOH 10:1) to give compounds **12** and **17**:

*4.1.5.1. 6-Aminopropyl-9-fluoroquinobenzothiazine (**12**).* (270 mg, 83%), an oil. ^{1}H NMR ($CDCl_3$) δ: 2.17 (m, 2H, CH_2), 2.91 (m, 2H, CH_2), 4.35 (m, 2H, NCH_2), 6.84 (m, 2H, H-7, H-8), 6.90 (d, 1H, H-10), 7.25 (t, 1H, H-2), 7.49 (t, 1H, H-3), 7.50 (d, 1H, H-1), 7.61 (s, 1H, H-12), 7.70 (d, 1H, H-4). FAB MS *m/z*: 326 (M + 1, 20), 309 (M + 1–NH_3, 100), 295 (M-CH_2NH_2, 40), 281 (M-$C_2H_4NH_2$, 90), 268 (M + 1–$C_3H_6NH_2$, 75). Anal. calcd. for $C_{18}H_{16}FN_3S$: C 66.44, H 4.96, N 12.91. Found: C 66.21, H 4.91, N 12.69.

*4.1.5.2. 6-Aminobutyl-9-fluoroquinobenzothiazine (**17**).* (255 mg, 75%), an oil. ^{1}H NMR ($CDCl_3$) δ: 1.73 (m, 2H, CH_2), 1.89 (m, 2H, CH_2), 2.85 (m, 2H, CH_2), 4.21 (m, 2H, NCH_2), 6.83 (m, 3H, H-7, H-8, H-10), 7.24 (t, 1H, H-2), 7.48 (d, 1H, H-1), 7.49 (t, 1H, H-3), 7.58 (s, 1H, H-12), 7.70 (d, 1H, H-4). FAB MS *m/z*: 340 (M + 1, 45), 323 (M–NH_2, 25), 295 (M-$C_2H_4NH_2$, 20), 281 (M-$C_3H_6NH_2$, 60), 268 (M-

$C_4H_8NH_2$, 100). Anal. calcd. for $C_{19}H_{18}FN_3S$: C 67.23, H 5.35, N 12.38. Found: C 67.02, H 5.30, N 12.17.

*4.1.6. Synthesis of 6-acetylaminoalkyl-9-fluoroquinobenzothiazines (**13** and **18**)*

To a suspension of aminopropyl **12** (163 mg, 0.5 mmol) or aminobutyl derivative **17** (170 mg, 0.5 mmol) in pyridine (3 mL) acetic anhydride (3 mL, 32 mmol) was added and the mixture was stirred at rt for 24 h. The reaction mixture was poured into water (10 mL) and the resulting solid was filtered off, washed with water, air-dried and purified by column chromatography (Al_2O_3, $CHCl_3$) to give compounds **13** and **18**:

*4.1.6.1. 6-Acetylaminopropyl-9-fluoroquinobenzothiazine (**13**).* (150 mg, 82%), mp 155–156 °C. ^{1}H NMR ($CDCl_3$) δ: 1.99 (s, 3H, CH_3), 2.07 (m, 2H, CH_2), 3.43 (m, 2H, NCH_2), 4.36 (m, 2H, NCH_2), 6.85 (d, 1H, H-7), 6.88 (m, 2H, H-8, H-10), 7.32 (t, 1H, H-2), 7.52 (t, 1H, H-3), 7.54 (d, 1H, H-1), 7.65 (s, 1H, H-12), 7.74 (d, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 23.38 (CH_3), 26.40 (CH_2), 37.47 (NCH_2), 42.94 (NCH_2), 113.93 (d, $J_{C\text{-}F}$ = 23.3 Hz, C-10), 114.23 (d, $J_{C\text{-}F}$ = 21.8 Hz, C-8), 116.69 (d, $J_{C\text{-}F}$ = 7.5 Hz, C-7), 117.84 (C-11a), 122.51 (d, $J_{C\text{-}F}$ = 8.3 Hz, C-10a), 124.58 (C-2), 125.89 (C-12a), 126.41 (C-1), 126.54 (C-4), 129.68 (C-3), 132.51 (C-12), 137.23 (d, $J_{C\text{-}F}$ = 2.3 Hz, C-6a), 145.16 (C-4a), 152.37 (C-5a), 158.60 (d, $J_{C\text{-}F}$ = 243.0 Hz, C-9), 170.20 (CO). FAB MS *m*/*z*: 368 (M + 1, 100), 309 (M-$NHCOCH_3$, 15), 295 (M-$CH_2NHCOCH_3$, 20), 281 (M-$C_2H_5NHCOCH_3$, 30), 268 (M-$C_3H_6NHCOCH_3$, 60). Anal. calcd. for $C_{20}H_{18}FN_3OS$: C 65.38, H 4.94, N 11.44. Found: C 65.17, H 4.89, N 11.21.

