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# Apoptosis of human malignant glioma-derived cell cultures treated with clomipramine hydrochloride, as detected by Annexin-V assay

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***Background.*** *Previous research in our laboratories has shown that Clomipramine Hydrochloride (CLOM), a tricyclic antidepressant in use for over thirty years, selectively kills neoplastic glial cells in vitro whilst leaving normal brain cells unaffected. The purpose of this study was to evaluate whether a range of early passage cell cultures and established cell lines, derived from a number of patients with malignant glioma, would display different sensitivities when exposed to CLOM. The particular assay of interest, following our discovery that CLOM targets the mitochondria of tumour cells and triggers Caspase 3 mitochondrially-mediated apoptosis, was Annexin-V flow cytometry. This assay was used to determine the mechanism of cell death, either necrosis or apoptosis, according to drug concentration and period of incubation.*

***Method.*** *Cells grown to 90% confluence in 25cm$^3$ flasks were incubated with concentrations of CLOM from 20μM – 100μM, for up to 6 hours. Cells were harvested and resuspended in calcium binding buffer, which triggers translocation of calcium-regulated phosphatidylserine residues to the nuclear envelope, before removing 500μl of the single cell suspension to a Facs tube. Controls used in the analysis were performed by omission of the drug incubation in one flask, and addition of 1μM staurosporine to one flask. These served as negative and positive controls respectively. Annexin-V FITC and propidium iodide were added to all tubes and incubated for 15 minutes at room temperature, in the dark. Subsequent to this, binding buffer was added to each tube and analysed using a BD FACScalibur.*

***Results.*** *Results show that, of the five malignant gliomas tested, the two established cell lines had the lower apoptotic threshold, with a significant percentage of apoptotic cells present at 60μM and above when compared to the control sample. The three early passage cultures, developed 'in house' from biopsy, had higher apoptotic thresholds, withstanding up to 100μM CLOM incubation for six hours. Normal human astrocytes were assayed in parallel, and show that CLOM does not cause cell death at the concentrations tested.*

***Conclusions.*** *It may be possible, in a larger study, to predict individual patient response to CLOM using the Annexin-V assay, alongside Bcl-2 analysis and CYP gene testing, on the individual patient's tumour cells. The difference in sensitivities between glioma, in this small study, indicates the importance of analysing early passage cultures, which retain original morphology and characteristics to a greater extent, alongside established cell lines.*

*Key words: annexin V; apoptosis; brain neoplasms - drug therapy; glioma; clomipramine; tumour cells, cultured*

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## Introduction

Previous research in our laboratories has shown that Clomipramine Hydrochloride (CLOM), a tricyclic antidepressant in use for over thirty years, has the ability to induce apoptosis in malignant glioma cells *in vitro*.[1] Thus, following the initiation of a clinical trial based at King's College Hospital, London (LREC 01-235), it is important to be able to predict the outcome of a given drug treatment, especially in the case of glioma which are often characterised by a poor prognosis.

Cell death is often defined as occurring by either apoptosis or necrosis. Whilst necrosis is a relatively passive process, involving loss of membrane integrity and cell membrane rupture, which leads to the release of intracellular debris and eventual inflammatory response, apoptosis is an active process resulting in cell shrinkage, plasma membrane blebbing and chromatin condensation which produces apoptotic bodies, rapidly recognised and phagocytosed by macrophages. This 'clean' mechanism of cell death, avoiding an inflammatory response, ensures minimal tissue damage to the surrounding brain parenchyma.

Apoptosis after CLOM treatment is associated with the intrinsic pathway of mitochondrial cytochrome C release[1,2] following a rapid increase in p-c-Jun[2], and activation of caspase-3.[3] The two main pathways of apoptosis can be identified in mammalian cells, both are controlled by caspases and eventually converge on 'executioner' caspase-3[4], which is responsible for the cleavage of structural cytoplasmic and nuclear proteins, with consequent cell death and collapse.[5]

Phosphatidylserine (PS), made by the two PS synthases PSS1 and PSS2, is normally located on the inner leaflet of the plasma membrane, but undergoes transbilayer movement during apoptosis and becomes exposed on the cell surface.[6] Annexin-V FITC (BD Biosciences) binds to the exposed PS residues in a calcium dependent manner, after a rise in nuclear calcium concentration that causes the translocation of the calcium regulated proteins to the nuclear envelope.[7] This mechanism, usually a pivotal step in the recognition and removal of apoptotic cells by phagocytes[8], allows the attachment of the Annexin V-FITC antibody and allows us to visualise apoptotic cells via flow cytometry.

