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Original article

N-Aryl-*N*′-(chroman-4-yl)ureas and thioureas display *in vitro* anticancer activity and selectivity on apoptosis-resistant glioblastoma cells: Screening, synthesis of simplified derivatives, and structure–activity relationship analysis

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ABSTRACT

A series of chroman derivatives previously reported as potassium channel openers, as well as some newly synthesized simplified structures, were examined for their *in vitro* effects on the growth of three human high-grade glioma cell lines: U373, T98G, and Hs683. Significant *in vitro* growth inhibitory activity was observed with 2,2-dimethylchroman-type nitro-substituted phenylthioureas, such as compounds **4o** and **4p**. Interestingly, most tested phenylureas were found to be slightly less active, but more cell selective (normal versus tumor glial cells, such as **3d**, **3e**, and **3g**), thus less toxic, than the corresponding phenylthioureas. No significant differences were observed in terms of chroman-derivative-induced growth inhibitory effects between glioma cells sensitive to pro-apoptotic stimuli (Hs683 glioma cells) and glioma cells associated with various levels of resistance to pro-apoptotic stimuli (U373 and T98G glioma cells), a feature that suggests non-apoptotic-mediated growth inhibition. Flow cytometry analyses confirmed the absence of pro-apoptotic effects for phenylthioureas and phenylureas when analyzed in U373 glioma cells and demonstrated U373 cell cycle arrest in the G0/G1 phase. Computer-assisted phase-contrast videomicroscopy revealed that **3d** and **3g** displayed cytostatic effects, while **3e** displayed cytotoxic ones. As a result, this work identified phenylurea-type 2,2-dimethylchromans as a new class of antitumor agents to be further explored for an innovative therapeutic approach for high-grade glioma and/or for a possible new mechanism of action.

1. Introduction

High-grade (anaplastic astrocytoma and glioblastoma (GBM)) glioma patients are associated with dismal prognoses because of: (i) the marked diffuse invasion of glioma cells into the brain parenchyma [1,2], rendering elusive complete surgical resection [3], and (ii) the intrinsic resistance of glioma cells to pro-apoptotic stimuli [2], thus to conventional radiotherapy and chemotherapy [4]. The standard treatment for high-grade glioma patients consists of maximal surgical resection followed by radiotherapy and chemotherapy with temozolomide [4,5].

Temozolomide contributes therapeutic benefits for glioma patients because it induces sustained pro-autophagic effects [6] that then lead to apoptotic-related cell death [7] as a consequence but not caused by the temozolomide-induced anti-glioma effects [4]. These temozolomide-induced sustained pro-autophagic effects circumvent, at least partly, the intrinsic resistance of glioma cells to direct pro-apoptotic insults [2,4]. Temozolomide also contributes therapeutic benefits in glioma patients because of its

Abbreviations: GBM, glioblastoma; ATP, adenosine triphosphate; SUR, sulfonylurea receptor; Kir channel, potassium inwardly rectifying channel; K_{ATP} channel, ATP-sensitive potassium channel; TRPM channel, transient receptor potential "melastatin" channel; TRPV1 channel, transient receptor potential vanilloid-1 channel.

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anti-angiogenic effects [8]. However, the 5-year survival of GBM patients remains as low as 10% [5] and new types of treatment are mandatory to improve GBM-patient-related survivals. One possibility would be to target some ion channels that are overexpressed and/or overactivated in glioma cells as compared to normal glial cells or neurons. Indeed, glioma cells release neurotransmitters, their transporters, and ion channels to support their unusual growth and invasive migration [9–12]. We have already demonstrated that a significant proportion of glioblastoma overexpress the alpha-1 subunit of Na^+/K^+-ATPase, whose inhibition leads to pronounced autophagy-related cell death in glioma cells [13]. Ongoing clinical trials demonstrate that an inhibitor of chloride channels may be a therapy for the treatment of gliomas [14].

In our efforts to develop new chroman derivatives as cromakalim-related potassium-channel openers, we previously synthesized a large number of compounds, some of which showed very interesting pharmacological profiles as activators of the ATP-sensitive potassium channels (K_{ATP} channels) [15–20]. Such potassium-channel openers are known to interact with the sulfonylurea receptor (SUR) subunit of the K_{ATP} channel. The latter channel is an octameric complex resulting from the combination of four SUR subunits with four Kir (potassium inwardly rectifying) subunits, both of which exist in several isoforms (SUR1, SUR2A, and SUR2B; Kir6.1 and Kir6.2) [21]. Kir channels are also implicated in glioma cell biology [22,23]. Recently, it was reported that the SUR1 subunit associated to the transient receptor potential "melastatin" 4 (TRPM4) channel protein (forming the NC_{Ca-ATP} channel) was overexpressed in glial cells under ischemic conditions [24]. It is tempting to speculate that the SUR1/TRPM4 channel could also be implicated in the metabolism of glial tumor cells. Therefore, it was decided to examine the *in vitro* effects of these chroman-type potassium-channel openers and related structures on the growth of three human high-grade glioma cell lines, i.e. U373 and T98G from astroglial origin [25–27] and Hs683 from oligodendroglial origin [25,26,28]. While the Hs683 model displays actual sensitivity to pro-apoptotic stimuli [25,29], the U373 [25,29] and T98G [29] models do not.

The effects of various compounds were monitored by means of quantitative videomicroscopy [30] using the U373 GBM astroglial model in order to grossly decipher the mechanisms of action of these compounds, i.e. to determine whether they display cytotoxic versus cytostatic effects. Moreover, their effect on cell cycle was investigated by flow cytometry. Lastly, the bioselectivity (thus toxicity) between normal astrocytes and glioma cells in terms of *in vitro* growth inhibition was also investigated for several compounds of interest.

2. Chemistry

The synthesis of compounds **1**–**4** (Fig. 1) has been described previously [15–20]. *N*-(3-Chlorophenyl)methylthioureas **6a**–**b** were prepared by condensation between 3-chlorobenzylamine **5** and the appropriate isothiocyanate (Scheme 1). For the *N*-(5-chloro-2-methoxyphenyl)methylthioureas **9a**–**b**, 5-chloro-2-methoxybenzonitrile **7** was first reduced to produce the corresponding benzylamine derivative **8**, which was then condensed with the appropriate isothiocyanate (Scheme 2).

6-Chlorochromans **16a**–**b** were obtained from *p*-chlorophenol in six steps (Scheme 3). *p*-Chlorophenol **10** was first alkylated using 3-bromopropionic acid under alkaline conditions. The reaction never came to completion but the expected compound was easily separated from the starting material using extraction from an ethyl acetate solution by a solution of sodium hydrogen carbonate, re-acidification of the aqueous alkaline solution, and collection by filtration of the resulting precipitate consisting of compound **11**. A ring closure reaction of 4-chlorophenoxypropanoic acid **11** by means of concentrated sulfuric acid produced the expected core structure **12** as described previously [31].

After reduction of the ketone to alcohol **13** using sodium borohydride [32], a Ritter reaction produced the corresponding 4-acetylamino derivative **14** using acetonitrile and concentrated sulfuric acid. The subsequent hydrolysis of **14** with a hydro-ethanolic solution of hydrochloric acid led to the 4-aminochroman **15**. The latter was finally converted into the expected thioureas after reaction with the appropriate isothiocyanates.

All the compounds were crystallized from appropriate solvents and characterized by ^1H NMR. Their purity was assessed by elemental analysis to obtain the final materials with the chemical purity required prior to pharmacological evaluations.

3. Results and discussion

3.1. Growth inhibitory activity (MTT colorimetric assay) on glioma cells

The first group of 6-chlorochroman compounds (Table 1) tested on glioma cells were characterized by the presence of a variety of functional groups at the 4-position and mainly included ureas (**1b**, **1c**, **1f**, and **1h**) and thioureas (**1d**, **1e**, and **1i**), but also an acetamide (**1a**), a sulfonylurea (**1g**), and a carbamate (**1j**) compound. None of them showed marked *in vitro* growth inhibitory activity on the three glioma cell lines ($IC_{50} > 50$ μM) but compounds **1f**, **1h**, and **1i** revealed that the presence of a phenyl ring increased the growth inhibition (see for example IC_{50} on T98G between 15 and 37 μM).

The second group of tested compounds (Table 2) included benzylureas and benzylthioureas structurally related to **1f** and examined the influence of the nature of the halogen atom at the 6-position. It was found that 6-F (**2a**, **2c**) and 6-Br (**2b**, **2e**) compounds showed a higher glioma growth inhibitory effect than 6-Cl (**1f**, **2d**), while no significant differences were observed between ureas and thioureas (Table 2).

