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Laboratory note

Cytotoxic effects, alkylating properties and molecular modelling of coumarin derivatives and their phosphonic analogues

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Abstract

The cytotoxic effects and alkylating activity of a series of 3-[1-(alkylamino)-ethylidene]-chroman-2,4-dione (**4a**–**4c**), 2-methoxy-3-[1-(alkylamino)-ethylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[*e*][1,2] oxaphosphinane (**5a**–**5c**) and [2-oxo-4-phenyl(alkyl)-2*H*-chromen-3-yl]-phosphonic acids dimethyl ester (**6a**–**6c**) on the two leukemia cell lines HL-60 and NALM-6 have been determined. The test compounds are much more toxic to NALM-6 cells than to HL-60 cells. IC_{50} data are up to nine times lower for the NALM-6 than for the HL-60 cell lines. As determined in an in vitro Preussmann test phosphonic derivatives **6a**–**6c** possess very high (+++) alkylating activity, phosphoric derivatives **5a**–**5c** are less active (++) while the derivatives **4a**–**4c** can be included in the group of low activity (+) alkylating agents. Using regression analysis QSAR we found a relationship between biological activity and the physicochemical properties of the test compounds. Their cytotoxic effect increases with an increase of the hydrophobic parameters in the region of the substituents at the 2-, 3- and 4-positions of the benzopyrone skeleton of **4**–**6**.

Keywords: Phosphonic derivatives; Coumarin; Alkylating activity; Cytotoxic properties; Molecular modelling

1. Introduction

Coumarin and chromone derivatives are of great interest due to their biological properties [1]. In particular, their physiological [2], bacteriostatic [3] and antitumor activity [4–6] makes both molecules attractive for further backbone derivatisation and screening as novel therapeutic agents. Weber et al. [7] have shown that coumarin and its metabolite 7-hydroxycoumarin have antitumor activity against several human tumor cell lines. In addition it has been shown that 4-hydroxycoumarin and 7-hydroxycoumarin inhibited cell proliferation in a gastric carcinoma cell line [8].

In the search for new therapeutics we have already synthesised several phosphonic analogues of coumarin [9] and chromone [10]. Here we present our study on some biological properties of the benzopyrone derivatives **4**, **5** and **6**, in particular, their cytotoxicity on some human leukaemia cell lines as well as their alkylating properties. Molecular modelling studies were also carried out to probe the relationship between the structure and alkylating and cytotoxic effect of the test compounds.

2. Chemistry

The general procedures for preparation of compounds **5** and **6** were described in our previous reports [9,10]. Substrate **1** (2-methyl-4-oxo-4*H*-chromene-3-carboxylic acid methyl ester) was obtained according to the described procedure [11] and treated with primary amines to give in excellent yields (85–90%) derivatives **4a**–**c** {3-[1-(alkylamino)-ethylidene]-chroman-2,4-dione), where alkyl is Me (a), 2-hydroxyethyl (b) and benzyl (c); Fig. 1. The structures of **4a**–**c** were confirmed by elementary and spectral (IR, ^{1}H-, ^{13}C-NMR, MS) analyses. Details are given in Section 7. Derivative **4c** was first synthesised by Strakov [12], by the reaction of 3-acyl-4-hydroxycoumarin with benzylamine, however,

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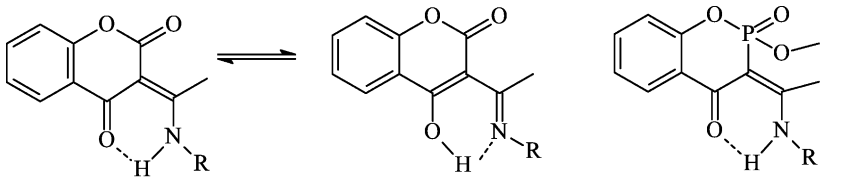


4a R = -CH_3, **4'a** R = -CH_3 **5a** R = -CH_3
4b R =-CH_2CH_2OH, **4'b** R =-CH_2CH_2OH **5b** R = -CH_2CH_2OH
4c R = -CH_2Ph **4'c** R =-CH_2Ph **5c** R = -CH_2Ph

