Selective Serotonin Reuptake Inhibitors Directly Signal for Apoptosis in Biopsylike Burkitt Lymphoma Cells

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Summary
Selective serotonin reuptake inhibitors (SSRI) are the treatment of choice for clinical depression and a range of anxiety-related disorders. They are well tolerated over extended periods with more than 50 million people worldwide benefiting from their use. Here we show that three structurally distinct SSRI – fluoxetine (Prozac®), paroxetine (Paxil®), and citalopram (Celexa®) – act directly on Burkitt lymphoma (BL) cells to trigger rapid and extensive programmed cell death. SSRI unexpectedly stimulated calcium flux, tyrosine phosphorylation, and down-regulation of the c-myc and nm23 genes in Burkitt lymphoma cells remaining faithful to the biopsy phenotype. Resultant SSRI-induced apoptosis was preceded by caspase activation, PARP-1 cleavage, DNA fragmentation, a loss of mitochondrial membrane potential, the externalization of phosphatidylserine, and reversed by the over-expression of bcl-2. Normal peripheral blood mononuclear cells and tonsil B cells, whether resting or stimulated into cycle, were largely resistant to SSRI-induced death as were five non-BL lymphoid cell lines tested. We discuss these findings within the context of whether the SSRI class of antidepressants could find future application as potential therapeutics for the highly aggressive and – due to its association with AIDS – increasingly more common Burkitt lymphoma.

Introduction
Burkitt lymphoma (BL) is a highly malignant tumor characterized uniquely by a 100 percent mitotic index among the constituent cells. The tumor – which tends to develop extra-nodally, with a preference for the jaw and abdomen – can double in size within a day and remains a serious health problem in those areas where it is endemic: namely, the malarial belts of equatorial Africa, North-eastern Brazil, and Papua New Guinea.
often limited medical resources in these regions can compromise the survival rates which can be achieved with current protocols of aggressive combination chemotherapy continued over several months and requiring frequent hospitalisations\(^3\). Outside of endemic regions, the incidence of Burkitt lymphoma has increased dramatically in the past two decades due to its association with HIV infection such that within North America, for example, there is a 1000-fold increased incidence among individuals with AIDS\(^4,5\). The median survival of HIV-positive patients with non-Hodgkin's lymphomas, of which BL comprises some 20%, is only 6 months\(^6\). Even among HIV-negative individuals where there is a 3-year relapse-free survival rate of approximately 80% for BL patients with local disease, those with disseminated tumor respond poorly to chemotherapy and have lower survival rates\(^3\).

Irrespective of geographical origin or HIV-association, BL is characterised by translocations of one c-myc allele to one of the immunoglobulin loci and the extraordinarily high growth rate that characterises these tumours reflects the pro-proliferative actions of the translocated gene\(^7-9\). However, despite translocation to immunoglobulin loci, the BL-associated constitutive expression of c-myc remains dependent on the binding of the recognised c-myc transcription factor Nm23-H2 to a PuF site within the regulatory sequence of the translocated gene\(^10\). A recent report has identified that expression of Nm23-H1 may also be linked to c-myc expression and the high expression of this gene has been correlated with poor responses to treatment in high-grade lymphoma in general\(^11-12\). Deregulated c-myc expression likely contributes to what appears an apparent dichotomy in the pathobiology of BL: namely aggressive,
uncontrolled proliferation coupled with high rate apoptosis. The latter attribute gives rise to the classical “starry sky” histology that characterizes BL, this reflecting the presence of large tingible body macrophages that have been mobilized to the tumor in order to clear the large burden of apoptotic cells generated at these sites\(^1,13,14\). Reflecting their normal physiological counterpart – the B cell proliferating in the germinal centers of secondary lymphoid tissues – BL cells \textit{in situ} are negative for, or only weakly express, the anti-apoptotic survival gene \textit{bcl-2}\(^14-17\). This feature is retained in BL lines held in early passage. Indeed, “biopsylike” (so-called “Group I”) BL cell lines stay remarkably faithful to the \textit{in situ} phenotype and thereby provide excellent models for examining treatments that might serve to shift the damaging imbalance between proliferation and apoptosis in the tumor in such a way as to favor the latter\(^18-19\).

We have recently reported on the serotonin transporter (SERT) as an unexpected candidate target for therapeutic attack in Burkitt lymphoma\(^20\). BL cells were shown to carry immunoreactive SERT and to transport 5-HT (serotonin) with appropriate first order kinetics. Moreover, a capacity for 5-HT to drive rapid and extensive apoptosis in biopsylike BL cells was largely reversed by the class of antidepressants known as the Selective Serotonin Reuptake Inhibitors (SSRI) that act by blocking the active uptake of 5-HT into SERT-carrying cells\(^20\). We now report the surprising finding that the SSRI themselves are capable of provoking apoptotic death in biopsylike BL cell lines.
Material and methods

Cells and cell lines

Early passage Group I BL lines L3055 (EBV−ve), Namalwa (EBV−ve), BL2 (EBV−ve), Elijah (EBV−ve), and Mutu I (EBV+ve), the late passage Group III BL line Mutu III (EBV+ve), NALM6 (“pre-B”), RPMI 8226 (“plasmacytoid”) and three T cell lines – Jurkat, J10 and CEM – were maintained in RPMI 1640 medium supplemented with 10% Serum Supreme (BioWhittaker, Wokingham, UK), 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin as previously described. Stable bcl-2 transfectants of L3055 cells, together with those carrying the mammalian expression vector pEF-MC1 neopA alone as controls, were as detailed elsewhere. Resting B cells were isolated by negative depletion of tonsilar mononuclear cells using a magnetic cell separator to remove those bearing CD3, CD14 or CD38 exactly as described previously.