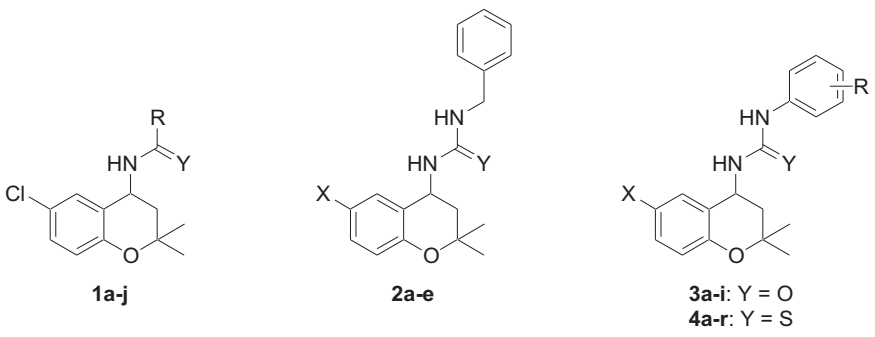
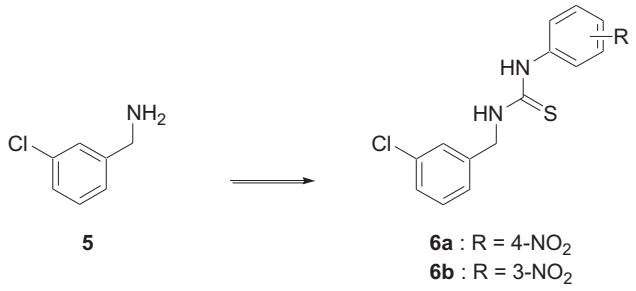


Fig. 1. Previously described chromans as potassium channel openers.



Scheme 1. Synthesis of *N*-(3-chlorophenyl)methylthioureas. Reagant: 4- or 3-nitrophenyl isothiocyanate, CH_2Cl_2.

The third group included diversely substituted phenylureas and phenylthioureas structurally related to **1h** and **1i** (Table 3). The monosubstitution of the phenyl ring by a chlorine atom at the three possible positions (*ortho*, *meta*, and *para*) as well as the presence of various halogen atoms at the 6-position were examined first. Most of the phenylureas (**3a**–**i**) except **3h** were found to be more active (in terms of glioma growth inhibition *in vitro*) than **1h** in the three glioma cell lines under study, but none of them showed an $IC_{50} < 10$ μM (Table 3).

The phenylthioureas **4a**–**c** structurally related to **1i** were equally or more potent than their corresponding phenylureas **3g**–**i**, some of them expressing IC_{50} values <10 μM on specific cancer cell lines (**4a** on T98G, **4b** on the three cell lines, **4c** on U373 and Hs683; see Table 3).

As a result, we focused on phenylthioureas bearing either an electron-donating group (**4d**–**f**) or an electron-withdrawing group (**4g**–**r**) (Table 3). While the introduction of an electron-donating group (methoxy) on the phenyl ring (**4d**–**f**) did not induce any marked improvement of the *in vitro* growth inhibitory activity compared to the unsubstituted derivative **1i**, the introduction of an electron-withdrawing group (cyano or nitro) (**4g**–**r**) markedly increased this growth inhibitory activity (Table 3). Most of the compounds showed $IC_{50} \leq 10$ μM on glioma cells and the best results ($IC_{50} < 5$ μM) were obtained with nitro-substituted compounds in the 6-Cl series (**4o** and **4p**), showing IC_{50} values between 2 and 4 μM on the three cell lines and reaching the effects displayed by cisplatin (Table 3). Temozolomide and cisplatin were used as reference compounds because temozolomide is currently the preferred drug used for the treatment of glioblastoma [4–7] and platinum derivatives are used to treat recurrent gliomas that relapse during temozolomide treatment [33]. Temozolomide revealed a modest *in vitro* antiproliferative activity on glioma cells (Table 3).

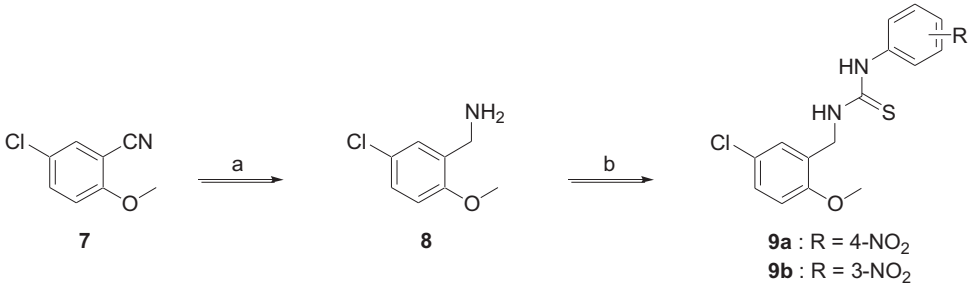
As **4o** and **4p** were the most potent compounds, it was decided to investigate the importance of the integrity of the 2,2-dimethylchroman core structure for activity against glioma cells. Simplified derivatives were synthesized and the biological results are shown in Table 4. After each step of simplification [C-2 demethylation (see **16a**–**b**), ring opening by C-3 removal (see **9a**–**b**), demethoxylation (see **6a**–**b**)], a gradual decrease of the activity was observed, with IC_{50} around 6–10 μM for the non-methylated compounds **16a** and **16b**, around 15–35 μM for compounds **9a** and **9b**, and around 30–60 μM for the fully open cores **6a** and **6b** (Table 4).

These results highlight the critical importance of the integrity of the 2,2-dimethylchroman core structure for activity on cancer cells. Even if this nucleus is well known in the literature, little has appeared on examples of such compounds expressing *in vitro* antitumor activity. Recently, however, a series of chromans bearing a piperidine ring linked through a spiro carbon atom at the 2-position, such as the 4-acylamino-substituted compound **17** (Fig. 2), were described as histone deacetylase inhibitors and were found to exert marked antiproliferative activity [34]. Concerning more specifically *N*-aryl-*N*′-(chroman-4-yl)ureas or thioureas, two recent works described compounds like **18** and **19** (Fig. 2) as potent TRPV1 receptor antagonists [35,36]. For the moment, it is not known if such compounds also exert antiproliferative activity on glioma cells and/or if the antagonism of TRPV1 receptors could explain the antiproliferative activity of our tightly related chromans.

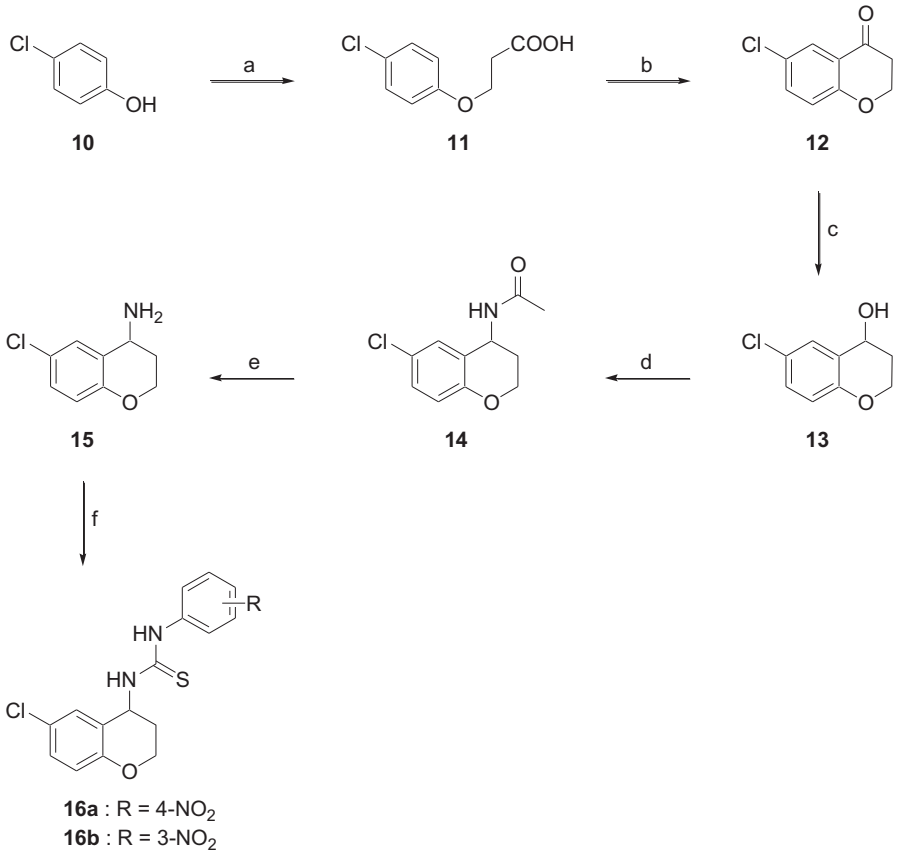
Considering that the active drugs have to cross the blood brain barrier (BBB) and to reach the central nervous system (CNS), it was important to consider their lipophilic character, which can be appreciated by examining their log *P* values. Although it is difficult to predict the optimal log *P* value required for a drug to cross the BBB and to penetrate into the CNS, literature reports that the mean clog *P* (calculated log *P*) value established for CNS drugs is higher than that of non-CNS drugs (3.43 versus 2.78) [37] and, in accordance with the Lipinski rule of five, may not exceed a value of 5 [38,39].