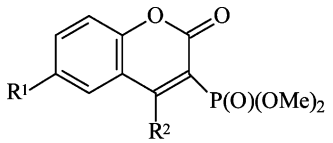
Fig. 1. Coumarin derivatives **4a**–**c** and their phosphonic analogues **5a**–**c**.

the authors suggested that the obtained product represents enol-structure 3-(1-alkylimino-ethyl)-4-hydroxy-chromen-2-one (**4c**′ Fig. 1). Reaction of **1** with aliphatic amines proceeded analogously to the previously described reaction of 3-phosphonic derivatives of chromone with primary amines [13]. Reaction of dimethyl 2-methyl-4-oxo-4*H*-chromen-3-yl-phosphonate (**2**) with primary amines gave satisfactory yields (50–80%) of the desired derivatives **5a**–**c** (Fig. 1). Coumarin derivatives **6a**–**c** (Fig. 2), with a phosphonic substituent at position C-3, were obtained according to the previously described procedure [9] by reaction of trimethylphosphite with bromo-derivatives **3a**–**c**. The spectral data of derivatives **5a**–**c** and **6a**–**c** are in an accordance to those described in the literature [9,13].

3. Pharmacology

3.1. Cytotoxicity

The cytotoxicity of the benzopyrone derivatives **4**–**6** was determined on the two human leukemia cell lines, promyelocytic HL-60 and lymphoblastic NALM-6. Warfarin was used as a reference. The viability of cells exposed continuously to test compounds was estimated by the trypan-blue exclusion assay. The values of IC_{50} (the concentration of test compounds required to reduce the cell survival fraction to 50% of the control) were calculated from dose response curves and used as a measure of cellular sensitivity to a given treatment. Cytotoxicity toward HL-60 and NALM-6 was determined for three different concentrations: 10, 100 and



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6a $R^1 = CH_3$, $R^2 = CH_3$
6b $R^1 = H$, $R^2 = Ph$
6c $R^1 = H$, $R^2 = (CH_2)_2P$

Fig. 2. The coumarin derivatives **6a**–**c**.

1000 μM of compounds **4a**–**c**, **5a**–**c** and **6a**–**c**. IC_{50} values cover a concentration range of 54.4–801 μM.

3.2. Alkylating activity

Compounds **4a**–**c**, **5a**–**c** and **6a**–**c** were screened for their alkylating activity toward 4-(4′-nitrobenzyl)pyridine (NBP). The level of alkylation was quantified spectrophotometrically at 560 nm. The screening was carried out with a concentration of 0.005 mmol mL^{-1} of the test compound in 2-methoxyethanol.

4. Molecular modeling

QSAR analyses of the cytotoxic and alkylation activities of benzopyrane derivatives **4a**–**c**–**6a**–**c** were carried out. All the structures of the studied compounds were geometrically optimised by the use of semi-empirical method AM1 [algorithm Polak–Ribiere, RMS grad = 0.01 kcal/(Å mol), in vacuo, HyperChem 5.1] [14]. The systematic conformational analysis was not used. The physicochemical parameters derived from the quantum mechanical calculations of the chemical structures are summarised in Table 1. Physicochemical parameters of **4a**–**c**, **5a**–**c** and **6a**–**c** were evaluated for entire molecules and, in addition, for their particular substituents at the 2-, 3- and 4-positions. The following parameters were used for mathematical analyses: van der Waals volume (*V*), molar refraction (MR), electric charge focused on an electronegative atom of the substituent at 2-, 3- and 4-positions (Q), dipole moment (DM), the value of lipophilicity (log *P*) and the molecular weight-MW (pMW). The regression analysis was carried out using a STATISTICA 5.1 program. The use of more than one variable in a multivariate equation was justified by an inter-correlation study.