Reagents

Fluoxetine (Prozac®), 5-HT, fura-2, thapsigargin, and Ca2+-free (low) medium were purchased from Sigma (Dorset, UK). Paroxetine (Paxil®) and citalopram (Celexa®) are from Smith-Kline Beecham (Harlow, Essex, UK) and Lundbeck (Copenhagen, Denmark) respectively. Affinity-purified goat F(ab’)2 antibody fragment to human IgM was purchased from ICN (Ohio, USA). Soluble recombinant human CD40 ligand (CD40L) was kindly provided by R.J. Armitage (Immunex Corporation, Seattle, USA). Staphylococcus aureus Cowan 1 (SAC) and ionomycin were from CN Biosciences (Nottingham, UK). The JC-1 (5,5’6,6’-tetrachlorol, 1’,3’,3’-tetraethylbenzimidazolyl-
carbocyanine iodide) cationic dye and Syto 16 was purchased from Molecular Probes (Leiden, Holland). The 4G10 anti-phosphotyrosine antibody was from Upstate biotechnology. Murine antibody to Bcl-2 (clone 124) was from Dako (Ely, UK), PARP-1 antibody (clone A6.4.12) was from Insight Biotechnology (Wembley, UK). The supersignal chemiluminescence reagent and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody were from Pierce (Chester, UK). All other chemicals were obtained from Sigma (Poole, UK), and were of the best grade available. Where reagents were added to cell culture, the appropriate vehicle to the same dilution, was used as control.

**Assessment of DNA synthesis and cell cycle status**

DNA synthesis was determined by measuring $^3$H-thymidine ($[^3$H]Tdr; Amersham, UK) incorporation into cellular DNA as documented elsewhere.$^{21}$ Cell cycle analysis of propidium iodide (PI) stained cells was performed exactly as described previously with results expressed as percentage of viable cells residing in each phase of the cycle.$^{24}$

**Viability assays**

Changes in the viability of cells cultured under conditions indicated were quantified by assessment of forward and 90° (side) light scatter of cells using an EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA) with cells being gated into two populations (viable and dead) as described elsewhere.$^{20}$ Cells were also analysed in order to assess the percentages within treated populations of those that were viable, apoptotic, or necrotic as described in Milner et al.$^{25}$ Analysis by FACS generates a 2D-plot of syto
16 fluorescence versus PI fluorescence: syto 16 is taken up only by viable cells whereas PI exclusively enters necrotic cells - syto 16<sup>-ve</sup>/PI<sup>-ve</sup> cells are deemed apoptotic<sup>20,25</sup>.

**Measures of apoptosis**

Apoptosis was assessed by staining cells with acridine orange and visualizing nuclear morphology exactly as described previously<sup>24</sup>. Viable cells display a homogeneous chromatin-staining pattern whereas apoptotic cells characteristically show condensed and fragmented chromatin. Mitochondrial depolarisation was assessed using the JC-1 cationic dye<sup>20</sup>. JC-1 exhibits potential-dependent accumulation in mitochondria accompanied by a shift in fluorescence emission from 525 nm (green) to 590 nm (red). Mitochondrial depolarisation is indicated by a decrease in the red/green fluorescence ratio as described previously<sup>20</sup>. Caspase activity was detected using the CaspaTag Caspace Activity Kit (Intergen, Oxford, UK). Cells cultured under the conditions indicated in the text were stained using FAM-VAD-FMK together with PI (to distinguish dead cells from live cells) then analysed on an EPICS XL-MCL flow cytometer or by confocal microscopy (with the addition of Hoechst 33342 stain) exactly as described previously<sup>20</sup>. Phosphatidylserine exposure was measured using the Annexin V FITC Apoptosis Detection Kit from CN Biosciences (Nottingham, UK) while TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling) was performed using the ApopTag Fluorescein Direct In situ Apoptosis Detection Kit from Flowgen (Ashby de la Zouch, UK), both in accord with the manufacturer’s instructions.

**Calcium measurements**
5 x 10^7/ml cells were resuspended in culture medium, and loaded with 1 μM of fura-2 for 45 min at 37°C. At the end of the incubation they were washed twice with HBSS containing 10% FCS and 10 mM HEPES (pH 7.2), resuspended at 4 X 10^6/ml, and stored on ice until used. Measurements were performed using a Hitachi F2500 fluorescence spectrophotometer, with excitation wavelengths of 340 nm and 380 nm and emission wavelengths of 510 nm. Cells were incubated at 37°C for 5 minutes prior to use. Maximum response was obtained following addition of 0.05% TX100 plus 0.01 mM DETAPAC; minimum values were obtained following addition of 10 mM EGTA plus 30 mM Tris pH 7.2. In preliminary experiments it was established that there was no significant dye leakage for at least 2 h after loading of the cells, and that the SSRI did not exhibit significant autofluorescence.