*4.1.6.2. 6-Acetylaminobutyl-9-fluoroquinobenzothiazine (**18**).* (150 mg, 79%), mp 128–129 °C. ^{1}H NMR ($CDCl_3$) δ: 1.70 (m, 2H, CH_2), 1.91 (m, 2H, CH_2), 1.93 (s, 3H, CH_3), 3.37 (m, 2H, NCH_2), 4.26 (t, 2H, NCH_2), 5.67 (s, 1H, NH), 6.87 (m, 3H, H-7, H-8, H-10), 7.28 (t, 1H, H-2), 7.52 (t, 1H, H-3), 7.53 (d, 1H, H-1), 7.64 (s, 1H, H-12), 7.71 (d, 1H, H-4). FAB MS *m*/*z*: 382 (M + 1, 100), 295 (M-$C_2H_4NHCOCH_3$, 20), 281 (M-$C_3H_6NHCOCH_3$, 50), 281 (M-$C_4H_7NHCOCH_3$, 95). Anal. calcd. for $C_{21}H_{20}FN_3OS$: C 66.12, H 5.28, N 11.02. Found: C 65.85, H 5.22, N 10.79.

*4.1.7. Synthesis of 6-chloroethylureidoalkyl-9-fluoroquinobenzothiazines (**14** and **19**)*

To a stirred solution of aminopropyl **12** (163 mg, 0.5 mmol) or aminobutyl derivative **17** (170 mg, 0.5 mmol) in dry ethanol (12 mL) at 0 °C 2-chloroethyl isocyanate (0.08 mL, 1 mmol) was added. The mixture was stirred at 0 °C for 1 h and at rt for 24 h. After evaporation of EtOH the residue was purified by column chromatography (Al_2O_3, $CHCl_3$) to give compounds **14** and **19**:

*4.1.7.1. 6-Chloroethylureidopropyl-9-fluoroquinobenzothiazine (**14**).* (165 mg, 77%), mp 169–170 °C. ^{1}H NMR ($CDCl_3$) δ: 2.09 (m, 2H, CH_2), 3.39 (m, 2H, CH_2), 3.52 (m, 2H, CH_2), 3.60 (m, 2H, CH_2), 4.43 (m, 2H, NH_2), 4.77 (m, 1H, NH), 5.15 (m, 1H, NH), 6.88 (m, 3H, H-7, H-8, H-10), 7.31 (t, 1H, H-2), 7.53 (t, 1H, H-3), 7.54 (d, 1H, H-1), 7.66 (s, 1H, H-12), 7.76 (d, 1H, H-4). FAB MS *m*/*z*: 431 (M + 1, 32), 368 (M + 1−CH_2CH_2Cl, 38), 309 (M + 1−$NH_2CONHCH_2CH_2Cl$, 83), 268 (M + 1−$C_3H_6NHCONHCH_2CH_2Cl$, 100). Anal. calcd. for $C_{21}H_{20}ClFN_4OS$: C 58.53, H 4.68, N 13.00. Found: C 58.26, H 4.62, N 12.79.

