Abstract. Background: Among seventeen compounds derived from chalcones investigated as potential anticancer drugs towards LN229 glioblastoma cell line, only two were effective. Materials and Methods: Anticancer activity was investigated by evaluating the cell growth, cell cycle, mitotic index and the cell death. Results: Two compounds, namely C2 and C12, inhibited cell proliferation associated with a blockade in the G2/M phase of the cell cycle and arrested the growth of tumour spheroid mimicking in vivo tumour. C2 blocked cells in the G2 phase whereas C12 blocked cells in the M phase of the cell cycle. C12 and C2 killed 40% and 95% of the cells respectively using complex mechanisms. The two compounds increased the fluorescence of rhodamine-123 and N-acetylcysteine inhibited their activity, suggesting a role for reactive oxygen species in cell death mediated by these two compounds. Conclusion: C2 and C12 are markedly cytostatic and cytolytic to glioblastoma cells and act through different pathways.

Glioblastoma represents the most common type of primary tumour of the central nervous system and has a poor prognosis (less than 12 month), requiring a multidisciplinary approach, including surgery, radiotherapy and chemotherapy (1, 2). The classical regimen is radiotherapy and chemotherapy using temozolomide. After disease relapse, some patients can benefit from new schedule, such as inhibitors of topoisomerase I and antiangiogenic in combination, which lead to a moderate increase of survival (3). In this context, the need for new drugs is still highly apparent. For this reason, we investigated the antitumour activity of chalcone-like compounds on a human glioblastoma cell line (LN229).

Chalcones are naturally occurring compounds belonging to the flavonoid family. They have been investigated in different pharmacological areas, especially for their anti-proliferative effect (4-9). The antitumour activity of chalcones has been found to be dependent on the presence, the number and the positions of methoxyl groups substituted at both A and B -rings. Recently, we reported the anticancer activity of diversely substituted chalcones on different cellular models (6, 9-11). Indole-containing compounds are known for their antitumour activity and some such molecules are clinically used as major anticancer drugs (12). In the search for new potential anticancer agents, we report the investigation of a series of 17 chalcone derivatives. After primary screening measuring the cell cycle arrest on LN229 cells, two chalcone derivatives, namely C2 and C12, were selected and their activity tested using various targets including cell proliferation, the cell cycle, mitotic index and cell death.

Materials and Methods

C2 and C12 synthesis. All the compounds were obtained from the laboratory of Pharmacology of the Grenoble University. To synthesize C2 and C12 compounds, a solution of 2',4',6'-trimethoxyacetophenone (for C2) or 2',4'-dimethoxyacetophenone (for C12) in a mixture of 1:1 EtOH: KOH (from 50% in water) (10 mL/mmol) was treated with 6-methoxy-3-nicotinaldehyde (for C2) or N-ethylpyrrol-2-carboxaldehyde (for C12). The solution was stirred at 60˚C overnight, then, EtOH was evaporated under reduced pressure. The crude was diluted in water then neutralized by adding HCl (10%) until reaching a pH~6. The solution was extracted with ethyl acetate and the organic layer was separated, washed with water, dried over sodium sulfate and evaporated. The crude product was purified by chromatography column on silica gel eluted with cyclohexanecetyl acetate (2:1) to provide C2 and C12 as pure compounds as evidenced by nuclear magnetic resonance and mass spectrometry analysis. The dry compounds were stored at room temperature in the dark until use.

Human glioblastoma cell line culture. Human glioblastoma-derived cell line LN229 was obtained from the German Collection of Human and Microorganisms and Cell Cultures (Braunschweig, Germany).
Cells were maintained in RPMI-1640 medium with 10% (v/v) inactivated foetal calf serum (FCS) (Gibco BRL, Eragny, France), antibiotics (penicillin 100 IU ml⁻¹ and streptomycin 100 μg ml⁻¹), and L glutamine (2 mM) (Roche, Meylan, France). Cells were seeded at 0.3x10⁶ cells ml⁻¹ for different times (24 to 72 h) in the presence of different doses of the compound (5 to 50 μM). In control culture, cells were cultured in presence of dimethylsulfoxide (DMSO) at 0.5% (control vehicle).

