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The serotonin transporter (SLC6A4) is present in B-cell clones of diverse malignant origin: probing a potential antitumor target for psychotropics

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ABSTRACT

Following our previous description of the serotonin transporter (SERT) acting as a conduit to 5-hydroxytryptamine (5-HT)-mediated apoptosis, specifically in Burkitt's lymphoma, we now detail its expression among a broad spectrum of B cell malignancy, while exploring additional SERT substrates for potential therapeutic activity. SERT was readily detected in derived B cell lines with origins as diverse as B cell precursor acute lymphoblastic leukemia, mantle cell lymphoma, diffuse large B cell lymphoma, and multiple myeloma. Concentration and timecourse kinetics for the antiproliferative and proapoptotic activities of the amphetamine derivatives fenfluramine (an appetite suppressant) and 3,4-methylenedioxymethamphetamine (MDMA; "Ecstasy") revealed them as being similar to the endogenous indoleamine. A tricyclic antidepressant, clomipramine, instead mirrored the behavior of the selective serotonin reuptake inhibitor fluoxetine, both being effective in the low micromolar range. A majority of neoplastic clones were sensitive to one or more of the serotonergic compounds. Dysregulated *bcl*-2 expression, either by t(14;18)(q32;q21) translocation or its introduction as a constitutively active transgene, provided protection from proapoptotic but not antiproliferative outcomes. These data indicate a potential for SERT as a novel anti-tumor target for amphetamine analogs, while evidence is presented that the seemingly more promising antidepressants are likely impacting malignant B cells independently of the transporter itself.

Key words: 5-hydroxytryptamine • Burkitt's lymphoma • B cell lymphoma

In the synaptic cleft of the brain, the serotonin transporter (SERT; solute carrier family 6 member 4 – SLC6A4) serves to limit the bioavailability of the pleiotropic monoamine neurotransmitter, 5-hydroxytryptamine (5-HT; serotonin) by promoting its active sequestration back into presynaptic terminals—a process accompanied by storage and/or degradation of the indolamine (1). Despite major roles for central nervous system (CNS) serotonin in regulating functions as diverse as sleep, appetite, sexual drive, and motor activity, greater than 90% of body 5-HT is produced in the periphery, primarily by enterochromaffin cells in the gut (2). Correspondingly, subsets of peripheral cells have been found to express functional SERT, platelets being the best-studied example (3). Evidence has been presented for SERT acting not merely as a transport protein but as a signal transducer in its own right when contacting substrate (4, 5). Moreover, SERT-delivered 5-HT was recently shown to impact signal transduction directly by a novel modification, the "serotonylation" of small GTPases (6). Thus, SERT appears equipped to modify a cell's functional behavior in potentially diverse ways.

We recently reviewed the evidence for lymphocytes expressing the serotonin transporter (7). This was prompted by our finding of SERT protein and functional 5-HT uptake in Burkitt's lymphoma (BL) cells, the consequence of which was apoptosis (8). Selective serotonin reuptake inhibitors (SSRI), while initially reversing the monoamine's actions were at higher concentrations themselves proapoptotic (9). The direct actions of SSRIs were encouraging, as serotonin itself is not a suitable therapeutic drug. However, although concentrations of fluoxetine (the SSRI for which there is the best bio-distribution data) required to elicit BL cell apoptosis in vitro were comparable to those achieved in brains of patients on standard antidepressant regimens, they were higher than levels reached in plasma (10).