**Western Blots**

For assessment of tyrosine phosphorylation cells were lysed in RIPA buffer, separated on SDS PAGE, transferred to PVDF membranes, and probed with the 4G10 anti-phosphotyrosine antibody. 25 μg of protein was loaded onto each lane. Blots were developed with an HRP-conjugated anti-mouse secondary antibody and visualised using an enhanced chemiluminescence method. Bcl-2 and PARP-1 blots were performed exactly as described elsewhere.

**Real-time PCR quantification of gene expression**

Reactions were performed using an ABI Prism 7700 sequence detector (Applied Biosystems, CA, USA) with 18S ribosomal RNA as the internal standard. Gene specific
primers were synthesized by Alta Bioscience (University of Birmingham, UK) and probes by Applied Biosystems (Roche, USA). Each reaction contained 900 nM of gene specific 5’ and 3’ primers, 1X Mastermix (containing pre-optimised dNTP, MgCl₂ and buffer concentrations; Applied Biosystems), 125-175 nM gene specific probe (5’-6-FAM, 3’-TAMRA labeled), 50 nM 18S 5’ and 3’ primers and 200 nM 18S probe (5’-VIC, 3’-TAMRA labelled) and 0.25 µl of cDNA (equivalent to 12.5 ng of reverse transcribed RNA), in a total volume of 25 µl. Reactions were replicated 3 times on a 96-well plate format. *nm23-H1* reactions used 5’ primer 5’-GGCCTGGTGAATACATGCA-3’, 3’ primer 5’-GGCCCGTCTTCACCACAT-3’ and probe 5’-6-FAM-CTCCCAGACCATGGCAACTACGG-TAMRA-3’. *nm23-H2* reactions used 5’ primer 5’-CTGGTGGGCGAGATCATCA-3’, 3’ primer 5’-TGTAGTGCTGCTGCTTCAGGT-3’ and probe 5’-6-FAM-CCATGAAGTTCCTCCGGGCCTCTG-TAMRA-3’. *C-myc* reactions used 5’ primer TCAAGAGGTGCCACGTCTCC-3’, 3’ primer 5’-TCTTGGCAGCAGGATAGTCCTT-3’ and probe 5’-6-FAM-CAGCACAACATACGCGCAGCCTCC-TAMRA-3’. Regulation of *C-myc*, *nm23-H1* and *nm23-H2* was compared with that of the house-keeping cyclophilin A. For these assays commercially available primers and probes were used as described by the manufacturers instructions (Applied Biosystems Cat no. 4310883E).

Cycle threshold (Ct) values were obtained graphically for test genes and 18S internal standard. Gene expression was normalized to the 18S and represented as ΔCt values. Comparison of gene expression between control and treated samples was derived from subtraction of control ΔCt values from treatment ΔCt values to give a ΔΔCt value and
relative gene expression was calculated as $2^{\Delta \Delta Ct}$ and normalized to controls. Where statistical comparisons have been made we have used ΔCt values and paired t-Tests.

Results

SSRI inhibit DNA synthesis in biopsylike BL cells

BL cells kept in early passage closely resemble those of the original biopsy. This includes a tendency for background (spontaneous) apoptosis coupled to a remarkably high rate of proliferation\textsuperscript{18,19,27}. Results illustrated in Figure 1(a-c) demonstrate that the chemically distinct SSRI, fluoxetine, paroxetine, and citalopram, each inhibited – in a concentration-dependent fashion – DNA synthesis occurring in the prototypic BL cell line, L3055. The potencies of the SSRI in this regard are lower than their EC\textsubscript{50} for SERT blockade\textsuperscript{28}. Nonetheless, similar inhibition was found in 5/5 distinct BL cell lines held in early passage (Fig. 1d). This included the Mutu I cell line that on long-term in vitro propagation drifts to what is known as a “Group III” phenotype due to the activation of endogenous Epstein-Barr virus (EBV) transforming genes that are otherwise latent in the original biopsy and early passage cells\textsuperscript{18,19}. As has been found for other anti-proliferative signals\textsuperscript{20,24,27,29}, progression to the Group III status – as exemplified here by Mutu III – also renders BL cells refractory to the inhibitory actions of the SSRI (Fig. 1d).
SSRI-inhibited DNA synthesis within biopsylike BL cultures was accompanied by an accumulation of cells at the G_0/G_1 stage of cell cycle similar to that previously described following cross-linking of the B cell antigen receptor (BCR) and achieved here using antibody to \( \mu \) heavy chain of surface IgM (Fig. 1e)\(^ {24} \). Cell cycle arrest in BL cells on BCR cross-linking is followed by entry into apoptosis: this can be reversed through CD40 on the BL cells engaging CD40 ligand (CD40L/CD154)\(^ {17,21,22,24} \). The results in Figure 1f show that, while CD40L had a minimal effect on the cessation of DNA synthesis promoted by SSRI (illustrated here by fluoxetine), it substantially reversed the cell death that was otherwise occurring on fluoxetine exposure (as judged by
characteristic changes in light scatter properties in otherwise homogenous viable BL populations\textsuperscript{30}).