5. Results and discussion

Chromone **1** (2-methyl-4-oxo-4*H*-chromene-3-carboxylic acid methyl ester) and its phosphonic analogue **2** were used as substrates for reaction with primary amines. Nucleophilic addition at position C-2 followed

Table 1
The physicochemical parameters of the test compounds used for molecular modelling

No	Parameter values in analysis QSAR (independent variables)									
	V	log P	MR	DM	pMW	V_{2-4}	MR_{2-4}	pMW_{2-4}		
4a	191.98	0.11	59.90	4.043	−2.337	98.16	33.54	−2.097		
4b	215.86	−0.34	66.19	4.405	−2.393	122.10	39.83	−2.191		
4c	266.36	1.88	84.51	4.778	−2.467	172.66	58.15	−2.304		
5a	223.71	−0.44	69.28	4.943	−2.427	133.82	42.91	−2.243		
5b	247.55	−0.88	75.57	5.313	−2.473	157.75	49.21	−2.312		
5c	298.20	1.34	93.89	5.813	−2.536	208.32	67.53	−2.400		
6a	237.01	1.44	71.09	7.788	−2.451	127.61	39.69	−2.246		
6b	278.12	2.19	86.15	7.620	−2.519	185.37	59.78	−2.377		
6c	311.58	2.98	95.35	7.152	−2.554	218.62	68.14	−2.424		
W [a]	278.51	2.73	86.57	7.799	−2.489	183.32	60.21	−2.335		
	pMW_2	V_2	pMW_3	V_3	pMW_4	V_4	Q_1	Q_2	Q_3	Q_4
4a	−1.447	11.59	−1.840	74.98	−1.447	11.59	−0.192	−0.340	−0.248	−0.379
4b	−1.447	11.59	−1.996	98.92	−1.447	11.59	−0.192	−0.339	−0.285	−0.382
4c	−1.447	11.59	−2.162	149.48	−1.447	11.59	−0.192	−0.342	−0.280	−0.378
5a	−1.892	47.25	−1.840	74.98	−1.447	11.59	−0.665	−1.123	−0.263	−0.366
5b	−1.892	47.25	−1.996	98.92	−1.447	11.59	−0.664	−1.121	−0.264	−0.368
5c	−1.892	47.25	−2.162	149.48	−1.447	11.59	−0.664	−1.123	−0.258	−0.366
6a	−1.447	11.59	−2.083	87.13	−1.432	28.89	−0.152	−0.315	−1.116	–
6b	−1.447	11.59	−2.083	87.13	−1.950	86.65	−0.153	−0.309	−1.117	–
6c	−1.447	11.59	−2.083	87.13	−2.069	119.90	−0.152	−0.314	−1.117	–
W [a]	−1.447	11.59	−2.202	154.69	−1.463	17.04	−0.190	−0.281	−0.291	−0.242

[a] Warfarin.

by intramolecular cyclisation resulted in two groups of compounds, **4** and **5**, respectively. Product **4** was for the first time obtained in the reaction of 3-acetyl-4-hydroxycoumarine with benzyloamine with the yield of 20% [12]. In our method, the synthesis of 3-[1-(alkylamino)-ethylidene]-chroman-2,4-dione (**4**) was performed by the reaction of 2-methyl-4-oxo-4*H*-chromene-3-carboxylic acid methyl ester with corresponding aliphatic amines (methylamine, 2-hydroxyethylamine and benzylamine). The yields of these reactions were rather satisfactory (76–91%). We suggest that compound **4** exist in keto-form shown at Fig. 1. This is in contrary to the enol-type structure **4′** suggested by Strakov [12]. The evidence that keto–enol equilibrium of **4** is shifted toward its keto-form is evidenced by the presence in ^{1}H-NMR spectrum of the signal of N–H proton with the chemical shift about 14 ppm. In addition doublet signal at 4.89 ppm, characteristic for benzyl methylene group next to amine function was observed in the spectrum of **4c** taken in DMSO. This signal became a singlet after treatment of the sample with D_2O. This results confirms attachment of exchangeable proton to amine function of keto-form of **4** rather than to hydroxyl function of enol-form **4′**. Additional evidence was obtained from the ^{13}C-NMR spectrum, in which signal of carbonyl group carbon atom (C-4) was observed at 180 ppm (for **4c**). For an enol-form of **4′** the respective chemical shift of C-4 should be observed at about 171 ppm [15]. We did not observed such signal. Finally, we succeeded in solving the X-ray structure of **4b** and **4c** (data not shown). Both these compounds in solid state exist in keto-form. Crystallographic structures of **4b** and **4c** will be presented elsewhere.