Table 3 reports the AClog *P* values (calculated log *P* values according to the ALOGPS 2.1 software [40]) of the most potent chromans and of the reference drug temozolomide. The latter drug is known to reach the CNS and to act on tumor glial cells. Surprisingly, the AClog *P* value of −1.14 obtained with temozolomide is representative of a hydrophilic rather than a lipophilic drug. On the contrary, most of the chromans were expected to be highly lipophilic drugs with AClog *P* values calculated between 4.0 and 5.4. At least, it is concluded that the chroman-type non-ionic small molecules express appropriate theoretical druglikeness to become centrally active compounds.

Interestingly, recent literature reported that chroman-type K_{ATP} channel openers such as bimakalim (AClog $P = 2.71$) need to cross the phospholipid bilayer of cell membranes to penetrate into the cell and then to interact with their receptors on the SUR subunit via the cytosol [41]. Since most of the active chromans on glioma cells are also known to be active as K_{ATP} channel openers, it is tempting to speculate that they are able to easily cross the hydrophobic

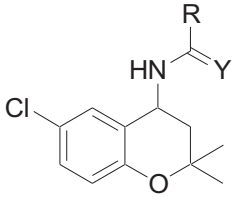


Scheme 2. Synthesis of *N*-(5-chloro-2-methoxyphenyl)methylthioureas. Reagents: (a) $LiAlH_4$, THF; (b) 4- or 3-nitrophenyl isothiocyanate, CH_2Cl_2.



Scheme 3. Synthesis of 6-chlorochromans. Reagents: (a) $BrCH_2CH_2COOH$, KOH; (b) H_2SO_4; (c) $NaBH_4$, CH_3OH; (d) CH_3CN, H_2SO_4; (e) HCl 25% in ethanol–water; (f) 4- or 3-nitrophenyl isothiocyanate, CH_2Cl_2.

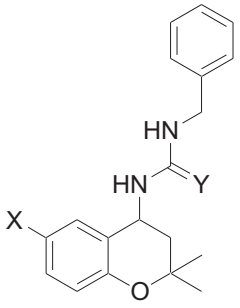
Table 1
In vitro growth inhibitory activity on three human glioma cell lines.



Compounds	Y	R	*In vitro* IC_{50} growth inhibitory concentrations (μM)[a]			
			U373	T98G	Hs683	Mean ± SEM
1a[b]	O	CH_3	>100	–[e]	–	–
1b[b]	O	$NHCH_2CH_3$	>100	–	–	–
1c[b]	O	$NHCH(CH_3)_2$	62 ± 2	>100	85 ± 2	> 82
1d[b]	S	$NHCH_2CH_3$	96 ± 4	>100	72 ± 3	> 89
1e[b]	S	$NHCH(CH_3)_2$	57 ± 1	91 ± 3	34 ± 2	61 ± 16
1f[b]	O	$NHCH_2C_6H_5$	66 ± 11	15 ± 3	47 ± 4	43 ± 15
1g[b]	O	$NHSO_2C_6H_5$	96 ± 4	>100	>100	> 99
1h[c]	O	NHC_6H_5	68 ± 8	34 ± 2	33 ± 3	45 ± 12
1i[d]	S	NHC_6H_5	46 ± 1	37 ± 1	32 ± 2	38 ± 4
1j[b]	O	OCH_2CH_3	>100	–	–	–

[a] IC_{50} values (μM): concentration of drug (μM) responsible for the inhibition of 50% of the growth of the specified cell line after $t = 72$ h [Mean ± s.e.m. ($n = 6$)].
[b] Published compounds in Ref. [15].
[c] Published compounds in Ref. [16].
[d] Published compounds in Ref. [17].
[e] "–" = not determined.

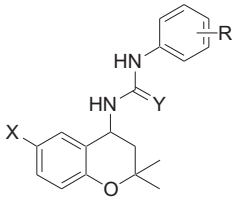
Table 2
In vitro growth inhibitory activity on three human glioma cell lines.



Compounds	Y	X	*In vitro* IC_{50} growth inhibitory concentrations (μM)[a]			
			U373	T98G	Hs683	Mean ± SEM
2a[b]	O	F	11 ± 2	49 ± 3	19 ± 1	26 ± 12
1f[b]	O	Cl	66 ± 11	15 ± 3	47 ± 4	43 ± 15
2b[b]	O	Br	27 ± 1	38 ± 12	38 ± 3	34 ± 4
2c[b]	S	F	27 ± 1	40 ± 1	25 ± 1	31 ± 5
2d[b]	S	Cl	64 ± 14	99 ± 11	43 ± 3	69 ± 16
2e[b]	S	Br	29 ± 2	54 ± 5	34 ± 3	39 ± 8

[a] IC_{50} values (μM): concentration of drug (μM) responsible for the inhibition of 50% of the growth of the specified cell line after $t = 72$ h [Mean ± s.e.m. ($n = 6$)].
[b] Published compounds in Ref. [15].

Table 3
In vitro growth inhibitory activity on three human glioma cell lines and cell selectivity.



Compounds	X	Y	R	*In vitro* IC50 growth inhibitory concentrations (μM)[a]			Mean ± SEM	B16F10 melanoma mouse	Sel.[e]	AClog *P*[f]
				U373 glioma human	T98G glioma human	Hs683 glioma human				
1h[b]	Cl	O	H	68 ± 8	34 ± 2	33 ± 3	45 ± 6	–	–[g]	4.44
1i[c]	Cl	S	H	46 ± 1	37 ± 1	32 ± 2	38 ± 4	–	–	4.74
3a[b]	F	O	2-Cl	40 ± 1	27 ± 1	29 ± 1	32 ± 4	–	–	4.50
3b[b]	F	O	3-Cl	39 ± 2	45 ± 1	38 ± 2	41 ± 2	–	–	4.50
3c[b]	F	O	4-Cl	24 ± 1	30 ± 1	25 ± 1	26 ± 2	–	–	4.50
3d[b]	Cl	O	2-Cl	17 ± 2	20 ± 3	25 ± 2	20 ± 3	20 ± 3	>10	5.05
3e[b]	Cl	O	3-Cl	21 ± 1	25 ± 1	17 ± 2	21 ± 2	25 ± 1	>10	5.05
3f[b]	Cl	O	4-Cl	19 ± 2	33 ± 1	22 ± 1	25 ± 4	32 ± 2	~5	5.05
3g[b]	Br	O	2-Cl	19 ± 2	11 ± 3	22 ± 2	17 ± 3	22 ± 3	>10	5.14
3h[b]	Br	O	3-Cl	69 ± 3	67 ± 3	61 ± 4	66 ± 2	–	–	5.14
3i[b]	Br	O	4-Cl	25 ± 3	27 ± 1	24 ± 2	25 ± 1	9 ± 1	~5	5.14
4a[c]	Br	S	2-Cl	34 ± 2	8 ± 1	22 ± 2	21 ± 8	17 ± 2	<5	5.43
4b[c]	Br	S	3-Cl	8 ± 1	9 ± 1	8 ± 1	8 ± 1	7 ± 1	<2	5.43
4c[c]	Br	S	4-Cl	7 ± 1	22 ± 1	9 ± 3	13 ± 5	7 ± 1	~2	5.43
4d[c]	Br	S	2-OCH_3	22 ± 1	42 ± 3	40 ± 1	35 ± 6	–	–	4.72
4e[c]	Br	S	3-OCH_3	66 ± 3	87 ± 1	81 ± 1	78 ± 6	–	–	4.72
4f[c]	Br	S	4-OCH_3	40 ± 2	33 ± 1	29 ± 1	34 ± 3	–	–	4.72
4g[d]	F	S	3-CN	30 ± 3	39 ± 2	33 ± 1	34 ± 3	35 ± 3	–	3.99
4h[d]	F	S	4-CN	11 ± 1	19 ± 1	14 ± 2	15 ± 3	5 ± 1	~3	3.99
4i[d]	Cl	S	3-CN	7 ± 1	10 ± 1	6 ± 2	8 ± 1	6 ± 1	<5	4.55
4j[d]	Cl	S	4-CN	10 ± 1	10 ± 1	6 ± 1	9 ± 1	7 ± 1	~3	4.55
4k[d]	Br	S	3-CN	6 ± 1	9 ± 1	4 ± 1	6 ± 1	7 ± 1	<5	4.63
4l[d]	Br	S	4-CN	22 ± 2	24 ± 1	15 ± 3	20 ± 3	–	~2	4.63
4m[d]	F	S	3-NO_2	6 ± 1	9 ± 1	6 ± 1	7 ± 1	6 ± 1	~2	4.19
4n[d]	F	S	4-NO_2	3 ± 1	5 ± 1	3 ± 1	3 ± 1	4 ± 1	~5	4.19
4o[d]	Cl	S	3-NO_2	3 ± 1	4 ± 1	3 ± 1	3 ± 1	6 ± 1	~3	4.74
4p[d]	Cl	S	4-NO_2	3 ± 1	4 ± 1	2 ± 1	3 ± 1	3 ± 1	~2	4.74
4q[d]	Br	S	3-NO_2	4 ± 1	4 ± 1	3 ± 1	4 ± 1	5 ± 1	~2	4.83
4r[d]	Br	S	4-NO_2	9 ± 4	5 ± 1	3 ± 1	6 ± 1	3 ± 1	<2	4.83
Temozolomide				220 ± 48	879 ± 34	956 ± 35	685 ± 23	258 ± 52	–	−1.14
Cisplatin				4 ± 1	12 ± 4	4 ± 1	7 ± 3	24 ± 4	–	–