*4.1.7.2. 6-Chloroethylureidobutyl-9-fluoroquinobenzothiazine (**19**).* (167 mg, 75%), mp 179–180 °C. ^{1}H NMR ($CDCl_3$) δ: 1.72 (m, 2H, CH_2), 1.92 (m, 2H, CH_2), 3.23 (m, 2H, CH_2), 3.52 (m, 2H, CH_2), 3.61 (m, 2H, CH_2), 4.26 (t, 2H, NCH_2), 4.63 (m, 2H, NH_2), 6.87 (m, 3H, H-7, H-8, H-10), 7.28 (t, 2H, H-2), 7.53 (t, 1H, H-3), 7.54 (d, 1H, H-1), 7.65 (s, 1H, H-12), 7.72 (d, 1H, H-4). FAB MS *m*/*z*: 445 (M + 1, 14), 409 (M + 1−HCl, 16), 340 (M + 1−$OCNCH_2CH_2Cl$, 100), 268 (M + 1−$C_4H_8NHCONHCH_2CH_2Cl$, 88). Anal. calcd. for $C_{22}H_{22}ClFN_4OS$: C 59.39, H 4.98, N 12.59. Found: C 59.20, H 4.96, N 12.40.

*4.1.8. Synthesis of 9-fluoro-6-methanesulfonylaminoalkylquinobenzothiazines (**15** and **20**)*

To a stirred solution of aminopropyl **12** (163 mg, 0.5 mmol) or aminobutyl derivative **17** (170 mg, 0.5 mmol) in a mixture of CH_2Cl_2 (5 mL) and 10% Na_2CO_3 solution (7 mL), a solution of methanesulfonyl chloride (0.06 mL, 0.75 mmol) was added. The mixture was stirred at rt for 24 h. The organic phase was separated and aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined extracts were washed with water (2 × 10 mL) and dried over Na_2SO_4. The drying agent was filtered off and filtrate was evaporated. The resulting residue was purified by column chromatography (Al_2O_3, $CHCl_3$) to give compounds **15** and **20**:

*4.1.8.1. 9-Fluoro-6-methanesulfonylaminopropylquinobenzothiazine (**15**).* (161 mg, 80%), mp 138–139 °C. ^{1}H NMR ($CDCl_3$) δ: 2.17 (m, 2H, CH_2), 2.86 (s, 3H, CH_3), 3.33 (m, 2H, CH_2), 4.43 (m, 2H, NCH_2), 5.91 (s, 1H, NH), 6.87 (d, 1H, H-7), 6.96 (m, 2H, H-8, H-10), 7.32 (t, 1H, H-2), 7.54 (d, 1H, H-1), 7.58 (t, 1H, H-3), 7.67 (s, 1H, H-12), 7.93 (d, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 27.35 (CCH_2), 40.02 (CH_3), 40.71 (CH_2), 42.99 (NCH_2), 113.95 (d, $J_{C\text{-}F}$ = 24.8 Hz, C-10), 114.33 (d, $J_{C\text{-}F}$ = 22.5 Hz, C-8), 116.88 (d, $J_{C\text{-}F}$ = 8.3 Hz, C-7), 117.68 (C-11a), 122.34 (d, $J_{C\text{-}F}$ = 8.3 Hz, C-10a), 125.06 (C-2), 125.73 (C-12a), 125.84 (C-1), 126.36 (C-4), 130.35 (C-3), 133.01 (C-12), 136.60 (C-6a), 144.47 (C-4a), 151.83 (C-5a), 158.80 (d, $J_{C\text{-}F}$ = 243.8 Hz, C-9). FAB MS *m*/*z*: 404 (M + 1, 100), 373 (M-CH_2O, 10), 309 (M-$NHSO_2CH_3$, 25), 295 (M-$CH_2NHSO_2CH_3$, 35), 281 (M-$C_2H_5NHSO_2CH_3$, 45), 268 (M + 1−$C_3H_6NHSO_2CH_3$, 85). Anal. calcd. for $C_{19}H_{18}FN_3O_2S_2$: C 56.56, H 4.50, N 10.41. Found: C 56.44, H 4.45, N 10.26.