Crystal structure of **5b** [2-methoxy-3-[1-(ethanolamino)-ethylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[*e*][1,2]oxaphosphinane] confirmed univocally the presence of a C-4 carbonyl group [16]. The coumarin derivatives **6a**–**c**, with a phosphonic substituent at the C-3, were obtained according to the previously described procedure, by the reaction of trimethyl phosphite with bromo-derivatives **3a**–**c** (Fig. 2) [9].

The cytotoxicity of benzopyran derivatives **4a**–**c**–**6a**–**c** was determined on two human leukemia (HL-60 and NALM-6) cell lines. Warfarin was used as a reference substance. The choice was made by the fact that warfarin, possesses coumarin skeleton. In addition, it is known that warfarin influences the growth of tumour cells [17] and inhibits metastasis of Mtln3 rat mammary carcinoma without affecting primary tumour growth [18]. The cytotoxicity and IC_{50} values are presented in Table 2. IC_{50} data for the NALM-6 cell line are much lower (up to nine times) than for the HL-60 cell line. Such results indicate that all the test compounds are less toxic to the promyelocytic HL-60 cell line. They can be divided into three groups based on their cytotoxicity to HL-60 cell lines. The group with moderate cytotoxic activity ($IC_{50} = 408.0–486.0$ μM) contains compounds **4a**, **6b** and warfarin, while derivatives **4b**, **4c**, **5a**, **5b** and

Table 2
Cytotoxicity of warfarin and of benzopyran derivatives **4a**–**c**–**6a**–**c** on promyelocytic HL-60 leukemia cells and lymphoblasic NALM-6 leukemia cells

Compound	HL-60					NALM-6				
	viability (%)			IC_{50}	pIC_{50} (M)	viability (%)			IC_{50}	pIC_{50} (M)
	10 (μM)	100 (μM)	1000 (μM)	$\times 10^{-6}$ (M)		10 (μM)	100 (μM)	1000 (μM)	$\times 10^{-6}$ (M)	
4a	87	67	17	408.0	3.3893	86	62	0	276.4	3.5585
4b	91	88	19	590.8	3.2286	94	44	0	89.4	4.0487
4c	106	103	35	801.0	3.0964	93	40	0	83.0	4.0809
5a	101	96	20	649.3	3.1876	93	43	0	87.9	4.0560
5b	101	89	36	772.4	3.1122	107	77	1	420.0	3.3768
5c	86	29	0	66.8	4.1752	91	8	0	54.4	4.2644
6a	102	92	7	542.3	3.2658	89	40	0	81.9	4.0867
6b	87	78	1	428.2	3.3684	84	52	0	133.8	3.8735
6c	83	45	6	88.0	4.0555	99	37	3	80.9	4.0921
Warfarin	84	83	5	486.0	3.3134	95	41	1	74.8	4.1261

Data for both experiments are collected in triplicate.

6a belong to a low cytotoxicity group. The remaining compounds are rather toxic with IC_{50} values in the range of 67 and 88 μM, for **5c** and **6c**, respectively. In the case of the NALM-6 cell line two groups of compounds can be distinguished, a very toxic grove (**4b**, **4c**, **5a**, **5c**, **6a**, **6c**, and warfarin) with the IC_{50} = 54.4–89.4 μM and a group of less toxic compounds (**4a**, **5b**, **6b**) with IC_{50} in the range of 133.8–420.0 μM. Two benzyl derivatives of chromone, compounds **5c** and **6c**, are highly toxic to both cell lines with similar level of toxicity (IC_{50} in the range of 54.4–88.0 μM). The different sensitivities of both cell lines to compounds **4a**–**c**–**6a**–**c** may be due to different activity of the membrane proteins responsible for transport of xenobiotics out of the cells [19,20].