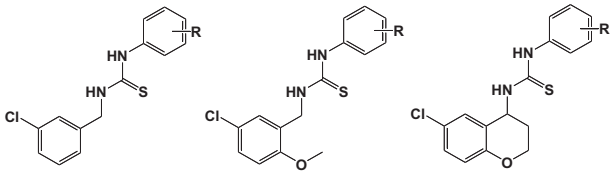
[a] IC_{50} values (μM): concentration of drug (μM) responsible for the inhibition of 50% of the growth of the specified cell line after $t = 72$ h [Mean ± s.e.m. ($n = 6$)].
[b] Published compounds in Ref. [16].
[c] Published compounds in Ref. [17].
[d] Published compounds in Ref. [18].
[e] "Sel" means the level of selectivity, i.e. IC_{50} concentrations obtained on normal astrocytes ($n = 3$)/IC_{50} concentrations obtained on tumor astrocytes ($n = 3$).
[f] AClog *P*: calculated log *P* values according to the ALOGPS 2.1 software [40].
[g] "–" = not determined.

phospholipid bilayer of cell membranes, and, by extension to cross the BBB.

3.2. Comparison between activity on K_{ATP}-channel-expressing cells and glioma cells

Taken together, the data reported in Tables 1–3 tentatively indicated a relationship between the efficacy of 2,2-dimethylchromans on pancreatic β-cells (K_{ATP}-channel-expressing tissue) and on glioma cells (with unknown K_{ATP}-channel-expressing patterns of expression). Indeed, previous works have revealed that among several series of chromans distinguished by the nature of the chemical moiety at the 4-position (acetamides, arylsulfonylureas, carbamates, aralkylureas/thioureas, arylureas/thioureas), arylureas and arylthioureas were found to be the most potent inhibitors of insulin release, as a result of their opening activity on the SUR1-type K_{ATP} channels [15–18]. Interestingly, the same observation was made with respect to glioma cell growth rates in that arylurea-/arylthiourea-type chromans were also found to be the most efficient *in vitro* growth inhibitory agents, supporting the view that a putative SUR1-type K channel, affected by such molecules, could be expressed by astroglial tumor cells and involved in their cell survival and proliferation (Table 3). After careful examination, however, some discrepancies were noticed with respect to the nature and the position (*ortho*, *meta*, or *para*) of the substituent on the phenyl ring of arylureas and arylthioureas. Indeed, methoxy-substituted compounds were found to be less active on β-cells as well as on glioma cells than chloro-, cyano-, and nitro-substituted compounds [17,18]. However, chloro-, cyano-, and nitro-substituted compounds were almost equally effective on β-cells [17,18], while the rank order of potency on glioma cells appeared to be nitro- > cyano- > chloro-compounds (Table 3). Moreover, looking at the chloro-compounds in the arylurea-type chroman series, although the *ortho* position was less favorable for activity on pancreatic β-cells [16,17], no clear differences were found between *ortho*-, *meta*-, and *para*-substituted chloro-compounds for growth inhibitory activity on glioma cells (Table 3).

Table 4
In vitro growth inhibitory activity on three human glioma cell lines (simplified derivatives).



6a-b **9a-b** **16a-b**

Compounds	R	*In vitro* IC_{50} growth inhibitory concentrations (μM)[a]		
		U373	T98G	Hs683
6a	4-NO_2	27 ± 1	20 ± 5	27 ± 2
6b	3-NO_2	32 ± 1	61 ± 2	41 ± 2
9a	4-NO_2	22 ± 1	35 ± 2	15 ± 2
9b	3-NO_2	32 ± 2	–	23 ± 2
16a	4-NO_2	8 ± 1	12 ± 1	6 ± 1
16b	3-NO_2	8 ± 1	10 ± 1	7 ± 1

[a] IC_{50} values (μM): concentration of drug (μM) responsible for the inhibition of 50% of the growth of the specified cell line after $t = 72$ h [Mean ± s.e.m. ($n = 6$)]; "–" = not determined.

To confirm or refute the possible link between the K_{ATP}-channel-opening potency of drugs and their growth inhibitory activity, we tested several reference compounds known to be K_{ATP}-channel openers or blockers. Thus, 6-chloro-7-fluoro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (a strong SUR1-type K_{ATP}-channel opener [19]), 7-chloro-3-(1,1-dimethylpropyl)amino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (expected to be a SUR2B-type K_{ATP}-channel opener [20]), *R*/*S*-pinacidil (a SUR2B-type K_{ATP}-channel opener), and glibenclamide (a K_{ATP}-channel blocker) were assayed in terms of glioma growth inhibitory activity by means of the MTT colorimetric assay in human U373 glioblastoma cells. None of these reference compounds (three openers and one blocker) expressed a marked growth inhibitory activity ($IC_{50} > 100$ μM) (data not shown), supporting the view that K_{ATP} channels were probably not involved in the *in vitro* anti-glioma growth inhibitory activity of arylurea- and arylthiourea-type chromans.

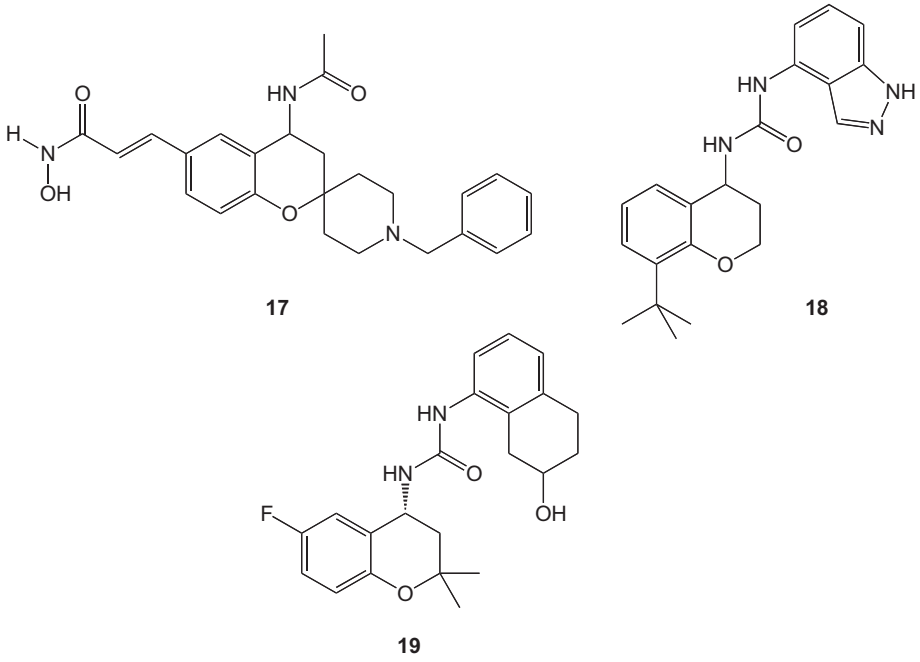
3.3. Selectivity for tumor glial cells versus normal glial cells

For compounds whose mean IC_{50} value was found to be <30 μM on the three human glioma cell lines, we analyzed their selectivity by quantification of their activity on the *in vitro* growth inhibition in normal mouse astrocytes (Table 3). The results of this analysis showed a clear distinction of cell selectivity between phenylureas and phenylthioureas (Table 3). All phenylurea derivatives presented a selectivity ratio ≥5 when dividing the mean IC_{50} growth inhibitory concentrations calculated on normal astrocytes by those IC_{50} concentrations calculated on the human glioma cell lines (Table 3). This selectivity index increased to >10 for **3d**, **3e**, and **3g** (Table 3). On the contrary, all thioureas showed a selectivity ratio below 5 (Table 3).

As the *in vitro* growth inhibitory activity of the compounds under study were analyzed on human glioma cell lines, while the bioselectivity was determined on mouse normal astrocytes, we also analyzed the *in vitro* growth inhibitory activity of the bioselective compounds detailed in Table 3 on a mouse melanoma pseudometastasis brain model [42] in order to rule out differences that could relate to interspecies features. The data in Table 3 clearly indicate that mouse and human cancer cells display similar sensitivity to the compounds under study.