*4.1.8.2. 9-Fluoro-6-methanesulfonylaminobutylquinobenzothiazine (**20**).* (160 mg, 77%), mp 124–125 °C. ^{1}H NMR ($CDCl_3$) δ: 1.56 (m, 2H, CH_2), 1.96 (m, 2H, CH_2), 2.94 (s, 3H, CH_3), 3.25 (m, 2H, CH_2), 4.34 (m, 2H, NCH_2), 6.89 (m, 3H, H-7, H-8, H-10), 7.30 (t, 1H, H-2), 7.53 (d, 1H, H-1), 7.54 (t, 1H, H-3), 7.68 (s, 1H, H-12), 7.86 (m, 2H, H-4). FAB MS *m*/*z*: 418 (M + 1, 100), 323 (M-$NHSO_2CH_3$, 15), 295 (M-$C_2H_5NHSO_2CH_3$, 20), 281 (M-$C_3H_6NHSO_2CH_3$, 55), 268 (M-$C_4H_8NHSO_2CH_3$, 100). Anal. calcd. for $C_{20}H_{20}FN_3O_2S_2$: C 57.54, H 4.83, N 10.06. Found: C 57.44, H 4.77, N 9.91.

4.2. Biological assays

4.2.1. Reagents

Fetal calf serum (FCS), RPMI-1640 and Hanks' medium were purchased from CytoGen GmbH (Sinn, Germany). Lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS), phytohemagglutinin (PHA), dimethyl sulfoxide (DMSO) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), indomethacin and prostaglandin receptor antagonists L-798106, AH23848 and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

4.2.2. Preparation of the compounds for biological assays

The compounds were dissolved in DMSO (10 mg/mL) and subsequently diluted in RPMI-1640 cell culture medium (see below). Indomethacin and prostaglandin receptor antagonists were initially dissolved in DMSO (5 mg/mL) and subsequently in the culture medium.

4.2.3. Isolation of the peripheral blood mononuclear cells (PBMC)

Venous blood from a single donor was withdrawn into heparinized syringes and diluted twice with PBS. PBMC were isolated by

centrifugation on Ficoll-Uropoline gradient (density 1.077 g/mL) and centrifuged at 800 × g for 20 min at 4 °C. The interphase cells, consisting of lymphocytes (20%) and monocytes (80%) were then washed three times with Hanks' medium and re-suspended in a culture medium, referred to below as the culture medium, consisting of RPMI-1640, supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics, at density of 2×10^6 cells/mL.

4.2.4. Cytotoxicity of the compounds against human PBMC

PBMC were washed three times with Hanks' medium and re-suspended in the culture medium at density of 2×10^6 cells/mL. 2×10^5 cells/100 μL/well were incubated with the tested compounds at doses 100, 10 and 1 μg/mL for 24 h in a cell culture incubator. Then the cell viability was evaluated using MTT colorimetric method [31]. The results, originally calculated as OD values, were presented as percent inhibition in comparison to appropriate DMSO control.

4.2.5. PHA-induced proliferation of human PBMC

PBMC were distributed into 96-well flat-bottom plates in 100 μL aliquots (2×10^5 cells/well). PHA was added at a concentration of 5 μg/mL. The compounds were tested at doses of 50, 10 and 1 μg/mL. DMSO at appropriate dilutions served as control. After a 4-day incubation in a cell culture incubator, the proliferative response of the cells was determined by the colorimetric MTT method [31]. The results, originally calculated as OD values, were presented as percent inhibition in comparison to appropriate DMSO control.