**Characteristics of SSRI-promoted cell death in BL cells**

To address the nature of BL cell death promoted by the SSRI we turned to a number of approaches. Figure 2a shows that fluoxetine, in a concentration-dependent manner, drastically reduced the number of cells remaining viable at 24 h as determined by a loss of cells capable of taking up the vital dye, syto 16 [green]. This loss of viability was accompanied by an increasing number of cells becoming apoptotic (syto 16\textsuperscript{−ve}/PI\textsuperscript{−ve} [blue]) and/or necrotic (syto 16\textsuperscript{−ve}/PI\textsuperscript{+ve} [red]), such determination being based on the differential ability of propidium iodide (PI) to be excluded from or to enter cells undergoing apoptosis or necrosis respectively. The appearance of cells in the “necrotic” gate in response to SSRI treatment likely reflects necrosis secondary to apoptosis (in the absence of cells that would otherwise elicit phagocytic clearance in an \textit{in vivo} setting).

Results from Figure 2b confirm the importance of the apoptotic pathway in the SSRI-induced cell death as over-expression of anti-apoptotic Bcl-2 protected the BL cells from all 3 drugs tested. Thus while cells carrying empty (control) vector lost the light scatter characteristics of the viable subpopulation (indicated in black) and moved to the dead gate (indicated in red) in response to SSRI those expressing the vector containing \textit{bcl-2} remained in the viable sector.
Each of the SSRI promoted caspase activation in the BL cells as assessed by the carboxyfluorescent probe FAM-VAD-FMK that binds irreversibly to caspases in their active configuration. Increased activation of caspases over background levels in L3055 Group I BL cells following 6 h of treatment with the SSRI can be seen both by FACS-based analysis (Fig. 2c) and by laser-scanning confocal microscopy where the treated cells – counterstained by the Hoechst nuclear dye (blue) and with PI to reveal dead cells (red) – exhibit green cytoplasmic staining for active caspases (Fig. 2d).

Staining of SSRI-treated L3055 cells with the carbocyanine liquid crystal forming probe JC-1 highlighted a loss of mitochondrial membrane potential accompanying the drug-
induced apoptosis of Group I BL cells. Thus, following exposure to each of the SSRI, there is a complete disappearance of the intense red emission from JC-1 aggregates which in control cells form within mitochondria maintaining their membrane potential integrity; instead, SSRI-treated cells develop a diffuse green emission that results from the disseminated distribution of relatively unconcentrated JC-1 monomers (Fig.2e). The classic apoptotic morphology of condensed and fragmented nuclei revealed by staining cells with acridine orange was also evident after SSRI exposure (Fig.2f). Again, this was reversed by Bcl-2 over-expression (Fig.2g).

Attempts to address whether the SSRI-promoted caspase activation as shown in Figure 2c,d was leading directly to the observed apoptosis using the pan-caspase inhibitor Z-VAD-FMK were thwarted due to deleterious effects on these cells by the inhibitor itself (Gordon, unpublished observation, 2001). We therefore asked whether a major downstream substrate of the caspase pathway, Poly(ADP-ribose) polymerase-1 (PARP-1), undergoes cleavage in biopsylike BL cells as a result of fluoxetine action. Figure 2h shows the degree of PARP-1 cleavage following exposure of L3055 cells to either anti-IgM or fluoxetine for 17 hours. Whereas intact 112 kDa PARP-1 was the almost exclusive form in control cells, anti-IgM treatment resulted in the majority of PARP-1 being cleaved to the p89 fragment; 20µM fluoxetine similarly promoted extensive PARP-1 cleavage.

Annexin V binding to phosphatidylserine exposed on the outer leaflet of cells to signal for phagocytic engulfment is another commonly used measure of programmed death
although caution should be applied when using as a sole indicator for assessing apoptosis in B cells \(^{31,32}\). We found that while untreated L3055 cells displayed low level Annexin V binding on approximately half the cells, the majority of cells developed a higher level of binding following their culture with either anti-IgM or fluoxetine (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Annexin V binding</th>
<th>% TUNEL positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% positive</td>
<td>MFI</td>
</tr>
<tr>
<td>Control</td>
<td>52.3±1.8</td>
<td>3.9±0.2</td>
</tr>
<tr>
<td>anti-IgM</td>
<td>94.5±1.0</td>
<td>71.1±12.1</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>81.1±3.9</td>
<td>26.1±8.4</td>
</tr>
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\(^{a}\)L3055 cells were cultured at \(10^6/\text{ml}\) for 17 h in control medium, with anti-IgM (\(\mu\) chain) at 10 \(\mu\)g/ml or Fluoxetine at 20 \(\mu\)M.

\(^{b}\)Samples analysed by FACS to generate % positive cells within the Annexin V\(^{+ve}/\text{PI}^{-ve}\) “apoptotic” gate together with Mean Fluorescent Intensity (MFI) of stain. Results are Means ± SE of 3 separate experiments.

\(^{c}\)Samples analysed by FACS to generate % non-subdiploid cells (as assessed by PI stain) positive by TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling). Results are Means ± SE of 3 separate experiments.

Neither BL cells nor their normal germinal center B cell equivalents tend to produce the extensive “DNA ladders” that can be seen as a result of DNA fragmentation when other cells (e.g. thymocytes) undergo apoptosis \(^{33,34}\). Nevertheless, we asked whether fluoxetine might be inducing the type of DNA breaks associated with apoptosis by using a TUNEL-
based method where cells are first treated with DNA ligase before assessing the incorporation by TdT of a fluorescent-labeled nucleotide into DNA with strand breaks having blunt ends or single base overhangs. The results showed a discernible (though not dramatic) increase in the number of L3055 cells with such breaks following their treatment with either anti-IgM or fluoxetine (Table 1).