3.4. Cell cycle analysis

We noticed no differences in terms of growth inhibitory activity (as revealed by the MTT colorimetric assay; Tables 1–4) for all compounds under study when comparing glioma cells actually sensitive to pro-apoptotic stimuli (the Hs683 glioma cells [25,29]) to glioma cells displaying various levels of resistance to pro-apoptotic stimuli (the U373 [25,29] and T98G [29] glioma cells). These data thus suggest that the compounds under study exert their effects through non-apoptotic pathways. Cell cycle kinetics of U373 glioma cells were thus performed to provide insights about the mechanism of action between the most active and bioselective compounds, i.e. **3d**, **3e**, and **3g**, versus the most



17 **18** **19**

Fig. 2. 4-Acylamino/ureido-substituted chromans reported as histone deacetylase inhibitors (**17**) or TRPV1 receptor antagonists (**18** and **19**).

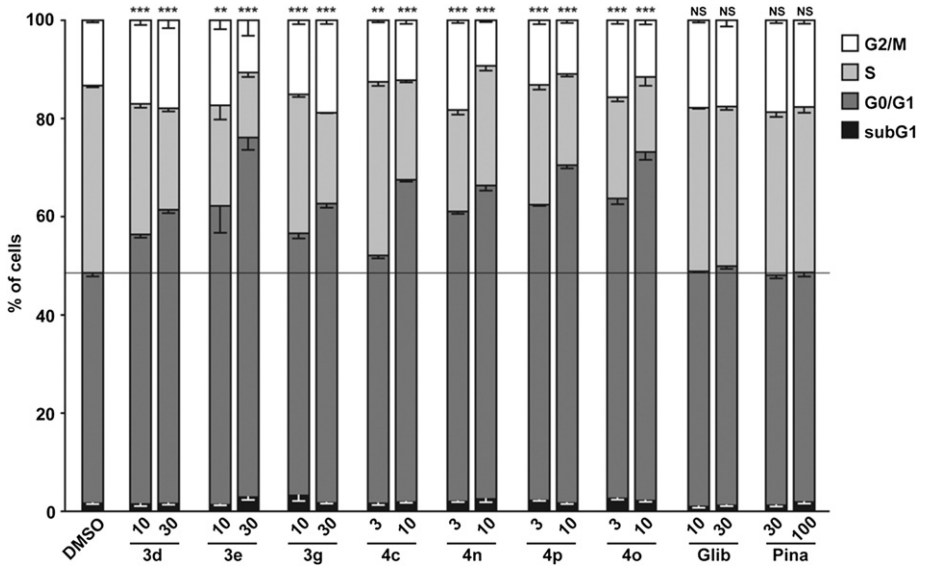


Fig. 3. Cell cycle analysis by flow cytometry. Human U373 glioblastoma cells were treated with the indicated chroman derivatives at the indicated concentrations (corresponding approximately to half and double of the IC_{50} growth inhibitory concentration revealed by the MTT colorimetric assay) for 24 h. Percentage of cells in G0/G1 phase is significantly higher than cells treated with vehicle only with p value < 0.01 (**) or with p value< 0.001 (***); $n = 3$.

active compounds, while not actually bioselective (thus toxic), i.e. **4c**, **4n**, **4o**, and **4p** (with $IC_{50} \leq 7$ μM) (Table 3). The data in Fig. 3 confirm the non-apoptotic-mediated growth inhibitory effects of the most active compounds under study according to the fact that none of the compounds markedly increased the percentage of subG1 U373 glioma cells.

The cell cycle kinetic analyses did not allow a clear distinction to be made between all these compounds because all of them blocked more or less the cell cycle in the G0/G1 phase (Fig. 3). In contrast, cell cycle analysis provided important information. Indeed, because the compounds under study are known to modify K_{ATP}-channel activity (as detailed in the Introduction), we also analyzed the effect of the K_{ATP}-channel opener pinacidil (30 and 100 μM) and the K_{ATP}-channel blocker glibenclamide (10 and 30 μM) on U373 cell cycle kinetics. The data in Fig. 3 show that these two compounds did not modify U373 cell cycle kinetics. These data therefore suggest that the compounds under study exert their growth inhibitory effects on glioma cell growth independently of K_{ATP}-channel activity through an as yet unknown mechanism.

3.5. Quantitative videomicroscopy-related analyses

In addition to the cell cycle kinetic analyses, computer-assisted phase-contrast microscopy (quantitative videomicroscopy) was used to provide further information about mechanisms of action. Thus, **3d**, **3e**, and **3g**, i.e. the three compounds under study among the most active ones associated with the highest bioselectivity (Table 3), were analyzed to discover if they displayed cytotoxic or

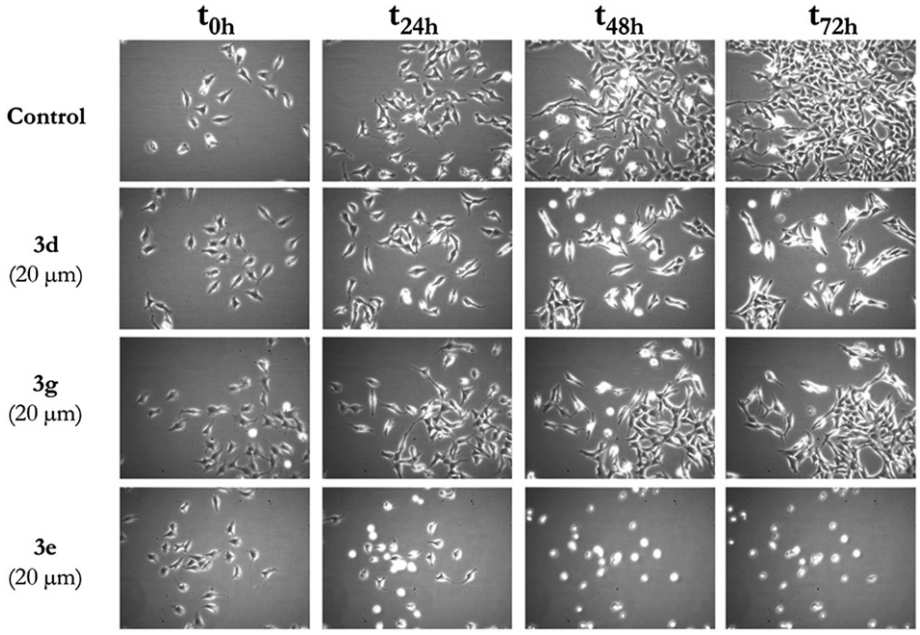


Fig. 4. Quantitative videomicroscopy-related illustrations of the cytostatic effects induced by **3d** (20 μM) and **3g** (20 μM) in human U373 glioblastoma cells, while **3e** (20 μM) induces cytotoxic effects (Gx200).

rather cytostatic effects. Each compound was assayed on the U373 glioma cell lines at 20 μM, i.e. a concentration close to their IC_{50} growth inhibitory concentration as revealed by the MTT colorimetric assay (Table 3). This 20 μM concentration turned out to correspond to an IC_{80} (quantitative videomicroscopy) rather than to an IC_{50} (MTT colorimetric assay) growth inhibitory concentration with respect to each compound analyzed (data not shown). The IC_{80} concentration was determined as follows: in both control and treated conditions, U373 glioma cell growth levels were evaluated by the ratio between the numbers of cells counted in the last and first frames of the image sequences. A global growth ratio (GGR) was thus determined as the ratio between the two growth levels obtained in the treated and control conditions. GGR values were equal to 0.2 (thus to an IC_{80} growth inhibitory concentration) for each of the three compounds analyzed at 20 μM. The fact that 20 μM appeared more active for each of the three compounds analyzed under quantitative videomicroscopy than under MTT colorimetric assay could relate, at least partly, to distinct cell culture conditions. Indeed, in the MTT test, cells are cultured in 100 μL cell culture volumes in 96-well microplates, while they are cultured in 25 cm^2 flasks containing 7 mL culture medium in the other test. The morphological illustrations in Fig. 4 and the dynamic movies (data not shown) clearly indicate that **3d** and **3g** are cytostatic at their IC_{80} growth inhibitory concentrations, while **3e** is cytotoxic at its IC_{80} concentration. It thus seems that the position of the chlorine atom on the phenyl ring (*o*-, *m*- or *p*-position) is of critical importance since only the *ortho*-substituted chloro-compounds **3d** and **3g**, but not the *meta*-substituted chloro-compound **3e**, were found to be clearly cytostatic.

4. Conclusions

Based on the ongoing clinical study using a chloride-channel blocker as a treatment for glioma, we examined a series of 2,2-dimethylchromans previously reported as potassium-channel openers, as well as some newly synthesized simplified structures, for their *in vitro* growth inhibitory activity on three human high-grade glioma cell lines, U373, T98G, and Hs683. A marked growth inhibitory activity was observed with 2,2-dimethylchroman-type phenylureas and phenylthioureas, among which nitro-substituted phenylthioureas, such as compounds **4o** and **4p**, were recognized as the most efficient (but also poorly selective) derivatives on glioma cells. Selected phenylureas (**3d**, **3e**, **3g**) were found to be less active, but more cell selective (normal versus tumor glial cells), thus less toxic, than the corresponding phenylthioureas. Furthermore, these most active and bioselective phenylureas (**3d**, **3e**, **3g**) displayed similar growth inhibitory concentrations between glioma cells that display actual sensitivity to pro-apoptotic stimuli (the Hs683 glioma model) and glioma cells that display various levels of resistance to pro-apoptotic stimuli (the U373 and T98G glioma models). In future studies of new active substances belonging to this class of compounds, it will be important to find the best equilibrium between activity and selectivity.