4.2.6. Lipopolysaccharide-induced TNF-a production in whole blood cell culture

Human whole blood was diluted 5 × with RPMI 1640 medium and distributed to 24-well culture plates in 1 mL aliquots. The cultures were stimulated with LPS (1 μg/mL) and the studied compounds were added at a concentrations of 25 and 5 μg/mL. Control cultures contained DMSO in appropriate concentration. After an overnight incubation the supernatants were harvested and frozen at −80 °C until cytokine determination. TNF-α levels were determined in the supernatants by using ELISA kit from eBioscience, in a presence of TNF-α standard, and originally expressed in pg/mL. The inhibition of TNF-α production (in percentage) was calculated from comparison with DMSO controls and was presented in the table.

4.2.7. Growth inhibition of tumor cell lines

A-431 epidermal cell line, L1210 lymphoma, CX-1 and SW948 colon tumor cell lines derived from the collection of cell lines of the Institute of Immunology and Experimental Therapy, Wrocław, Poland. The lines were re-suspended in the culture medium and distributed into 96-well flat-bottom culture plates (Nunc). L-1210 was incubated at 1.5×10^4 cells/100 μL/well while SW948, A-431 and CX-1 at 2.5×10^4 cells/100 μL/well. The preparations were added to the cultures at the concentration range 50–0.39 μg/mL. After 72 h incubation in a cell culture incubator the cell viability was determined using MTT colorimetric method and originally expressed as OD values [31]. The results are presented as GI_{50} as calculated from comparisons with DMSO controls.

4.2.8. Colorimetric MTT assay for cell growth and kill

The assay was performed according to Hansen et al. [31]. Briefly, 25 μL of MTT (5 mg/mL) stock solution was added per well at the end of cell incubation period and the plates were incubated for additional 3 h in a cell culture incubator. Then, 100 μL of the extraction buffer (20% SDS with 50% DMF, pH 4.7) was added. After an overnight incubation the OD was measured at 550 nm with the reference wavelength of 630 nm in a Dynatech 5000 spectrophotometer.

4.2.9. Two-way MLR

The compounds were dissolved in DMSO (1 mg/100 μL DMSO) and further diluted in culture medium. For the assay combinations of untreated PBMC from two individuals were used. 2×10^5 cells from each individual (total volume of 100 μL) were combined in each well of 96-well flat bottom culture plates. The compounds were used at a final concentrations of: 50, 10 and 1 μg/mL. CsA was used as a reference drug. Appropriate DMSO dilutions in the culture medium served as control cultures. After 120 h incubation the cell proliferation was determined by MTT colorimetric method and the results presented as mean OD values ±SE.

4.2.10. CTLL-2 assay

CTLL-2 cells (2×10^3/well/100 μL) were distributed in flat bottom 96-well plates. Interleukin 2 (ImmunoTools, Germany), was added at a dose of 8 ng/mL and compound **4** was used at 10–0.3 μg/mL concentration range. After 24 h incubation the cell viability was determined by MTT colorimetric method and the results presented as mean OD values ± SE.

*4.2.11. Testing the role of prostanoids in the action of compound **4***

In this experiment the following protocol was used. PBMC were preincubated for 15 min with indomethacin (5 μg/mL) or prostaglandin receptor antagonists (L-798106 or AH23848) at the concentration of 5 μg/mL, followed by 30 min incubation with compound **4** (10 μg/mL). Then, PHA was added to all cultures except of the negative control. Additional control cultures, except PHA, contained appropriate DMSO concentrations corresponding to concentrations of DMSO used for dissolution of compound **4**, indomethacin and prostaglandin receptor antagonists. After 4-day incubation the degree of cell proliferation was determined by MTT colorimetric method. The results are shown as mean OD values ± SE.

4.2.12. Statistics

The results are presented as mean values ± standard error (SE). Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by post hoc comparisons with the Tukey's test to estimate the significance of the difference between groups. Nonparametric data were evaluated with the Kruskal–Wallis' analysis of variance. Significance was determined at $p < 0.05$. Statistical analysis was performed using STATISTICA 6.1 for Windows.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.10.070.

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