The purpose of this study was to evaluate whether a range of both early passage cultures and established cell lines, derived from patients with malignant glioma, would display different sensitivities, with regard to apoptotic cell death, when exposed to CLOM. This, combined with other assays previously carried out by the research group, could go some way towards defining *in vitro* markers for patient response to CLOM.

## Materials and methods

*Cells; the following cell cultures were used:*

SNB-19 – an established glioblastoma muetiforme GBM (grade IV) cell line p20-24, derived from a 47-year old male (DSMZ Cell Bank)

DK-MG – an established GBM (grade IV) cell line p23-27, derived from 67-year old female (DSMZ Cell Bank)

UPAB – a primary GBM (grade IV) cell culture set up 'in house', p11-14, derived from a 73 year-old male

UPMC – a primary GBM (grade IV) cell culture set up 'in house', p9-12, derived from a 69 year-old female

UPJM – a primary astrocytoma (grade II) cell culture set up 'in-house', p7-10, derived from 42 year-old male

CC-2565 – a normal human astrocyte cell line, p4-6, derived from an 18 year-old male (Cambrex Biosciences).

*Annexin V analysis*

The apoptosis assay was used to determine the mechanism of cell death according to drug concentration and period of incubation. Cells grown to 90% confluence in $25cm^3$ flasks were incubated with 10X concentrations of CLOM (20, 40, 60, 80 & 100$\mu M$), added to flasks at 1:10, for up to 6 hours. Cells were harvested by firstly removing the complete media to centrifuge tubes (to ensure that all cells are subject to analysis), before adding 1.0ml of clear Tryplexpress (a non-enzymatic rapid dissociation solution; Gibco). During the two-minute dissociation period, flasks were placed in the incubator (37°C, 5% $CO_2$) to maintain the optimum temperature. The Tryplexpress was removed by centrifugation at $200g_{av}$ after neutralisation with 10% complete media.

Following staining the cell pellet was resuspended in 1ml of 1X calcium binding buffer, which triggers translocation of calcium-regulated phosphatidylserine residues to the nuclear envelope, before removing 500µl of the single cell suspension to a FACS tube. Controls used in the analysis were performed by omission of the drug incubation in one flask, and addition of 1µM staurosporine to one flask. These served as negative and positive controls respectively. Five microlitres of annexin V FITC and 5µl of propidium iodide were added to all tubes, by placing a drop of the fluorochrome on the side of the tube and inverting it. The tubes were incubated for 15 minutes at room temperature, in the dark. Subsequent to this, 400µl of binding buffer was added to each tube and analysed by the BD FACScalibur within 1 hour.

## Results

After a six-hour incubation with CLOM the cell lines/cultures undergoing a marked degree of apoptosis, when compared against negative controls were DK-MG and SNB-19. Because of the slow-growing nature of the lower grade astrocytoma UPJM, some samples were not achieved due to lack of cells (20,000 minimum required for analysis). Although the percentage of apoptosis in UPMC appears high, when compared to the control values it demonstrates that CLOM does not exert any effect at the concentrations tested (see Table 1 and Figure 1).

From the five malignant gliomas tested, the two established cell lines had the lower apoptotic threshold, with a significantly higher percentage of apoptotic cells present at 60µM CLOM and above. The three early passage cultures, developed 'in-house' from biopsy, had higher apoptotic thresholds, withstanding up to 100µM CLOM incubation for six hours.

The normal human astrocytes, tested in parallel, demonstrated that CLOM did not cause cell death at the concentrations tested.

The cells at the highest passage number (DK-MG) are the most responsive to Clomipramine in this assay; this could be due, in part, to the homogeneity of the sample population. Also, it is of interest to note that the CLOM was less effective at causing apoptosis in the CC-2565 cell line than the staurosporine in the positive control sample (Table 1).