Arylurea-/arylthiourea-type chromans, known to interact with SUR1-type K_{ATP} channels, were found to be the most efficient *in vitro* growth inhibitory agents, supporting the view that a putative SUR1-type K channel, affected by such molecules, could be expressed on astroglial tumor cells and involved in their antitumor activity. However, none of the reference K_{ATP}-channel openers and blockers, tested on the U373 cell line to confirm such an assumption, revealed a significant growth inhibitory activity, demonstrating that no clear relationship could be found between the potency of chromans as potassium channel openers and their growth inhibitory activity on glioma cells. Computer-assisted phase-contrast videomicroscopy indicated that minor chemical modifications were sufficient to transform cytostatic compounds into cytotoxic ones and flow cytometry showed that these compounds exert their growth inhibitory effects by blocking the cell cycle in the G0/G1 phase. As a result, this work identified phenylurea- and phenylthiourea-type 2,2-dimethylchromans as new classes of antitumor agents to be further explored for an innovative therapeutic approach of high-grade glioma in humans.

5. Experimental section

5.1. Chemistry

Melting points were determined on a Stuart SMP3 capillary apparatus and are uncorrected. The ^1H NMR spectra were recorded on a Bruker Avance (500 MHz) instrument using d_6-DMSO as the solvent with TMS as an internal standard; chemical shifts are reported in δ values (ppm) relative to that of internal TMS. The abbreviations s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, and b = broad are used throughout. Elemental analyses (C, H, N, S) were realized on a Thermo Scientific FlashEA 1112-elemental analyzer and were within ±0.4% of the theoretical values. This analytical method certified a purity ≥95% for each tested compound. All reactions were routinely checked by TLC on silica gel Merck 60 F_{254}.

5.1.1. *N-[(3-Chlorophenyl)methyl]-N′-(4-nitrophenyl)thiourea (**6a**)*

4-Nitrophenyl isothiocyanate (0.15 g, 0.85 mmol) was added to a solution of 3-chlorobenzylamine (0.1 g, 0.71 mmol) in methylene chloride (2 mL). After 20 min at room temperature, the resulting precipitate was collected by filtration, washed with hexane and dried (0.16 g, 70%): mp 130–132 °C; ^1H NMR (DMSO-d_6) δ 4.77 (s, 2H, CH_2), 7.31–7.41 (m, 4H, C_6H_4Cl), 7.85 (d, J = 9 Hz, 2H, 2′-*H*/6′-*H*), 8.19 (d, J = 9 Hz, 2H, 3′-*H*/5′-*H*), 8.72 (bs, 1H, CH_2NH), 10.28 (bs, 1H, $NO_2C_6H_4NH$); ^{13}C NMR (DMSO-d_6) δ 46.7 (CH_2NH), 120.8 (C-2′/C-6′), 124.5 (C-3′/C-5′), 126.2, 126.9, 127.2 (C-2/C-4/C-6), 130.2 (C-3), 132.9 (C-5), 141.0 (C-1), 142.0 (C-4′), 146.2 (C-1′), 180.6 (NHCSNH). Anal. ($C_{14}H_{12}ClN_3O_2S$) theoretical: C, 52.26; H, 3.76; N, 13.06; S, 9.96. Found: C, 52.30; H, 3.80; N, 13.09; S: 9.60.

5.1.2. *N-[(3-Chlorophenyl)methyl]-N′-(3-nitrophenyl)thiourea (**6b**)*

The title compound was obtained as described for **6a**, starting from 3-chlorobenzylamine (0.1 g, 0.71 mmol) and 3-nitrophenyl isothiocyanate (0.15 g, 0.85 mmol) (0.19 g, 83%): mp 149.5–151.5 °C; ^1H NMR (DMSO-d_6) δ 4.78 (s, 2H, CH_2), 7.31–7.41 (m, 4H, C_6H_4Cl), 7.6 (t, J = 8.2 Hz, 1H, 5′-*H*), 7.85 (d, J = 8.2 Hz, 1H, 6′-*H*), 7.93 (d, J = 8.2 Hz, 1H, 4′-*H*), 8.59 (bs, 2H, 2′-*H*/CH_2NH), 10.09 (bs, 1H, $NO_2C_6H_4NH$); ^{13}C NMR (DMSO-d_6) δ 46.4 (CH_2NH), 116.7 (C-2′), 118.2 (C-4′), 126.1, 126.9, 127.1 (C-2/C-4/C-6), 128.7 (C-6′), 129.7 (C-5′), 130.2 (C-3), 132.9 (C-5), 140.9 (C-1), 141.3 (C-1′), 147.5 (C-3′), 181.1 (NHCSNH). Anal. ($C_{14}H_{12}ClN_3O_2S$) theoretical: C, 52.26; H, 3.76; N, 13.06; S, 9.96. Found: C, 52.08; H, 3.78; N, 13.05; S: 9.69.

5.1.3. *5-Chloro-2-methoxybenzylamine (**8**)*

Lithium aluminum hydride (0.34 g, 9 mmol) was added to a solution of 5-chloro-2-methoxybenzonitrile (0.5 g, 3 mmol) in tetrahydrofuran (10 mL) at 0 °C. The mixture was maintained at this temperature for a further 30 min and then stirred for an additional 1 h at 30 °C. After cooling at 0 °C, water (50 mL) was added and the amine was extracted twice with methylene chloride. The organic layers were dried over magnesium sulfate, filtered, and evaporated under reduced pressure. The crude residue of the title compound (0.35 g, 68%) was used directly in the next step (synthesis of **9a** and **9b**). ^1H NMR (DMSO-d_6) δ 1.85 (bs, 2H, NH_2), 3.64 (s, 2H, CH_2), 3.77 (s, 1H, OCH_3), 6.94 (d, J = 8.7 Hz, 1H, 3-*H*), 7.21 (dd, J = 8.7 Hz/2.7 Hz,

1H, 4-*H*), 7.38 (d, $J = 2.7$ Hz, 1H, 6-*H*); ^{13}C NMR (DMSO-d_6) δ 37.0 (CH_2NH_2), 56.0 (OCH_3), 112.7 (C-3), 123.8 (C-5), 129.5 (C-4), 129.8 (C-6), 156.0 (C-2).

5.1.4. N-[(5-Chloro-2-methoxyphenyl)methyl]-N′-(4-nitrophenyl) thiourea (**9a**)

The title compound was obtained as described for **6a**, starting from **8** (0.075 g, 0.44 mmol) and 4-nitrophenyl isothiocyanate (0.095 g, 0.53 mmol) (0.06 g, 40%): mp 188–190 °C; ^{1}H NMR (DMSO-d_6) δ 3.85 (s, 3H, OC*H*$_3$), 4.69 (s, 2H, C*H*$_2$), 7.05 (d, $J = 8.8$ Hz, 1H, 3-*H*), 7.25 (d, $J = 2.2$ Hz, 1H, 6-*H*), 7.32 (dd, $J = 9.1$ Hz/2.7 Hz, 1H, 4-*H*), 7.88 (d, $J = 9$ Hz, 2 H, 2′-*H*/6′-*H*), 8.19 (d, $J = 9$ Hz, 2H, 3′-*H*/5′-*H*), 8.55 (bs, 1H, CH_2N*H*), 10.3 (bs, 1H, $NO_2C_6H_4$N*H*); ^{13}C NMR (DMSO-d_6) δ 42.2 (CH_2NH), 55.8 (OCH_3), 112.4 (C-3), 120.5 (C-2′/C-6′), 123.9 (C-5), 124.5 (C-3′/C-5′), 127.7, 127.8 (C-4/C-6), 127.9 (C-1), 141.9 (C-4′), 146.3 (C-1′), 155.6 (C-2), 180.5 (NHCSNH). Anal. ($C_{15}H_{14}ClN_3O_3S$) theoretical: C, 51.21; H, 4.01; N, 11.94; S, 9.14. Found: C, 51.24; H, 4.04; N, 12.01; S: 8.74.