The aims of the present study were twofold. First, to establish whether B cell malignancies other than BL express SERT and second, to evaluate the efficacy of additional SERT substrates to impact lymphoma cell population dynamics with the emphasis on therapeutically deliverable compounds. In addition to the SSRI antidepressants, members of the tricyclic class can also demonstrate high affinity binding to SERT. Clomipramine, for example, has an affinity for SERT comparable to that of fluoxetine (7). Plasma levels of clomipramine are somewhat higher than those of fluoxetine under therapeutic dosing for depression and anxiety-related disorders: a figure of up to 1.4 μM being reported (11). Particularly exciting is the indication of clomipramine as a novel drug in the treatment of at least a subgroup of brain tumors (12). We therefore compared directly the efficacies of fluoxetine and clomipramine in the studies here. The amphetamine derivatives 3,4-methylenedioxymethamphetamine—MDMA, "Ecstasy"—and fenfluramine, until recently a widely used appetite suppressant, were also included. Both compounds bind to SERT and on active transport into target cells displace stored 5-HT, thereby causing its release (13). Direct effects have been reported for each of the amphetamine analogs, including the induction of apoptosis in nonlymphoid cells (14–17). Their capacity to impact—directly or indirectly—the immune system and its constituent cells was recently reviewed (7, 18). Likewise, cocaine, a relatively nonselective biogenic amine (5-HT, norepinephrine, dopamine) transporter antagonist, has also been implicated in immune modulation receiving particular attention owing to its link with HIV-infection and is included for study here (19).

MATERIALS AND METHODS

Reagents

The hydrochloride salts of serotonin (H9523), (+/–)-fenfluramine (F8507), (+/–)-3,4-methylenedioxymethamphetamine (M6403), cocaine (C5776), fluoxetine (F132), clomipramine (C7291), maprotiline (M9651) and GBR12909 (D-052) were purchased from Sigma-Aldrich (Dorset, UK). Drugs were dissolved in distilled water and sterile filtered (0.2 μM DynaGard filters, Microgen). Mouse monoclonal anti-human serotonin transporter antibody (ST51-1) and competing peptide were purchased from mAb Technologies, and mouse IgG1 isotype control were obtained from Caltag-MedSystems Ltd (Towcester, Northants, UK). PE-conjugated goat anti-mouse IgG, biotin-conjugated goat ant-mouse Immunoglobulins, FITC-conjugated mouse anti-human-Bcl-2 and FITC-conjugated mouse IgG1 negative control were obtained from Dako Ltd (High Wycombe, Bucks, UK). MRK16 mouse IgG2a anti-human P-gp monoclonal antibody was obtained from Kamiya Biomedical Co (Seattle, WA). PE-streptavidin was purchased from Becton Dickinson (Mountain View, CA).

Cells

A full description and derivation of the cell lines included for study and listed in Table 1 can be obtained on request. All lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 10% Serum Supreme (BioWhittaker, Wokingham, UK), 2 mM glutamine, 100 IU/ml penicillin and 100μg/ml streptomycin, as described previously (8). 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 (8). HEK-SERT transfectants were grown in DMEM containing 10% FCS and G418 sulfate (250 μg/ml final concentration). 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; germinal center B cells were prepared by depletion from the $CD3^{-ve}/CD14^{-ve}$ fraction of those bearing IgD and CD39 (20).

Measurement of intracellular Bcl-2 and SERT

For analysis of intracellular proteins, cells were incubated in 100 μl of 2% paraformaldehyde for 5 min on ice before washing in PBS then resuspended in 500μl of 0.1% saponin/PBS and left for 5 min on ice. After wash ×1 in 0.1% saponin/PBS, cells were incubated with 50-μl antibody for 30 min on ice before washing with 0.1% saponin/PBS and then with PBS to reseal membranes: for indirect stain, cells were incubated with 50 μl of secondary antibody (diluted in 0.1% saponin/PBS) for 30 min on ice before final wash. Cells were resuspended in 5% NGS/0.1% sodium azide/1% formaldehyde/PBS and analyzed on an EPICS XL flow cytometer (Beckman Coulter, Mountain View, CA). For Bcl-2 measurements, mouse anti-human Bcl-2 (FITC-conjugated, 1/100) was used with FITC-conjugated non-immune mouse IgG1 as negative control (1/100). For SERT, mouse anti-human serotonin transporter (1/1000) with unconjugated mouse IgG1 isotype control (1/1000) were used at the primary stage with PE-conjugated goat anti-mouse IgG (1/25) as secondary. For peptide blocking, mouse antibody and peptide were mixed at a ratio of 1:10 before staining, using the mixture at a concentration equivalent to that of antibody alone as above.