Therapeutics with alkylating properties belong to the first class of cytostatics used for cancer therapy [21]. Alkylating activity expressed as an effective transfer of an alkyl group from phosphonic esters to amines has been exhaustively described in many studies [22,23] and has been a subject of many patents [24–26]. Our aim was to determine the in vitro alkylating activity of the novel coumarin derivatives **4**–**6**. For this purpose we used an in vitro Preussmann test [27–30]. In this assay the tested compounds demonstrate their alkylating activity toward NBP molecule. Compounds containing acidic protons can also protonate the NBP ring. The reaction is reversible after addition of piperidine. Thus, in some cases the level of alkylation is lower than expected due to the side-reactions. An improvment of the Preussmann test could be achieved by collecting alkylating data in different pH conditions. In our case non-acidic compounds **4a**–**c**, **5a**–**c** and **6a**–**c** were screened for their alkylating activity in anhydrous conditions in 2-methoxyethanol solution.

The alkylating activity results are presented in Table 3. Phosphonic derivatives of coumarin, compounds **6a**–**c**, possess very high (+++) alkylating activity. The phosphoric derivatives **5a**–**c** are less active and can be included in the group of high (++) alkylating activity agents. Low (+) alkylation activity is shown by derivatives of 4-hydroxycoumarin **4a**–**c**. Interestingly, our reference compound, warfarin, does not show any alkylating activity (−). There is no close correlation between the alkylating activity of the test compounds and their toxicity on the leukemia cell lines. For example, highly toxic compound **5c** showed only moderate alkylating activity, while less toxic, in respect to both HL-60 and NALM-6 cell lines, compound **6b**, exhibited very high alkylating activity. Compound **6c** has high alkylating activity associated with a large cytotoxic potential.

To answer the question whether there is any relationship between the structural features of the benzopyran derivatives **4a**–**c**–**6a**–**c** (Figs. 1 and 2) and their cytotoxic and alkylating properties we used molecular modelling.

First, we analysed the relationship between the cytotoxicity and the alkylating activity of these compounds. Analysis of the data obtained from the pharmacological studies leads to the conclusion that there is no correlation between alkylating activity of particular derivatives **4**–**6** and their cytotoxic activity on HL-60 and NALM-6 cell lines (the calculated correlation coefficients (r) were 0.42 and 0.19, respectively). This result may indicate that the toxic effect is caused by a mechanism, which is independent of alkylation. Another explanation for the lack of any relationship between cytotoxicity and alkylating activity may be found in the low bioavailability of the target molecules (proteins, DNA) in the cell experiments.

Table 3
Alkylating activity of compounds **4a**–**c**–**6a**–**c**; NBP test results

Compounds	ε	Absorbance (A) [a] $\lambda_{max} = 560$ nm	Log A	Alkylation activity [c,d]
4a	62.9	0.0629	−1.2014	+
4b	59.4	0.0594	−1.2262	+
4c	55.1	0.0551	−1.2589	+
5a	208.3	0.2083 [b]	−0.6813	++
5b	121.3	0.1213 [b]	−0.9161	++
5c	226.3	0.2263 [b]	−0.6453	++
6a	1669.8	1.6698	0.2266	+++
6b	1014.1	1.0141	0.0061	+++
6c	1848.2	1.8482	0.2667	+++
Warfarin	41.4	0.0414	−1.3830	−

Data for reference derivative (warfarin) are also included.
[a] Average data from three determinations.
[b] According to Ref. [33].
[c] According to Ref. [27].
[d] (−) $A < 0.05$, (+) $A = 0.05$–0.1, (++) $A = 0.1$–0.5, (+++) $A > 0.5$.

The structural variability of derivatives **4a**–**c**–**6a**–**c** covers in various substituents at the 2-, 3- and 4-positions of the coumarin skeleton. The values of cytotoxic and alkylating effects were analysed by linear and multiple regression methods with respect to the structural features of the test compounds. In the multiple regression analysis we used exclusively those parameters not showing mutual inter-correlation (Table 4).