Importantly, normal peripheral blood mononuclear cells (PBMC) remained viable on exposure to SSRI. The viability of cells exposed for 24 h to 5µM fluoxetine and to 10µM fluoxetine was recorded as 97.3±0.88% and 97.3±0.33% respectively compared to 98.0±0.58% in control cultures. Even with 20µM fluoxetine, viability remained at 83.7±1.20%. A nearly identical outcome was noted with normal resting B cells isolated from tonsils where viabilities after 24 h of culture with control medium or fluoxetine at 5, 10, or 20µM were 97.4±0.53%, 97.0±0.41%, 96.5±0.88% and 85.9±1.07% respectively.

To assess the possibility that SSRI might selectively target B cells when actively cycling, tonsilar B cells were exposed overnight to fluoxetine following 2 days of stimulation either with SAC (alone or combined with soluble CD40L) or with PMA (alone or in combination with ionomycin). As can be seen from Figure 3a, little inhibition of DNA synthesis occurred in any of the actively cycling populations even with fluoxetine present at 20 µM. Similarly, the clear inhibitory effect of fluoxetine on DNA synthesis in biopsylike BL lines such as L3055 was not evident when assessed on 5 non-BL lines with either no, or only minor reductions, in the level of ³H-Thymidine incorporation observed in Nalm-6 (“pre-B”), RPMI 8226 (“plasmacytoid”), or the three T-cell lines: Jurkat, J10, and CEM (Figure 3b).
We wished to examine whether the observed resistance to SSRI-induced death among normal B cells related to a constitutive expression of Bcl-2 and how these levels compared to, and among, the biopsylike BL lines and the non-BL lines studied here.

Western blotting for Bcl-2 confirmed its low level expression in wild-type L3055 cells and in those carrying empty vector; those transfected with bcl-2 carried the expected high levels of the protein (Figure 3c). Two other Group I BL lines, Elijah and Mutu I carried undetectable levels of Bcl-2 whereas Namalwa carried detectable but still relatively low levels of the protein. Normal resting B cells from tonsils were confirmed as expressing high levels of Bcl-2 (Figure 3c). The 5 non-BL lines studied carried variable but readily detectable Bcl-2 (Figure 3d). When compared against the freshly isolated population,
tonsilar B cells that had been activated for 2 days by PMA and ionomycin showed a relative down-regulation of Bcl-2 expression although levels remained readily detectable. Independent measure of Bcl-2 content by FACS analysis performed as described previously fully confirmed the findings made by Western blotting and additionally showed that PBMC were at least as positive for Bcl-2 expression as purified resting tonsilar B cells (data not detailed). Although not available at the time of this analysis, it has been well documented that Mutu III cells carry Bcl-2 protein at a level considerably higher than those of its Mutu I counterpart; furthermore, a direct comparison of Bcl-2 levels in Mutu I, Mutu III, L3055 wild-type and L3055/bcl-2 transfectants as assessed by Western blotting was comprehensively detailed by two of us in a recent study. Likewise, we have previously demonstrated that BL2 cells carry low levels of Bcl-2 similar to L3055 cells.

**SSRI directly stimulate signal transduction pathways and downregulation of c-myc and nm23 genes in BL cells**

Apoptosis is an active process driven by diverse signal transduction pathways among which an elevation in the level of intracellular free Ca$^{2+}$ ([Ca$^{2+}$]$_i$) can be key. SSRI have variously been described as either direct inhibitors of Ca$^{2+}$ channels or inducers of Ca$^{2+}$ signalling in a diversity of cell types. Here, we found that fluoxetine prompted a rapid rise in basal Ca$^{2+}$ in BL cells with an EC$_{50}$ close to that obtained for inhibition of DNA synthesis (Fig.4a). All three SSRI promoted an increase in basal Ca$^{2+}$ with a similar shape and kinetics (Fig.4b-d). Further study with fluoxetine indicated that: (i) it acted on thapsigargin-sensitive endoplasmic reticulum Ca$^{2+}$ stores and; (ii) triggered Ca$^{2+}$
influx. Thus: (i) Fig.4e shows that, in low Ca\(^{2+}\) medium, fluoxetine addition failed to increase [Ca\(^{2+}\)], following the depletion of Ca\(^{2+}\) from stores sensitive to thapsigargin\(^{29}\); (ii) Fig.4f demonstrates that chelating extracellular Ca\(^{2+}\) with EGTA resulted in a substantial diminution of the rise in [Ca\(^{2+}\)], provoked on fluoxetine addition\(^{29}\).

**Figure 4**

The concentrations of SSRI required to promote elevations in intracellular free Ca\(^{2+}\), DNA synthesis cessation, and induction into apoptosis, appear incompatible with their pharmacology at the serotonin transporter\(^{28,44}\). In an attempt to address whether their actions were targeted on SERT or not, we made use of the competitive binding that exists between fluoxetine and 5-HT at the transporter\(^{45}\). While 5-HT, via SERT, can also drive apoptosis in BL cells\(^{20}\) it does so without increasing basal levels of intracellular free Ca\(^{2+}\)
The inability of even a large (50x) excess of 5-HT to attenuate the fluoxetine-driven change in intracellular Ca\textsuperscript{2+} indicates that the actions of the latter—as detailed in this study—are most likely SERT-independent (Figure 4g).