5.1.5. N-[(5-Chloro-2-methoxyphenyl)methyl]-N′-(3-nitrophenyl) thiourea (**9b**)

The title compound was obtained as described for **6a**, starting from **8** (0.2 g, 1.17 mmol) and 3-nitrophenyl isothiocyanate (0.25 g, 1.4 mmol) (0.3 g, 73%): mp 169–172 °C; ^{1}H NMR (DMSO-d_6) δ 3.84 (s, 3H, OC*H*$_3$), 4.7 (s, 2H, C*H*$_2$), 7.04 (d, $J = 8.8$ Hz, 1H, 3-*H*), 7.24 (d, $J = 2.2$ Hz, 1H, 6-*H*), 7.32 (dd, $J = 8.7$ Hz/2.6 Hz, 1H, 4-*H*), 7.59 (t, $J = 8.2$ Hz, 1H, 5′-*H*), 7.87 (d, $J = 7.6$ Hz, 1H, 6′-*H*), 7.93 (d, $J = 8.1$ Hz, 1H, 4′-*H*), 8.37 (bs, 1H, CH_2N*H*), 8.65 (s, 1H, 2′-*H*), 10.11 (bs, 1H, $NO_2C_6H_4$N*H*); ^{13}C NMR (DMSO-d_6) δ 42.1 (CH_2NH), 55.8 (OCH_3), 112.4 (C-3), 116.4 (C-2′), 118.1 (C-4′), 123.9 (C-5), 127.5, 127.7 (C-4/C-6), 128.2 (C-1), 128.4 (C-6′), 129.7 (C-5′), 141.0 (C-1′), 147.5 (C-3′), 155.5 (C-2) 181.0 (NHCSNH). Anal. ($C_{15}H_{14}ClN_3O_3S$) theoretical: C, 51.21; H, 4.01; N, 11.94; S, 9.14. Found: C, 50.89; H, 3.98; N, 11.95; S: *8.44*.

5.1.6. 3-(4-Chlorophenoxy)propanoic acid (**11**)

A mixture of 4-chlorophenol (10 g, 78.1 mmol), 3-bromopropionic acid (11.9 g, 78.1 mmol), potassium hydroxide (8 g, 0.2 mol) and ethanol (5 mL) in water (80 mL) were refluxed overnight. After cooling, the solution was acidified with concentrated hydrochloric acid to pH = 1 and extracted with ethyl acetate. The organic layer was washed with a saturated aqueous solution of sodium bicarbonate. The aqueous phase was then acidified with concentrated hydrochloric acid and extracted with ethyl acetate. The final organic layer was dried over magnesium sulfate, filtered, and evaporated under vacuum. The title product was recrystallized in ethyl acetate/hexane (1:5). The resulting precipitate was collected by filtration, washed with hexane, and dried (3.6 g, 23%): mp 136–138 °C; ^{1}H NMR (DMSO-d_6) δ 2.68 (t, $J = 6$ Hz, 2H, C*H*$_2$COOH), 4.15 (t, $J = 6$ Hz, 2H, OC*H*$_2$), 6.96 (d, $J = 9$ Hz, 2H, 2-*H*/6-*H*), 7.32 (d, $J = 9$ Hz, 2H, 3-*H*/5-*H*), 12.38 (bs, H, COO*H*); ^{13}C NMR (DMSO-d_6) δ 34.0 (*C*H$_2$COOH), 63.9 (OCH_2), 116.2 (C-2/C-6), 124.3 (C-4), 129.2 (C-3/C-5), 157.2 (C-1), 172.1 (COOH).

5.1.7. 6-Chlorochroman-4-one (**12**)

A mixture of **11** (2 g, 10 mmol) and concentrated sulfuric acid (20 mL) was stirred at room temperature for 1 h, poured onto ice and extracted with ethyl acetate. The organic layer was washed twice with saturated aqueous sodium bicarbonate, dried over magnesium sulfate, filtered, and evaporated under vacuum. The title product was recrystallized in ethyl acetate/hexane (1:5). The resulting precipitate was collected by filtration, washed with hexane, and dried (1 g, 55%): mp 98–101 °C (lit. [31]: mp 100–101 °C); ^{1}H NMR (DMSO-d_6) δ 2.82 (t, $J = 6.5$ Hz, 2H, 3-H_2), 4.56 (t, $J = 6.5$ Hz, 2H, 2-H_2), 7.1 (d, $J = 8.9$ Hz, 1H, 8-*H*), 7.6 (dd, $J = 8.9$ Hz/2.7 Hz, 1H, 7-*H*), 7.68 (d, $J = 2.7$ Hz, 1H, 5-*H*); ^{13}C NMR (DMSO-d_6) δ 36.8 (C-3), 66.9 (C-2), 120.3 (C-8), 122.0 (C-4a/C-6), 125.3 (C-5), 135.6 (C-7), 160.1 (C-8a), 190.6 (C-4).

5.1.8. R/S-6-Chlorochroman-4-ol (**13**)

Sodium borohydride (0.46 g, 12.1 mmol) was added to a stirred suspension of **12** (2 g, 11 mol) in methanol (30 mL) at 0 °C, and the mixture was maintained at this temperature for a further 30 min. The mixture was stirred for an additional 30 min at room temperature and the solvent was evaporated under vacuum. Water was added to the residue, the suspension was acidified by means of concentrated hydrochloric acid and the product was extracted twice with methylene chloride. The organic layers were dried over magnesium sulfate, filtered, and evaporated under vacuum. The title product was recrystallized in ethyl acetate/hexane (1:5). The resulting precipitate was collected by filtration, washed with hexane, and dried (1.7 g, 84%): mp 80–83 °C (lit. [32]: mp 91–93 °C); ^{1}H NMR (DMSO-d_6) δ 1.81–2 (m, 2H, 3-H_2), 4.18 (t, $J = 5.1$ Hz, 2H, 2-H_2), 4.61 (qd, $J = 5.1$ Hz, 1H, 4-*H*), 5.5 (d, $J = 5.1$ Hz, 1H, O*H*), 6.77 (d, $J = 8.7$ Hz, 1H, 8-*H*), 7.16 (dd, $J = 8.7$ Hz/2.6 Hz, 1H, 7-*H*), 7.32 (d, $J = 2.6$ Hz, 1H, 5-*H*); ^{13}C NMR (DMSO-d_6) δ 30.5 (C-3), 61.3 (C-4), 62.6 (C-2), 118.0 (C-8), 123.3 (C-6), 128.2 (C-4a), 128.3 (C-7), 128.9 (C-5), 152.9 (C-8a). Anal. ($C_9H_9ClO_2$) theoretical: C, 58.55; H, 4.91. Found: C, *57.83*; H, 4.82.

5.1.9. R/S-4-Acetamido-6-chlorochroman (**14**)

A suspension of **13** (1.7 g, 9.24 mmol) in acetonitrile (33 mL) was added dropwise to a stirred solution of 98% sulfuric acid (1.1 mL) in acetonitrile (7 mL) kept between −10 and 0 °C. The mixture was allowed to warm to room temperature during 1 h. The solution was poured into cold water, and the precipitate was collected by filtration, washed with water, and dried (2 g, 96%): mp 190–192 °C; ^{1}H NMR (DMSO-d_6) δ 1.81–2.05 (m, 2H, 3-H_2), 1.88 (s, 3H, NHCOC*H*$_3$), 4.2 (m, 2H, 2-H_2), 4.98 (qd, $J = 7$ Hz, 1H, 4-*H*), 6.81 (d, $J = 8.7$ Hz, 1H, 8-*H*), 7.15 (d, $J = 2.3$ Hz, 1H, 5-*H*), 7.18 (dd, $J = 8.7$ Hz/2.6 Hz, 1H, 7-*H*), 8.36 (d, $J = 8$ Hz, N*H*COCH$_3$); ^{13}C NMR (DMSO-d_6) δ 22.6 (CH_3), 28.2 (C-3), 42.2 (C-4), 63.5 (C-2), 118.3 (C-8), 123.7 (C-6), 125.6 (C-4a), 128.3 (C-7), 128.4 (C-5), 153.5 (C-8a), 168.9 (*C*OCH$_3$). Anal. ($C_{11}H_{12}ClNO_2$) theoretical: C, 58.55; H, 5.36; N, 6.21. Found: C, 58.63; H, 5.38; N, 6.24.

5.1.10. R/S-4-Amino-6-chlorochroman (**15**)

A solution of **14** (1 g, 4.44 mmol) in concentrated hydrochloric acid (14 mL) and ethanol (7 mL) was refluxed for 72 h. Hydrochloric acid and ethanol were removed under vacuum, and the residue was dissolved in hot water (20 mL). The solution was filtered, 10% aqueous sodium hydroxide was added to the filtrate until alkaline and the amine was extracted twice with methylene chloride. The organic layers were dried over magnesium sulfate, filtered, and evaporated under reduced pressure. The pure residue (0.6 g, 74%) was used directly in the next step (synthesis of **9a** and **9b**). ^{1}H NMR (DMSO-d_6) δ 1.68–2.01 (m, 2H, 3-H_2), 1.99 (bs, 2H, NH_2), 3.86 (t, $J = 5.8$ Hz, 1H, 4-*H*), 4.1–4.26 (m, 2H, 2-H_2), 6.73 (d, $J = 8.7$ Hz, 1H, 8-*H*), 7.1 (dd, $J = 8.7$ Hz/2.6 Hz, 1H, 7-*H*), 7.44 (d, $J = 2.6$ Hz, 1H, 5-*H*); ^{13}C NMR (DMSO-d_6) δ 25.9 (C-3), 43.2 (C-4), 62.0 (C-2), 118.9 (C-8), 120.7 (C-6), 123.8 (C-4a), 129.1 (C-5), 129.9 (C-7), 153.6 (C-8a).