**Proliferation, apoptosis and cell cycle analysis.** The number of total and viable cells was determined using Trypan blue (0.4%) exclusion in triplicate then confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described by the manufacturer (Sigma Aldrich, l’Isle d’Abeau, France) by using 5 to 50 μM of C2 or C12 for 24 to 72 h. Viability was confirmed using acridine orange method measured using a flow cytometer (FACS Canto II; Becton Dickinson, La Jolla, CA, USA) and analysed using FACS DIVA software (BD Sciences, Le Pont de Claix, France) (13). Cell DNA content was analysed using the CycleTest™ PLUS/DNA reagent kit (BD Sciences, San Jose, CA, USA) and apoptosis was confirmed using the annexin V-(FITC) (PI) method (Vybrant™ Apoptosis Assay kit, Molecular Probes, Eugene, OR, USA). Both were measured by flow cytometric analysis as previously described (10).

**Mitotic index and microphotography.** After exposure, cells were trypsinized then washed twice in phosphate-buffered saline (PBS). Mitotic index (MI) was determined by using cells fixed on glass slides by cytospin apparatus (Cytospin2; Shandon, Pittsburgh, PA, USA) with further May-Grunwald-Giemsa staining. The MI was determined as the percentage of mitotic figures of 200 cells from each sample with conventional morphological analysis using a Zeiss microscope (Oberkochen, Germany), as previously described (10). Photomicrographs were taken using a Canon EOS400 digital camera on a Zeiss microscope.

**Drug cytotoxicity measurements in tumor spheroids.** The tumour spheroids (TS) were prepared in a 96-well uncoated U-bottom tissue culture plate with low evaporation lid (Microtest™; Becton Dickinson) to allow TS to form. LN229 cells (taken from exponentially growing cultures) were cultured in EBM-2 medium (Clonetics®; Lonza, Walkerville, MD, USA) supplemented with 5% FCS, EGM-2MV (Lonza) and 0.2% (w/v) methylcellulose. Cell suspension (100 μl, 3x10³ cells) was placed in each well then the tissue culture plates were incubated at 37°C in 5% CO₂ for 3 days before the addition of the compounds. C2 and C12 were prepared in EB2 medium at the desired concentration to give a final concentration range of 10 μM to 50 μM. TS were grown for 10-12 days in normoxic conditions and the diameter of the TS was measured daily using a graduated calibrated inverted microscope (CK2, Olympus, Japan).

**Analysis of reactive oxygen species (ROS) generation.** LN229 cells were cultured in presence of each compound for 48 to 72 h, trypsinized, washed twice in PBS then used for the determination of mitochondrial ROS. Briefly, 10⁵ cells in PBS with MgCl₂ and CaCl₂ containing glucose (20 mM) and sodium azide (3 mM) were incubated for 15 min at 37°C in the dark in the presence of dihydrodihoradamine-123 (DHR; final concentration 50 μM). DHR is non-fluorescent and is oxidized to fluorescent rhodamine-123 by various ROS. Cell fluorescence intensity was measured using FACS Canto II cytometer. To confirm ROS generation, cells were cultured in the presence of N-acetylcysteine (NAC), an ROS scavenger, at 5 mM for 4 h prior to the addition of C2 or C12.

**Results**

Among 17 molecules tested and after 24 h exposure at 10 μM, only two compounds, C2 and C12, were found to arrest cells in G2/M phase as evidenced by accumulation of cells with 4n DNA content which was associated with an increase of the MI in the case of C12 (Table I). These results suggest that compound C2 blocks the cell cycle in the G2 phase, whereas compound C12 blocks it at the mitosis phase. LN229 cells were then cultured in the presence of different doses of C2 and C12 (5 to 50 μM) and for different incubation times (from 24 to 72 h). MTT test showed that the two compounds inhibited cell proliferation in a time- and dose-dependent manner (Figure 1). All the investigations were then carried out with a 20 μM concentration of compound and for different incubation times.