In addition to elevating Ca\textsuperscript{2+}, the SSRI (as illustrated by fluoxetine) also prompted tyrosine phosphorylation on a number of protein substrates of which two major ones—with apparent molecular weights of 85 and 105 kd—were common to those targeted by antibody to surface IgM (albeit with different kinetics), a treatment which similarly liberates Ca\textsuperscript{2+} from intracellular stores and stimulates Ca\textsuperscript{2+} influx\textsuperscript{29} (Fig.4h).

Despite its hallmark translocation to immunoglobulin loci, the BL-associated constitutive expression of c-myc remains dependent on the binding of the recognised c-myc transcription factor Nm23-H2 to a PuF site within the regulatory sequence of the translocated gene\textsuperscript{10}. Using real-time RT-PCR, we found that a 6 h exposure of biopsylike BL cells to fluoxetine resulted in an approximate 60% reduction in the levels of both nm23-H2 and c-myc (Fig.4i). We also observed a rapid 75% decrease in the expression of nm23-H1 mRNA, the product of which has also been linked to c-myc expression\textsuperscript{11}. In contrast expression of the house-keeping gene cyclophilin A was not significantly altered with levels remaining at 81.8% (SE=8.3%) of control values following fluoxetine treatment ($P=0.16$).
Discussion

Our findings reported herein demonstrate that the SSRI class of antidepressants are capable of driving programmed death among the constituent cells of Burkitt lymphoma, a highly aggressive tumor that remains problematic in areas where it is endemic and that has increased dramatically worldwide due to an association with AIDS\(^1^\text{-}^6,^4^6\). The present study was prompted by our recent finding that BL cells carry the serotonin transporter and are susceptible to programmed death resulting from the uptake of 5-HT; however, in itself, the monoamine is not a deliverable drug\(^2^0\). During those studies we noted that at concentrations only slightly higher than those required for blocking serotonin-promoted apoptosis, the SSRI began to exhibit what appeared to be direct inhibitory effects on biopsylike BL cells maintained in early passage\(^2^0\). This preliminary observation led us to explore possible pro-apoptotic activity of the SSRI in their own right.

Each of the three structurally distinct SSRI\(^4^4\) investigated were found capable of driving cessation of DNA synthesis in Group I BL cells remaining faithful to the original biopsy phenotype. Inhibition of S phase entry correlated with a relative accumulation of cells in the G\(_0\)/G\(_1\) phase of cell cycle and was accompanied by extensive cell death that displayed features of apoptosis: characteristic cell shrinkage with increased granularity; loss of viability with retained membrane integrity against propidium iodide; phosphatidylserine exposure; nuclear condensation and fragmentation; caspase activation; cleavage of PARP-1; DNA fragmentation; decreased mitochondrial membrane potential. SSRI-induced BL cell death could be effectively reversed either by engaging the CD40 pro-
survival pathway or ectopically expressing anti-apoptotic bcl-2. Similarly, the refractoriness of late passage Mutu III cells to the SSRI actions is inconsistent with the apoptotic pathway being activated in the sensitive Group I lines in that the turn on of EBV latent genes as cells drift to the Group III phenotype is accompanied by a general resistance to apoptosis-inducing stimuli. Both peripheral blood mononuclear cells and purified resting tonsilar B cells were largely refractory to SSRI-induced cell death which, again, may partly relate to a constitutive expression of the requisite survival genes although the role of other factors also needs to be considered. The effects of the SSRI against BL cells did not simply reflect the fact that they were cycling: 5 randomly chosen non-BL lymphoid lines were largely immune to the actions of the SSRI as were tonsilar B cells that had been stimulated into cycle by polyclonal mitogens. Germinal center B cells, the phenotypic normal counterparts to BL, enter apoptosis spontaneously \textit{ex vivo} and the preliminary indications are that SSRI do not perceptibly alter the kinetics of this intrinsic programmed death (unpublished observations, Serafeim, Holder and Gordon, 2001). Encouragingly, therefore, it appears that with respect to the apoptotic pathway of death the SSRI preferentially target the proliferating BL cell. Whether other B cell malignancies where the constitutive cells display a propensity for apoptosis induction (for example, chronic lymphocytic leukemia) might also be sensitive to the actions of SSRI is currently under investigation.