The moderate cytotoxic effect of derivatives **4a**–**c**–**6a**–**c** observed on the HL-60 cell line (Table 2) exhibits exclusive correlation within the value of the van der Waals volume for all substituents at the 2-, 3- and 4-positions (V_{2-4}) ($r = 0.66$, $n = 9$).

We can clearly see that an increase of the V_{2-4} value favours higher biological activity of the tested coumarin derivatives. In contrast, the high cytotoxic effect of **4**–**6** to lymphoblastic NALM-6 cells can be correlated with the extent of hydrophobic interaction of substituents at 2- and 3-positions (V_2 and pMW_3). For all test compounds this correlation is described by Eq. (1).

$$pIC_{50} = 1.546(\pm 0.709) + 0.007(\pm 0.002)V_2 - 1.140(\pm 0.338)pMW_3$$

Table 4
Inter-correlation among parameters used in the multiple regression analysis

Variable	V_2	pMW_3	Q_3	V_3
V_2	1.00	0.25	0.45	0.03
pMW_3	–	1.00	0.21	0.79
Q_3	–	–	1.00	0.41
V_3	–	–	–	1.00

Parameters used are as follows: molecular weight, (pMW); van der Waals volume, (V); and electric charge, (Q) characteristic for a given substituent numbered in a subscript.

$$r = 0.84;\ r^2 = 0.71;\ n = 9;\ F = 7.282;\ SEE = 0.125;\ P < 0.02 \quad (1)$$

Cytotoxicity goes up with an increase of the value of the hydrophobic parameters. After excluding from the calculations compounds **6a**, **6b** and **6c**, possessing an alkyl substituent at C-4, which is an additional steric element in the central part of the molecule, we could conclude that the cytotoxic effect correlates with the molecular weight (MW) of the substituents at positions: 2-, 3- and 4- (pMW_{2-4}). Eq. (2) describes this relationship.

$$pIC_{50} = -6.50(\pm 1.10) - 2.00(\pm 0.48)pMW_{2-4}$$

$$r = 0.90;\ r^2 = 0.81;\ n = 6;\ F = 17.087;\ SEE = 0.117;\ P < 0.01 \quad (2)$$

A distinct relationship between the structures of the test compounds **4a**–**c**–**6a**–**c** and their alkylating properties (log A where A is the absorption at 560 nm) can be observed. This activity is related to the significant electron density localised on the substituent at position 3, and to the van der Waals interactions in the region of the substituents at positions 2 and 3. The parameter crucial for the alkylating activity of the molecule is the value of the electrical charge localised on the electronegative oxygen or nitrogen atoms bound to the substituent at the 3-position (Q_3), which gives a measure of its donor–acceptor interaction potential [31]. The relationship between this parameter and alkylating activity can be expressed by Eq. (3).

$$\log A = 1.43(\pm 0.15) - 1.42(\pm 0.23)Q_3$$

$$r = 0.91;\ r^2 = 0.83;\ n = 10;\ F = 38.198;\ SEE = 0.281;\ P < 0.0002 \quad (3)$$

The best bivariate relationship, explaining 97% of the total variance, was obtained after inclusion of the second parameter, van der Waals volume of the substituent at the 2-position (Eq. (4))

$$\log A = -1.94(\pm 0.11) + 0.02(\pm 0.00)V_2 - 1.72(\pm 1.13)Q_3$$

$$r = 0.99;\ r^2 = 0.97;\ n = 10;\ F = 114.940;\ \text{SEE} = 0.124;\ P < 0.00000 \quad (4)$$

Eq. (5) describes the relationship between alkylating activity of the molecules and the range of their van der Waals interactions and the electrical charge in region of the substituent at 3-position:

$$\log A = -1.180(\pm 0.440) - 0.002(\pm 0.000)V_3 - 1.357(\pm 0.263)Q_3$$

$$r = 0.91;\ r^2 = 0.84;\ n = 10;\ F = 17.798;\ \text{SEE} = 0.293;\ P < 0.001 \quad (5)$$