Detection of P-glycoprotein

A three-stage indirect staining technique was devised for the detection of P-gp expression at lymphoid cell surfaces. 10^6 pelleted cells were resuspended in 50 µl of 1% BSA/PBS + 5 µl NGS (normal goat serum) before adding 2 µl MRK16 anti-human P-gp monoclonal antibody or non-immune mouse IgG2a as isotype control for 1 h at RT. After washing, the resuspended pellet was incubated with 50 µl biotin-conjugated goat anti-mouse immunoglobulins (1/100) + 5 µl NGS for 30 min at RT in the dark. Again, after washing, cells were resuspended with 50 µl streptavidin-PE diluted 1/100 in 1% BSA/PBS + 5µl NGS and incubated for 30 min at RT in the dark. Washed cells were resuspended in 500 µl PBS/1% BSA/1% formaldehyde before analysis on an EPICS XL flow cytometer.

Measurement of DNA synthesis, viability, and caspase activity

DNA synthesis was determined by measuring ^{3}H-thymidine ([^{3}H]Tdr; Amersham, UK) incorporation into cellular DNA as documented previously (8). Changes in cell viability were quantified by assessment of forward and 90° (side) light scatter of cells using flow cytometry with cells being gated into two populations (viable and dead) as described (9). Apoptosis was assessed by staining cells with the active caspase substrate PhiphiLux-G_1D_2 (Oncoimmunin Inc., College Park, MD) and propidium iodide (P4170, Sigma Aldrich, St. Louis, MO). Cells were seeded at an initial density of 2.5×10^5/ml, then incubated with or without drug for 6 h (37°C in 5% CO_2). After pelleting, cells were resuspended in 30 µl of PhiPhiLux-G_1D_2 then incubated in the dark for 1 h at 37°C before washing in cold PBS with resuspension in 350 µl of the flow cytometery buffer provided. Immediately before analysis, 4 µl of propidium iodide (100 µg/ml) was added and cells were monitored by flow cytometry for forward/side scatter characteristics plus intensity of PhiPhiLux fluorescence (FL-1) and propidium iodide fluorescence (FL-3).

Western blot analysis for detection of SERT

Immunodetection of SERT protein contained in cell lysates was performed by Western blotting as detailed elsewhere (8). For peptide blocking a 16aa peptide (PSPGAGDDTRHSIPAT) was synthesized, corresponding to a sequence from the N terminus of the human serotonin transporter used as immunogen for the monoclonal SERT antibody. In blocking experiments, SERT antibody was incubated with fivefold excess peptide (Alta Bioscience, Birmingham, UK) in PBS for 2 h at RT before adding to membranes.

RESULTS

Characterization of cell lines used in this study

Table 1 highlights some characteristics of the cell lines used in this study, including the L3055 BL line, where the presence of SERT and its response to 5-HT and SSRI antidepressants have been documented previously (8, 9). Another BL line, KHM-2B, was included but differed from L3055 cells by carrying, additional to the classic t(8;14) c-*myc* translocation, another at t(14;18) involving *bcl*-2. In terms of B cell differentiation stage, a spectrum from pre-B to plasma cell was covered with the inclusion of lines derived from B cell precursor acute lymphoblastic leukemia (BCP-ALL) through to multiple myeloma (MM). Prolymphocytic leukemia (PLL),

mantle cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL), and primary mediastinal B cell lymphoma (PMBCL) were also represented.

We have previously observed that high-level expression of Bcl-2 confers in transfected BL cells a resistance to the apoptotic actions of both 5-HT and SSRIs (8, 9); therefore, we determined the relative expression of Bcl-2 among the cell lines. Parental L3055 BL cells were confirmed as being very low for Bcl-2, while *bcl*-2-transfected counterparts carried the expectedly high levels of protein. A range of Bcl-2 levels was found among the remaining lines with those carrying the t(14;18) translocation being positive as expected. Besides L3055 BL cells, Rec-1, NCEB-1, RC-K8, Karpas 1106, and CTB1 were also relatively weak expressers.