All three SSRI were seen to provoke a rise in the basal level of intracellular free Ca$^{2+}$ in biopsylike BL cells. We examined this possibility, in part as it is known that a rise in [Ca$^{2+}$]$_i$ can be key to initiating the apoptotic cascade in BL cells – for example in
response to BCR cross-linking\textsuperscript{29} – but also from reports on other cell types indicating that SSRI can directly affect Ca\textsuperscript{2+} flux: either negatively or positively. Thus fluoxetine has been shown to block voltage-gated calcium channels in rat hippocampal pyramidal cells\textsuperscript{40} and in PC12 cells\textsuperscript{42} (derived from rat adrenal medulla chromaffin cells) with an IC\textsubscript{50} of 6.8 and 13.4 \(\mu\text{M}\) respectively while stimulating a Ca\textsuperscript{2+} flux in Madin-Darby canine kidney cells\textsuperscript{43} and bladder female transitional carcinoma (BFTC)\textsuperscript{41} cells with an EC\textsubscript{50} of 40 \(\mu\text{M}\) and 30 \(\mu\text{M}\) respectively. While the concentration of fluoxetine required to elicit a Ca\textsuperscript{2+} response in BFTC cells was more than twice that noted in the present study for BL cells, the characteristics were remarkably similar: notably the provoking of Ca\textsuperscript{2+} release from thapsigargin-sensitive intracellular stores and the induction of Ca\textsuperscript{2+} influx from the extracellular medium\textsuperscript{41}. This pattern also bears similarities to the elevation in Ca\textsuperscript{2+} prompted in BL cells on ligating BCR where a direct causal relationship has been established between this change and subsequent apoptosis\textsuperscript{29}. It was of interest that both exposure to SSRI and ligation of BCR stimulated in BL cells tyrosine phosphorylation with at least two of the protein substrates being common to the two triggers suggesting some parallels in the signalling pathways engaged.

The concentrations of SSRI required to promote change in biopsylike BL cells appear incompatible with their pharmacology at the serotonin transporter; indeed, the inability of even a 50-fold excess of 5-HT to compete out the fluoxetine-induced Ca\textsuperscript{2+} rise indicates that their actions reported herein are possibly SERT-independent\textsuperscript{28,44,45}. Non-serotonergic effects of fluoxetine have been reported\textsuperscript{47-49} and it will be of interest to determine the molecular target(s) for the direct actions of the SSRI on BL cells.
BL is characterised by translocations of one c-myc allele to one of the immunoglobulin loci and the extraordinarily high growth rate that characterises these tumours reflects the pro-proliferative actions of the translocated gene\(^7\). However, despite translocation to immunoglobulin loci, the BL-associated constitutive expression of c-myc remains dependent on the binding of the recognised c-myc transcription factor Nm23-H2 to a PuF site within the regulatory sequence of the translocated gene\(^{10}\). Here we found, using real-time RT-PCR that a 6 h exposure to fluoxetine resulted in reductions in the levels of both nm23-H2 and c-myc and also of nm23-H1. A recent report has identified that Nm23-H1 may also be linked to c-myc expression\(^{11}\). Thus the SSRI appear to influence the very genes that underpin the uncontrolled cell division normally associated with BL.

Furthermore, high expression of Nm23-H1 has been correlated with poor responses to treatment in high-grade lymphomas other than BL\(^{12}\). These observations may therefore have implications for the clinical exploitation of SSRI in the broader context of B cell lymphoma. Although a constitutively high bcl-2 expression could potentially compromise such hope, the possibility of combining the delivery of SSRI with – for example – targeted antisense strategies could keep this wider possibility open\(^{50}\).

The concentrations of SSRI shown to be active against BL cells (e.g. fluoxetine, EC\(_{50}\) = 9.3±2.3 µM for cessation of DNA synthesis) are higher than those seemingly achieved with the current therapeutic use of these drugs for depression and anxiety-related disorders\(^{44}\). After 30 days of dosing at 40 mg/day, plasma concentrations of fluoxetine, for example, reach no more than 1 µM\(^{44}\). However, SSRI are highly lipophilic and may
accumulate in tissues: for example, in one study an estimate of 20:1 was given for the relative partition of fluoxetine between brain and blood\textsuperscript{51}. Interestingly, while Bolo and colleagues\textsuperscript{52} reported a somewhat lower brain-to-plasma ratio of 10:1, the average concentration of fluoxetine found in brain for 12 subjects taking between 10 - 40 mg per day was 13 \(\mu\)M, a figure slightly above the EC\textsubscript{50} obtained for the ability of the drug to inhibit DNA synthesis in BL cells. Yet another study\textsuperscript{53} confirmed the high accumulation of fluoxetine into tissue spaces with an apparent distribution volume of 12 - 43 in brain relative to plasma. Moreover, SSRI can be administered at levels much higher than those currently prescribed without major side effects. From an extensive survey of the literature, Barbey and Roose concluded that the taking of amounts up to 30 times the common daily dose is associated with either minor or no undesirable symptoms; even ingestions of greater amounts typically resulted in – at worst – drowsiness, tremor, nausea, and vomiting\textsuperscript{54}. Only at >75 times the normal dosage did more serious adverse events occur. Indeed, one of the perceived benefits of SSRI over, for example, the tricyclic antidepressants is that fatalities through overdosing are extremely rare and usually result from combination with other drugs\textsuperscript{44,54}.

While the above pharmacokinetic considerations seem encouraging in relation to our \textit{in vitro} data, it is clear that more work is required before the SSRI can be considered as deliverable therapeutics for patients with Burkitt lymphoma: for example, issues such as whether autonomously produced CD40L could potentially counter the pro-apoptotic actions of SSRI \textit{in situ} need to be addressed\textsuperscript{38}. The availability of well characterized
animal models of BL will allow the necessary next stages of such a goal to be investigated in detail\textsuperscript{55,56}.