When LN229 cells were treated with compound C2, the percentage of cells in the G2 phase remained unchanged from 24 to 72 h (Figure 2) whereas the percentage of viable cells as determined by the Trypan blue exclusion (Table II) and acridine orange methods (Figure 3), decreased by less than 10%. However, in the same time, the percentage of cells in the sub-G₀/G₁ phase of the cell cycle did not change (Figure 2) suggesting a death process different from apoptosis. On the other hand, the percentage of early apoptotic and end-stage apoptotic/necrotic cells as determined by annexin V/FITC method increased to 90% (Figure 3). In all the cells treated with compound C2, the formation of vacuoles was observed and the nucleus condensed (Figure 2, left panel). All these results suggest that C2 was able to induce LN229 cell death via several mechanisms, including apoptosis and an autophagy-like process.

From 24 to 72 h of C12 treatment, the percentage of cells in the G2/M phase increased up to 24 h then did not statistically change up to 72 h, whereas the percentage of cells showing mitotic figures increased up to 24 h then strongly decreased to fewer than 2% at 72 h (Table II). This result suggests that C12 blocks cells in the M phase then in the G2 phase. However, after 48 h exposure, a sub-G₀/G₁ and a sub-G₂ phase appeared in the cell cycle suggesting an apoptotic process of the cells with 2n and 4n DNA content (Table II, Figure 2). On the other hand, the percentage of early apoptotic and end-stage apoptotic/necrotic cells as determined by annexin V/FITC method increased in a time-dependent manner to 40% (Table II, Figure 3). Cell morphological analysis confirmed the presence of apoptotic figures (Figure 2, left panel). Moreover, the effect of C2 and C12 on TS growth is dramatic (Figure 4). The TS control displayed sustained, exponential growth up to 10 days, when the spheroid diameter was 550 μm. In comparison, C2 and C12 inhibited the growth of the TS, although an increase in...
spheroid diameter was shown up to 48 h, probably in relation to the arrest of cells in the G2/M phase of the cell cycle.

On the other hand, C2 increased the rhodamine-123 fluorescence and NAC inhibited C2-induced cell death and reduced rhodamine-123 fluorescence. Similar results were observed in cells treated with the compound C12, although to a lesser extent (Table III). These findings indicate that C2- and probably C12-induced cell death is, in part, regulated by an ROS-mediated signalling pathway.

Discussion

Glioblastomas are highly aggressive tumours and their treatment is extremely challenging due to the resistance of these cells to apoptosis and the invasiveness of residual cells. The search for new therapeutic drugs able to overcome the resistance of glioblastoma cells to apoptosis is a worthwhile goal. In our investigation, the key finding is that among 17 chalcone derivatives tested, two, C2 and C12, inhibit cell proliferation, modulate the cell cycle and induce the cell death of LN229 glioblastoma cells. However, the mechanisms of how the two compounds affect these cell characteristics, are still unclear.

Table I. Chalcone structure activity relationship. LN229 glioblastoma cells were cultured for 24 h in RPMI-1640 with 10% foetal calf serum before the addition of compounds (10 μM). At the indicated time, cells were trypsinized, washed twice then used for the determination of several parameters. At 24 h induction time, cell cycle analysis using the CycleTest™ PLUS/DNA reagent kit and the mitotic index (MI) were determined. At 72 h induction time, the cell viability was determined using the acridine orange method. Results are expressed as a percentage of positive cells and are the mean±SD of three experiments.

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Figure 1. Effect of C2 and C12 on cell growth. LN229 glioblastoma cells were cultured for 24 h in RPMI 1640 + 10% FCS before the addition of either C2 (△) or C12 (■) for 24 h (-----), 48 h (— — —) and 72 h (-----) and with different doses (10, 20 and 50 μM). At the indicated time, MTT test was performed. Data are the results (mean±SD) of two experiments.
Figure 2. Effects of C2 and C12 on LN229 cells. LN229 cells were cultured with 0.5% DMSO (vehicle) (T) or with compound C2 (C2) or C12 (C12) both at 20 μM for 24 to 72 h. Left panel: Microphotography (48 h). In untreated cells (T), fewer than 3% of the cells showed mitotic figures, whereas in C12 treated cultures (C12), 30 to 40% of the cells showed mitotic figures (dotted arrows) and numerous cells showed apoptotic figures (arrows). In cells treated with compound C2, no mitotic figure was found but the formation of vacuoles (arrows) was observed. Right panel: Cell cycle distribution. After 24 and 72 h exposure, cell cycle distribution was analysed using the CycleTestTM PLUS/DNA reagent kit. Arrows: apoptosis.