6. Conclusions

In the in vitro Preussmann test phosphonic derivatives **6a**–**c** possess very high (+++) alkylating activity. Phosphoric derivatives **5a**–**5c** are less active and can be included in the group of high activity (++) alkylating agents. Low (+) alkylating activity is shown by derivatives of 4-ketocoumarin **4a**–**c**. The test derivatives **4**–**6** exhibit higher cytotoxicity on the NALM-6 leukemia cell line in comparison to the HL-60 cell line. IC_{50} coefficients for the former cells are two to nine times lower. Derivatives **5c** and **6c** exhibit the highest cytotoxic property and potential for alkylation. Using regression analysis we found a relationship between biological activity and the physicochemical properties of the test compounds. Their cytotoxic effect increases with an increase of the hydrophobic parameters in the region of the substituents at the 2-, 3- and 4-position of the coumarin skeleton (Eqs. (1) and (2)). Correlation between cytotoxicity and the alkylation effect was not found. A good univariate relationship of the alkylation effect involves a charge of the electronegative atom (N or O) bound to the substituent at the 3-position, and the van der Waals volume parameter of the substituent at the 3-position (Eqs. (3) and (5)). A good bivariate relationship involves the charge of the atom (N or O) at the 3-position and the steric parameter of the 2-substituent region (Eq. (4)).

7. Experimental

7.1. Chemistry

Chemicals were purchased from Sigma–Aldrich and Merck and solvents were from POCH, Poland. All reagents and solvents used in the experiment were of analytical grade. The melting points were measured using an Electrothermal 1A9100 apparatus and they are uncorrected. The IR spectra were recorded on a Pey-Unicam 200G Spectrophotometer in KBr. The ^{1}H-NMR spectra were registered at 300 MHz on a Varian Mercury spectrometer. The MS data were obtained on an LKB 2091 mass spectrometer (70 eV ionisation energy). Satisfactory elemental analyses (±0.4% of the calculated values) were obtained for the new compounds using a Perkin–Elmer PE 2400 CHNS analyser (Microanalytical Laboratory, Institute of Chemistry, Medical University of Lodz).

All compounds used for the experiments, except derivatives **4**, were synthesised according to the described procedures. Derivative **1** (2-methyl-4-oxo-4*H*-chromene-3-carboxylic acid methyl ester) was prepared according to Ref. [11], dimethyl 2-methyl-4-oxo-4*H*-chromen-3-yl-phosphonate (**2**) according to Ref. [12], 2-methoxy-3-[1-(alkylamino)ethylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[*e*][1,2]oxaphosphinane (**5**) according to Ref. [13], 2′-bromoacetoxy-5-methylacetophenone (**3a**), 2′-bromoacetoxybenzophenone (**3b**), 2′-bromoacetoxy-3-phenylpropiophenone (**3c**) and [2-oxo-4-phenyl(alkyl)-2*H*-chromen-3-yl]phosphonic acids (**6a**–**c**) were prepared according to Ref. [9].

7.1.1. General procedure for compounds *4a*–*c*

Methyl-, 2-hydroxyethyl- or benzylamine (20 mmol) in MeOH (0.5 mL) was added at room temperature to a solution of 2-methyl-4-oxo-4*H*-chromene-3-carboxylic acid methyl ester (**1**) (20 mmol) in MeOH (5 mL). The solid crude product, which precipitated after several minutes, was filtered off, dried, and crystallised from MeOH. Pure compounds **4a**–**c** were obtained as white solids.

*7.1.1.1. 3-(1-Methyl-2-methylamino-ethylidene)-chroman-2,4-dione (**4a**).* Yield: 3.78 g (87%). m.p. 181–183 °C; IR (KBr, ν cm^{-1}): 3393.3 (NH), 1700.6 (C=O), 1607.2, 1464.3; ^{1}H-NMR ($CDCl_3$, δ ppm): 2.74 (s, 3H, CH_3), 3.24 (d, 3H, $^3J_{HH} = 5.16$ Hz, N–CH_3), 7.20–8.06 (m, 4H, aromat), 14.21 (s, 1H, NH); ^{13}C-NMR ($CDCl_3$, δ ppm): 18.68 (C–CH_3), 31.10 (N–CH_3), 116.67, 123.65, 126.05, 133.86, 176.14, 180.75 (C=O). MS *m*/*z* (%): 217 (100, M^+), 202.1 (63), 127.2, 121 (44.5), 56(20). Anal. Found: C, 66.36; H, 5.11; N, 6.45. Calc. for $C_{12}H_{11}NO_3$ (217.22): C, 66.28; H, 5.22; N, 6.67%.