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References


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Figure 1. Fluoxetine promotes growth cessation in biopsy-like Burkitt’s lymphoma cells. L3055 cells were treated for 24 hours with: a, 0.5 - 30 µM of fluoxetine; b, 0.5 - 30 µM of paroxetine and; c, 1 - 150 µM of citalopram: EC50 values of DNA synthesis inhibition for each of the SSRI are indicated. d, five Group I BL lines and the Group III cell line “Mutu III” were treated for 24 h with fluoxetine (20 µM), paroxetine (20 µM), or citalopram (100 µM) and resultant DNA synthesis expressed as % control. e, L3055 cells cultured for 24 h either alone, with fluoxetine (20 µM), or with anti-µ chain antibody (10µg/ml) and viable population subjected to cell cycle analysis. f, L3055 cells pretreated for 1 h with soluble CD40L trimer were then cultured for 24 h before analysis of DNA synthesis (3[H]Thymidine) and cell viability (FACS); results are % change due to presence of 20 µM fluoxetine. All data are mean ± SEM: from 8 separate experiments for “a”, 6 for “b”, “c”, “d”; from 3 for “e” and “f”.

Figure 2. Characteristics of SSRI-induced apoptosis in group I BL cells. a, L3055 cells treated with fluoxetine for 24 h then double stained with syto 16 and PI for FACS analysis: % cells viable (green), apoptotic (blue), necrotic (red), indicated. b, L3055 transfected with either bcl-2, or empty vector treated for 24 h with control medium, 20
µM fluoxetine, 20 µM paroxetine, or 100 µM citalopram, before analysis for forward versus side scatter: % viable cells remaining (black dots) indicated; dead cells in red. c, d, e, L3055 cells treated with SSRI as for “b”: c,d, for 6 h then stained for active caspases before analysis by c, flow cytometry (% cells active caspase positive indicated) or d, confocal microscopy, counterstained with Hoechst nuclear dye (blue) and PI to detect dead and membrane-compromised cells (red) – activate caspases stain green; e, for 24 h, then stained with the cationic dye JC-1 for confocal microscopy – mitochondrial depolarisation is indicated by a shift from red to green fluorescence; f, for 24 h, then stained with the acridine orange for fluorescence microscopy; g, as for “f” but using L3055 bcl-2-transfectants; h, L3055 cells were cultured for 17 h with either control medium, anti-µ chain antibody (anti-IgM, 10µg/ml) or fluoxetine (20 µM), cell lysates prepared and equal amounts of protein (25µg) resolved on 10% SDS-PAGE before blotting for PARP-1, the intact 112 kDa and cleaved 89 kDa protein being indicated by arrows. Each result is representative of at least three individual experiments.

Figure 3. Fluoxetine actions on and Bcl-2 levels in normal B cells and non-BL cell lines. a, Resting tonsilar B cells were cultured at 10^5 cells per 200 µl of culture medium for 48 h in flat-bottom microtiter wells with either no addition (CM), Staphylococcus aureus Cowan 1 (SAC, 1:10,000), Phorbol 12-Myristate 13-Acetate (PMA, 5 ng/ml), SAC+sCD40L (1µg/ml), or PMA+ionomycin (PMA/I, 1 µg/ml) before addition of fluoxetine to 0 - 20 µM as indicated then cultured for a further 24 h, being pulsed with ^3[H]Thymidine for the last 7 h for the analysis of DNA synthesis; b, Cells from lines indicated were cultured for 24 h with fluoxetine as in “a” before assessment of DNA
synthesis as described in Fig.1a-d. Data are mean ± SEM from 3 separate experiments. Cell lysates were prepared from pelleted cells and equal amounts of protein (25µg) resolved on 12.5% SDS-PAGE before blotting for Bcl-2, the protein migrating with an apparent molecular weight of 26 kDa as indicated; c, is a representative blot of protein from L3055 (wild type), L3055 vector controls, L3055/Bcl-2 transfectants, Namalwa, Elijah, Mutu I, and resting tonsilar B cells; d, from Nalm 6, CEM, Jurkat, J10, RPMI8226, resting tonsilar B cells, B cells activated for 48 h with PMA+ionomycin.

Figure 4. SSRI trigger Ca2+ flux, tyrosine phosphorylation, and down-regulation of c-myc and nm-23 genes in group I BL cells. [*Ca^{2+}]* measurements in L3055 cells treated with: a, 0.5 - 80 µM of fluoxetine; b, 20 µM fluoxetine; c, 20 µM paroxetine; d; 100 µM citalopram; e, 100 nM of thapsigargin followed by 20 µM fluoxetine in Ca2+-low medium (addition of 20 µM fluoxetine alone in Ca2+-low medium indicated in insert); (f) 20µM fluoxetine in the presence or absence of 9 mM EGTA; g, 20µM fluoxetine in the presence or absence of 1mM of 5-HT. Each result either representative, or mean ± SEM, of three experiments. h, Western blotting analysis for protein phosphotyrosine in L3055 cells showing control (C) cultures and following treatment with 20µM fluoxetine (Fl) or 10µg/ml anti-µ chain antibody (Ig) for 60, 150 or 300 sec. Blot representative of 4 different experiments. i, Real-time quantitative PCR analysis of indicated genes after 6h exposure of L3055 cells to 20µM fluoxetine. Relative gene expressions normalized to L3055 control cultures (100%). Data are the mean ± SEM of four experiments each performed in triplicate.
Selective serotonin reuptake inhibitors directly signal for apoptosis in biopsylike Burkitt lymphoma cells

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