Figure 3. Effects of C2 and C12 on viability and apoptosis. LN229 tumour cells were cultured for 24 h in RPMI-1640 with 10% foetal calf serum before the addition of compounds (20 μM). After 48 or 72 h of exposure, the cells were trypsinized, washed twice then used for the determination of either the cell viability using acrine orange method or apoptosis and necrosis using annexin V/FITC-PI kit. Fluorescence intensity was measured using a FACSCanto II and analysed using FACS DIVA software.
If we take compound C12, our results suggest that this compound acts through a complex mechanism. It appears to block cells at two phases of the cell cycle. C12 blocks the cells in the last phase of the mitotic process (telophase) possibly via activity on tubulin, acting as a microtubule depolymerising agent (10, 12). These cells died through an apoptotic process. This phenomenon may explain the decrease of the MI from 24 to 72 h. The fact that the percentage of apoptotic cells was correlated to the MI (R=0.90) (data not shown) would appear to confirm our hypothesis. These results suggest that glioblastoma cells treated with C12 undergo cell death through both microtubule damage as demonstrated by the increase of the MI and mitotic catastrophe. C12 also blocks proliferating cells in the G2 phase via a tubulin-independent process. Only a small proportion of these cell died through an apoptotic/necrotic process (sub-G2 phase), without any correlation between the percentage of apoptotic cells and the percentage of cells arrested in the G2 phase (data not shown). The fact that only some of the cells died may explain the cell proliferation that continued after the arrest of the C12 treatment (data not shown). This latter process, not related to a microtubule-damaging mechanism, suggests that C12 may be involved in the control of the cell cycle process.

On the other hand, how C2 blocks the cell cycle in the G2 phase then induces cell death, is not fully understood. Based on recently reported results regarding flavonoids (structurally similar analogues), it has been shown that these compounds exert cell death in the absence of caspase-3 activation and typical apoptotic morphological features (15). In addition, in the cells treated with C2, the formation of vacuoles was observed, suggesting the induction of an autophagy-like phenomenon. Recently, Kuo et al. (16) showed that flavokawain B, a chalcone precursor, induced G2/M accumulation and, in addition to apoptosis, induced autophagy, suggesting that this compound activated multiple...
pathways to inhibit cell growth and induced cell death. Flavokawain B and C2 are chemically very similar: both possess the same A-ring and this could explain part of their effects on cell growth and cell death. It is also possible that C2 induced cell death through alternative (non-apoptotic) cell death pathways, including either a death associated with the accumulation of autophagosomes (17), or a caspase-independent cell death (oncrosis, necroptosis, paraptosis or methuosis) as recently described (18-21). Our results also indicate that C2- and probably C12-induced cell death is, in part, regulated by an ROS-mediated signalling pathway.

In summary, we selected two chalcone derivatives and studied their antiproliferative profile on the LN229 glioblastoma cell line. Taken together, our results show that the two compounds (C2 and C12) are markedly cytotstatic and cytolytic to glioblastoma cells and act through multiple pathways, including G2 and M accumulation, mitotic catastrophe, intracellular ROS generation, apoptosis and probably an autophagy-like process. An exciting finding of this study is that although C2 has a chemical formula closely related to C12, however the latter kills cells by using an apoptotic process, whereas C2 so preferentially using an autophagy-like process. Nevertheless, further investigations are necessary to understand the exact mechanisms controlling the activity of these two compounds and, due to the fact that the glial cells are protected by the brain-blood barrier, it will be interesting to analyze their effects in vivo on xenografts in nude mice.

References