5.1.11. R/S-N-[6-Chlorochroman-4-yl]-N′-(4-nitrophenyl)thiourea (**16a**)

The title compound was obtained as described for **6a**, starting from **15** (0.15 g, 0.82 mmol) and 4-nitrophenyl isothiocyanate (0.18 g, 1 mmol) (0.28 g, 94%): mp 204–206 °C; ^{1}H NMR (DMSO-d_6) δ 2.06–2.26 (m, 2H, 3-H_2), 4.25 (m, 2H, 2-H_2), 5.63 (m, 1H, 4-*H*), 6.86 (d, $J = 8.7$ Hz, 1H, 8-*H*), 7.24 (dd, $J = 8.7$ Hz/2.6 Hz, 1H, 7-*H*), 7.33 (d, $J = 2.6$ Hz, 1H, 5-*H*), 7.88 (d, $J = 9$ Hz, 2 H, 2′-*H*/6′-*H*), 8.19

(d, $J = 9$ Hz, 2H, 3′-*H*/5′-*H*), 8.77 (d, $J = 7.7$ Hz, 1H, CH_2NH), 10.09 (s, 1H, $NO_2C_6H_4NH$); ^{13}C NMR (DMSO-d_6) δ 27.4 (C-3), 47.3 (C-4), 63.8 (C-2), 118.5 (C-8), 120.8 (C-2′/C-6′), 123.8 (C-6), 124.4 (C-3′/C-5′), 124.5 (C-4a), 128.3 (C-5), 128.7 (C-7), 142.0 (C-4′), 146.2 (C-1′), 153.6 (C-8a), 180.6 (NHCSNH). Anal. ($C_{16}H_{14}ClN_3O_3S$) theoretical: C, 52.82; H, 3.88; N, 11.55; S, 8.81. Found: C, 52.58; H, 3.87; N, 11.47; S: *7.93*.

5.1.12. R/S-N-[6-Chlorochroman-4-yl]-N′-(3-nitrophenyl)thiourea (**16b**)

The title compound was obtained as described for **6a**, starting from **15** (0.15 g, 0.82 mmol) and 3-nitrophenyl isothiocyanate (0.18 g, 1 mmol) (0.19 g, 64%): mp 178–180 °C; ^1H NMR (DMSO-d_6) δ 2.06–2.24 (m, 2H, 3-H_2), 4.25 (m, 2H, 2-H_2), 5.66 (m, 1H, 4-*H*), 6.85 (d, $J = 8.7$ Hz, 1H, 8-*H*), 7.23 (dd, $J = 8.7$ Hz/2.6 Hz, 1H, 7-*H*), 7.32 (d, $J = 2.6$ Hz, 1H, 5-*H*), 7.59 (t, $J = 8.1$ Hz, 1H, 5′-*H*), 7.85 (d, $J = 8.1$ Hz, 1H, 6′-*H*), 7.94 (d, $J = 8.1$ Hz, 1H, 4′-*H*), 8.64 (bs, 2H, 2′-*H*/CH_2NH), 9.9 (bs, 1H, $NO_2C_6H_4NH$); ^{13}C NMR (DMSO-d_6) δ 27.5 (C-3), 47.4 (C-4), 63.9 (C-2), 116.7 (C-2′), 118.3 (C-4′), 118.5 (C-8), 123.8 (C-6), 124.8 (C-4a), 128.2 (C-5), 128.7 (C-7), 128.8 (C-6′), 129.7 (C-5′), 140.8 (C-1′), 147.5 (C-3′), 153.5 (C-8a), 180.4 (NHCSNH). Anal. ($C_{16}H_{14}ClN_3O_3S$) theoretical: C, 52.82; H, 3.88; N, 11.55; S, 8.81. Found: C, 52.63; H, 3.88; N, 11.55; S: *8.00*.

5.2. Pharmacology

5.2.1. Determination of in vitro growth inhibitory activity in human glioma cell lines

We have made use of three human glioma cell lines (Hs683, U373, T98G) and one mouse melanoma cell line (B16F10 that can develop as brain pseudometastases [31]). The U373 astroglioma cell line was obtained from the European Collection of Cell Culture (ECACC; code 96062201) and the remaining three cell lines from the American Type Culture Collection (ATCC), i.e. the T98G astroglioma cell line (code CRL-1690), the Hs683 oligodendroglioma (code HTB-138) and the mouse B16F10 melanoma (code CRL-6475) cell lines.

The influence of each compound under study on the *in vitro* growth rates of the three human glioma cell lines, and on some occasion of the mouse B16F10 melanoma, was determined using the colorimetric MTT (3-[4,5-dimethylthiazol-2yl])-diphenyl tetrazolium bromide, (Sigma, Belgium) assay as detailed previously [13,29,31]. The use of the MTT colorimetric assay is based on the capability of living cells to reduce the yellow product MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a blue product, formazan, by a reduction reaction occurring in the mitochondria. The number of living cells after 72 h of culture in the presence (or absence: control) of the various compounds is directly proportional to the intensity of the blue, which is quantitatively measured by spectrophotometry (Biorad Model 680XR; Biorad, Nazareth, Belgium) at a 570 nm wavelength (with a reference of 630 nm). Each experiment was carried out in six replicates.

The cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10,000–40,000 cells/mL culture medium depending on the cell type) to ensure adequate plating prior to cell growth determination. The cells were cultured in RPMI (Invitrogen, Merelbeke, Belgium) media supplemented with 10% heat inactivated fetal calf serum (Invitrogen). All culture media were supplemented with 4 mM glutamine, 100 μg/mL gentamicin, and penicillin-streptomycin (200 U/mL and 200 μg/mL) (Invitrogen).

5.2.2. In vitro growth inhibition analyses in normal astrocytes

Astrocyte culture. Astrocytes were obtained from neonatal NMRI mouse cortices that were freed of meninges, minced into small pieces of tissue with microscissors and then suspended in MEM (Invitrogen) supplemented with glucose 6 g/L and 10% Fetal Calf Serum (FCS) (Invitrogen) and successively filtered through a 225 μm-pore and a 25 μm-pore filter. The filtered cell suspension was plated on an uncoated T25 flask for 72 h. The medium was then changed and cells were grown until confluence.

MTT survival assay. The day before the test of various compounds, astrocytes are resuspended using a 0.25% trypsin/EDTA solution and seeded in 96 wells plates (Nunc, Thermo Scientific, VWR, Leuven, Belgium) at 30,000 cells/well density in 100 μL/well in the serum-containing MEM except in column A (control wells for the MTT assay). Various compounds are dissolved in a DMSO (dimethylsulfoxide) solution (Sigma–Aldrich, Bornem, Belgium) at a concentration that is 1000× of the final dilution. A dose response curve of the various tested solutions is set up in DMSO, allowing adding in each culture well the DMSO solution at a final concentration 1/1000. Each dilution is tested in column of the 96 wells plate or in octoplicates. A control column is set up by adding the DMSO solution (1/1000 final concentration) without any compound. After 72 h of culture, culture medium is replaced by a 50 μL MTT solution prepared at 0.15 mg/mL in MEM. MTT or (3-(4,5-dimethylyhiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma–Aldrich) was previously prepared as a 1.5 mg/mL stock solution in MEM, then passed on a 0.22 μm filter and aliquoted at −20 °C. The plates are then cultivated for 3 h at 37 °C. Then the medium is discarded, cells are washed with a sterile PBS solution and then 50 μL of an acidic isopropanol solution was added (20 mL isopropanol supplemented with 120 μL of 37 M HCl). The plates are read using a spectrophotometer using a 570 nm wavelength OD (with a reference of 630 nm).

5.2.3. Computer-assisted phase-contrast videomicroscopy (quantitative videomicroscopy)

As whether various compounds under study induced cytotoxic versus cytostatic effects on U373 glioma cells were characterized *in vitro* with computer-assisted phase-contrast videomicroscopy as described previously [27,30]. Cells were monitored for 72 h with one image digitized every 4 min and movies were constructed from the 1080 time-lapse image sequences, which enabled rapid screening for cell viability.

5.2.4. Cell cycle analysis by flow cytometry

Propidium iodide (PI; Sigma–Aldrich) was used for cell cycle analysis. Briefly, cells were trypsinized and fixed with 70% ethanol overnight at −20 °C, then pelleted before resuspending in PBS containing PI (50 μg/mL; Invitrogen), RNAse A (0.1 mg/mL; Sigma–Aldrich) and Triton X-100 (0.05%; Sigma–Aldrich) followed by cytofluorometric analysis with a FACSCalibur (Becton Dickinson, Erembodegem, Belgium).

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