Amphetamine derivatives and antidepressants have been variably reported to interact with P-glycoprotein (P-gp) (21, 22). We therefore used a novel three-stage technique to assess expression of this multidrug resistance protein by the different lines. Most cell lines were negative or very weak expressers of P-gp (as illustrated by the examples of L3055 and RL) with two exceptions: Rec-1 and RC-K8 as shown (Fig. 1).

Expression of the serotonin transporter among B cell lines of diverse malignant origin

Western blotting revealed that all B cell lines, regardless of derivation, carried immunoreactive SERT with two dominant bands of apparent molecular weights of ~60 and 70 kDa (Fig. 2*A*). The relative intensity of these bands varied between the different malignancies. For example, in the DLBCL cell line K422 and the PMBCL line K1106, the lower band dominated, while in the myeloma lines H929 and KMS11 the upper band was stronger. The two bands were also apparent in HEK 293 cells transfected with full-length neuronal SERT (HEK-SERT), though here the major molecular weight species was a diffuse 90-95 kDa band, probably representing heavily glycosylated SERT protein, as reported previously (23). A minor component of similar size occasionally appeared on blots of material from some B cell lines (e.g., PR-1, L3055/Bcl-2). Specific peptide blocking confirmed the specificity of each of these bands, both in HEK-SERT and L3055/Bcl-2 cells (Fig. 2*B*). In contrast to malignant B cell lines, normal tonsilar B cells carried undetectable levels of immunoreactive SERT in the case of germinal center cells or undetectable to low levels in the case of the resting, extrafollicular population (Fig. 2*C*). On stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, resting B cells could be induced to express high levels of immunoreactive SERT: appearing primarily as the 70-kDa component but with the 60-kDa band apparent with increasing time of activation (Fig. 2*C*).

As a complementary approach to Western blotting and to provide a quantitative assessment suitable for high throughput screening, we developed a FACS-based assay to measure total immunoreactive cellular SERT. Results depicted in Fig. 3 validate this approach, the high-level staining of HEK-SERT cells being reversed in the presence of blocking peptide. Similarly, SERT staining in L3055/Bcl-2 cells returned to control levels in the presence of inhibitory peptide. Typical staining profiles, together with the mean fluorescence intensity (MFI) of staining confirm that all the malignancies express readily detectable SERT, though the amount found could vary by up to threefold. In some lines—such as L3055, LILA, and Rec-1—there was clear evidence of bimodality resulting in two peaks of staining intensity. An impression that the level of SERT expression tended to reflect basal growth rate was borne out by linear regression analysis using control values of radioactive-thymidine uptake as an indicator for the latter.

Actions of serotonergic compounds modeled against Burkitt's lymphoma cells

Two SERT substrates that have found common use, either recreationally or medicinally, are the amphetamine derivatives 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") and fenfluramine (Fen, "Pondimin," a popular appetite suppressant before being voluntarily withdrawn by the manufacturers following concerns relating to valvular heart disease). When assessing antiproliferative activity against model L3055 BL cells, MDMA and Fen behaved similarly, both to each other and to 5-HT. As shown in Fig. 4*A*, an IC_{50} of 100–200 μM was obtained for each compound at 24 h, and this did not substantially alter when measured at day 3 or day 6. However, cell density-dependent differential sensitivities were recorded. Plating of L3055 cells at higher densities protected against the antiproliferative actions of 5-HT, whereas the response to Fen (shown) and MDMA (not shown) were unaltered at higher cell densities. Cocaine, a nonselective biogenic amine transporter antagonist, was also antiproliferative in a cell-concentration-independent fashion but at a significantly higher IC_{50} and requiring concentrations of >500 μM to achieve an appreciable effect.