| | | SNB-19 | DK-MG | UPAB | UPMC | UPJM | CC-2565 |
|---|---|---|---|---|---|---|---|
| | | | | *Apoptosis (%)* | | | |
| ***Control Sample*** | | *1.25* | *2.05* | *2.27* | *10.77* | *3.03* | *0.36* |
| **Staurosporine (6hr control)** | | *21.47* | *71.56* | *5.04* | *47.43* | *38.94* | *1.94* |
| **20μM** | 6h | 1.25 | 1.87 | 2.35 | 9.96 | 1.01 | 0.29 |
| | 5h | 0.92 | 1.50 | 3.21 | 10.81 | 3.20 | 4.68 |
| | 4h | 0.95 | 1.38 | 2.11 | 12.74 | 0.50 | 3.21 |
| | 3h | 1.29 | 1.18 | 3.00 | 11.72 | 0.88 | 2.51 |
| | 2h | 0.71 | 1.40 | 1.87 | 10.99 | 0.24 | 4.12 |
| | 1h | 1.45 | 1.03 | 1.46 | 10.13 | 1.31 | 2.98 |
| **40μM** | 6h | 3.67 | 1.95 | 2.84 | 11.67 | - | 3.62 |
| | 5h | 2.69 | 2.64 | 3.45 | 13.32 | - | 3.28 |
| | 4h | 2.13 | 1.30 | 4.19 | 14.11 | - | 2.92 |
| | 3h | 2.11 | 5.39 | 2.93 | 12.41 | - | 1.55 |
| | 2h | 2.64 | 4.26 | 3.00 | 12.31 | - | 3.74 |
| | 1h | 2.72 | 3.14 | 4.01 | 14.03 | - | 3.32 |
| **60μM** | 6h | 4.84 | **23.27** | 3.86 | 14.26 | - | 2.86 |
| | 5h | 5.14 | **21.44** | 3.40 | 12.16 | - | 0.03 |
| | 4h | **11.50** | **51.25** | 2.99 | 12.80 | - | 5.65 |
| | 3h | **10.98** | **18.75** | 3.71 | 13.62 | - | 0.32 |
| | 2h | **5.9** | **26.53** | 3.66 | 12.33 | - | 4.49 |
| | 1h | **5.31** | **19.75** | 3.08 | 13.47 | - | 3.52 |
| **80μM** | 6h | **5.89** | **27.13** | 3.00 | 14.22 | 12.32 | 0.23 |
| | 5h | **15.91** | **20.86** | 4.36 | 14.02 | 10.00 | 1.65 |
| | 4h | **10.55** | **38.92** | 3.18 | 15.88 | 10.47 | 0.15 |
| | 3h | **4.98** | **42.25** | 3.31 | 15.82 | - | 0.05 |
| | 2h | **9.18** | **23.76** | 2.51 | 13.19 | - | 0.02 |
| | 1h | **10.27** | **23.41** | 3.71 | 14.59 | - | 0.00 |
| **100μM** | 6h | **11.03** | **49.16** | 3.91 | 10.97 | 10.73 | 0.36 |
| | 5h | **2.57** | **23.18** | 2.77 | 12.91 | 8.64 | 0.01 |
| | 4h | **10.91** | **22.81** | 2.97 | 11.64 | 5.99 | 3.21 |
| | 3h | **12.58** | **17.74** | 4.98 | 11.94 | 10.28 | 0.04 |
| | 2h | **17.13** | **20.66** | 4.68 | 10.76 | 6.50 | 0.05 |
| | 1h | **11.00** | **30.84** | 2.79 | 9.41 | - | 0.38 |

**Table 1.** A summary of the apoptosis data obtained by Annexin V flow cytometry, highlighted are the samples at which apoptosis (defined as a sample with more apoptosis than the negative control) was achieved when compared to the negative and positive controls, which were cells with no drug and cells with staurosporine added respectively. This table illustrates the differences in apoptotic sensitivity of the cell lines; with DK-MG being the most responsive when compared to the control values.