*7.1.1.2. 3-[1-(2-Hydroxy-ethylamino)-ethylidene]-chroman-2,4-dione (**4b**).* Yield: 3.75 g (76%). m.p. 183–185 °C; IR (KBr, ν cm^{-1}): 3411.5 (NH), 1662.2 (C=O), 1615.3; ^{1}H-NMR ($CDCl_3$, δ ppm): 2.62 (s, 3H, CH_3), 3.64 (t, 2H, CH_2, $J_{HH} = 5.3$ Hz), 4.11 (t, 2H, CH_2, $J_{HH} = 5.3$ Hz), 7.22–7.91 (m, 4H, aromat), 13.79 (s, 1H, NH); ^{13}C-NMR ($CDCl_3$, δ ppm): 18.88 (CH_3), 46.98, 59.38, 96.55, 116.78, 134.62, 153.64, 176.61, 180.24. MS *m/z* (%): 248 (100, M^+), 145.3 (14), 127.2 (14), 121 (48), 57(98). Anal. Found: C, 62.86; H, 5.17; N, 5.79. Calc. for $C_{13}H_{13}NO_4$ (247.24): C, 63.15; H, 5.30; N, 5.67%.

*7.1.1.3. 3-(2-Benzylamino-1-methyl-ethylidene)-chroman-2,4-dione (**4c**).* Yield: 5.35 g (91%). m.p. = 162–163, 164–165 °C [12]; IR (KBr, ν cm^{-1}): 3382.3 (NH), 1698.3 (C=O), 1612.9, 1464.3; ^{1}H-NMR (DMSO-d_6, δ ppm): 2.76 (s, 3H, CH_3), 4.89 (d, 2H, $^3J_{HH} = 5.16$ Hz, CH_2Ph), 7.21–8.04 (m, 9H, aromat), 14.67 (s, 1H, NH); ^{13}C-NMR ($CDCl_3$, δ ppm): 18.82 (CH_3), 48.13, 97.43, 116.51, 133.83, 134.87, 153.69, 176.96, 181.76. MS *m/z* (%): 294 (78, M^+), 250.2 (6), 173.8 (7), 121 (100), 91 (98).

7.2. Pharmacology

7.2.1. Cell culture

The NALM-6 cell line was purchased from German Collection of Microorganisms and Cell Cultures [32]. HL-60 and NALM-6 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM glutamine, at 37 °C in a 5% CO_2/95% air atmosphere.

7.2.2. Cytotoxicity

Exponentially growing cells were seeded at 0.5×10^6 per each well of a 24-well plate (Nunc) and cells were then exposed to compounds **4**–**6**. Stock solutions of the test compounds in DMSO were used for serial dilutions in complete culture medium. Cells were exposed to the drugs for 48 h in a 5% CO_2/95% air atmosphere. The number of viable cells was counted in Bürker hemocytometer using the trypan-blue exclusion assay. Dose–response curves were determined. Values of IC_{50} were used as a measure of cellular sensitivity to given treatment [32].

7.2.3. Determination of alkylating properties (NBP test)

NBP (5% solution in 2-methoxyethanol, 1 mL) was added to the solution of the test compound (0.005 mmol) dissolved in 2-methoxyethanol (1 mL). The sample was heated at 100 ± 0.5 °C for 1 h and then quickly cooled to 20 °C. 2-Methoxyethanol (2.5 mL) and piperidine (0.5 mL) were added to the sample making a total volume of 5 mL and a final concentration of the test compound of 10^{-5} mol L^{-1}. After 90 s the absorbance was measured at $\lambda = 560$ nm in a quartz cell (1 cm). 2-Methoxyethanol was used as a reference.

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