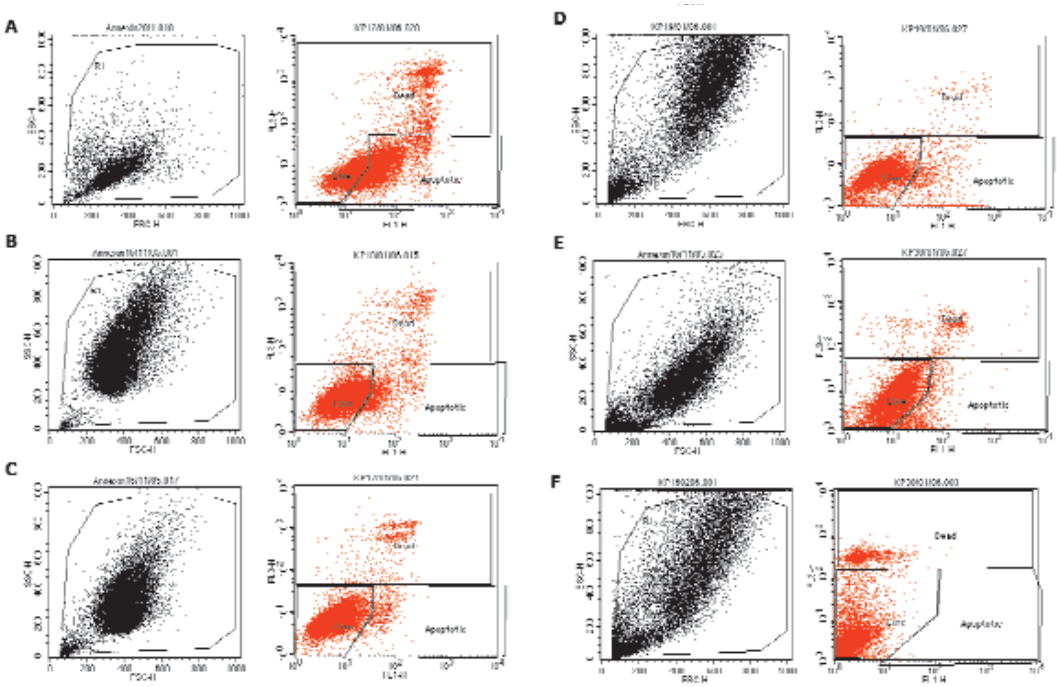


**Figure 1.** Annexin V Data for DK-MG (A), SNB-19 (B), UPAB (C), UPMC (D), UPJM (E) and CC-2565 (F). Plots showing the side scatter and gating selected for this assay (left) demonstrate the difference in cell size and heterogeneity of the samples. The plots of interest shown (right) are samples analysed after a 6-hour incubation with 100ĶM Clomipramine Hydrochloride. The FL-1 detector detected annexin-V binding; the FL-2 detector detected cells counterstained with isotonic propidium iodide. The percentage values (%) for apoptotic and dead cells, respectively, are as follows A = 49.16; 13.23, B= 11.03; 5.96, C=3.91; 4.76, D=10.97; 2.24, E= 10.73; 4.85, F=0.36; 11.28.

## Discussion

CLOM has previously been reported to exert an apoptotic effect by Xia *et al.*[9], on human myeloid leukaemia HL-60 cells (50μM), and Levkovitz *et al.*[2] on C6 glioma cells (25μM) and human neuroblastoma SH-SY5Y cells (20μM). Significant morphological changes following incubation with 12μM CLOM, represented by red (propidium iodide) fluorescence of fragmented apoptotic nuclei, were observed by Levkovitz *et al.*[2] when compared to blue (hoechst) fluorescence of the intact nuclei treated with vehicle (saline). Similar morphological findings were presented by Daley, E[10], when human malignant glioma cells were incubated with CLOM (maximum incubation period of 4 hours) and subsequently stained with ethidium bromide and acridine orange. Internucleosomal DNA fragmentation measured by electrophoresis in glioma cell lines was also demonstrated by Daley, E[10], confirming DNA laddering and hence condensation of chromatin, the 'classic' hallmark of apoptosis. These findings, from the literature on CLOM, confirm the potent apoptotic effect that CLOM has on tumour cells. They also observe the higher resistance of primary cell cultures[11] which can be accounted for by the high proportion of non-neoplastic cells maintained in these short-term, low passage, cultures.

This was reflected in the results of this study, whereby the control normal human

brain astrocytes (CC-2565) were unaffected by CLOM. It is tempting to postulate that a population of normal astrocytes remains in the primary cultures developed 'in-house'; further subculturing (leading to increased homogeneity of cell populations) and analysis may reveal passage-dependent apoptotic sensitivity to CLOM.

It may be possible, in a larger study, to predict individual patient response to CLOM using the Annexin-V assay, alongside Bcl-2 analysis and CYP gene testing, on the patients own tumour cells. Bcl-2 analysis, performed previously in our laboratories by Daley, E[10] demonstrated that Bcl-2 expression correlated with apoptotic rate. Bcl-2 prevents cytochrome C release, and hence glioma cell lines expressing a high percentage of endogenous Bcl-2 had the lowest apoptotic rate.

A common clinical observation within a patient cohort, diagnosed with the same tumour type, is the appearance of a few 'responders' who respond very well to the test agent, and a majority of non-responders who do not gain any advantage from the test agent.[12] One explanation for this could be the multidrug-resistant phenotype of brain tumours; Andersson *et al*.[13] found a large heterogeneity in the expression of different resistance markers (P-glycoprotein, PgP; Multidrug resistance protein, MRP1; lung-resistance related protein, LRP and O(6)-methylguanine-DNA methyltransferase, MGMT). The next steps in this research are to combine CLOM with other potentially synergistic agents to enhance to apoptotic effect. It may also be possible to isolate cancer stem cells and/or other 'clones' from heterogeneous primary glioma to test the resistance of subpopulations. The significance of further studies on Bcl-2, which acts upstream of the mitochondria, is that the expression may enhance the survival of cancer cells.[3] The difference in sensitivities between glioma, in this small study, indicates the importance of analysing early passage cultures, which retain original morphology and characteristics to a greater extent, alongside established cell lines.[14]

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