We previously documented that the SSRI fluoxetine triggers growth arrest and apoptosis in biopsy-like BL cells within 24 h (9). Here, we show that with increasing length of exposure, the efficacy of fluoxetine increases such that by day 6, the IC_{50} is in the region of 1 μM compared with 10 μM at day 1 (Fig. 4*B*). We next compared the actions of the SSRI with that of a tricyclic antidepressant, clomipramine. Dose-response and time course kinetics of clomipramine essentially mirrored those of fluoxetine. Neither the antiproliferative actions of fluoxetine (not shown) nor clomipramine (Fig. 4*B*) was affected by cell seeding density. Partly to gauge the degree of selectivity for the antidepressants' actions at SERT, we also assessed the efficacy in arresting L3055 cell proliferation of maprotiline and GBR12909, which act as selective uptake inhibitors at the transporters for norepinephrine and dopamine, respectively (24). Remarkably, each of these inefficient SERT inhibitors displayed similar impact on the model BL cells as the SSRI and TCA (Fig. 4*B*).

We wished to establish whether, as with 5-HT and SSRIs (8, 9), the antiproliferative actions of amphetamine derivatives and clomipramine was accompanied by cell death and, if so, whether there was evidence of apoptosis. Figure 5 illustrates the outcome for the amphetamine analog Fen (MDMA produced similar results; not shown). Even as short as 6 h exposure to Fen produced a concentration-dependent reduction in the number of L3055 cells found in the "viable gate", as indicated by forward vs. side scatter properties. This was associated with a reciprocal dose dependent increase in caspase activity and the identification of increased numbers of early apoptotic cells, as recognized by activated caspase combined with the capacity to exclude propidium iodide (Fig. 5). That the antiproliferative actions of Fen are associated with apoptosis was further supported by the observation that L3055 cells overexpressing *bcl*-2 were fully protected from caspase activation, whereas control transfectants were not.

Exposure of L3055 cells to clomipramine also resulted in rapid and concentration-dependent increases in the proportion of nonviable cells, in cells displaying caspase activity and cells recognized as early apoptotic by possessing increased caspase activity, while still able to exclude propidium iodide (Fig. 6). Again, *bcl*-2 overexpressing L3055 cells were protected against caspase activation, whereas control L3055 cells were not. We additionally asked whether along with an ability to protect from clomipramine's proapoptotic actions, high Bcl-2 afforded

concomitant resistance to its antiproliferative effect. Any protection from cessation in DNA synthesis was marginal at best: L3055 cells overexpressing *bcl*-2 being of almost equal sensitivity to clomipramine as those carrying an empty vector. An identical outcome was seen when using fluoxetine as the antiproliferative agent (not shown). In summary, the cellular processes engaged in BL cells by amphetamine derivatives and TCA antidepressants appear indistinguishable from those of SSRIs (9).

Response of diverse B cell malignancies to serotonergic compounds

Despite being a highly aggressive tumor, Burkitt's lymphoma is characterized by a high proportion of cells entering apoptosis (25). It was possible that BL might be uniquely sensitive to the serotonergic compounds studied here. Accordingly, the actions of 5-HT and representative SSRI (Fluox), TCA (Clomip), amphetamine derivatives (MDMA and Fen) and cocaine were assessed against each of the cell lines studied for SERT expression. Concentrations of each compound were selected, according to maximum/near-maximum effects against the model L3055 cells: the full concentration-dependent response of the L3055 cells to each of the compounds being detailed in Fig. 4 and from which it can be noted that a higher molarity is required for 5-HT and the amphetamine derivatives to be effective compared with the antidepressants. Table 2 details the outcome in terms of the rate of proliferation in response to each drug relative to vehicle control following 24-h exposure with an illustrative depiction of some of these data presented in Fig. 7*A*.

Of the 17 lines studied, 12 revealed an antiproliferative response to one or more of the compounds at the ≥50% level. With rare exception, the prototype group I BL line L3055 displayed the highest level of sensitivity to each of the serotonergic compounds. Notably, the DLBCL line OZ was more sensitive than L3055 cells to both 5-HT and fenfluramine.

The relative sensitivities of the two lines studied side by side are detailed in Fig. 7*B*. In general (though not universally), sensitivity of lines to 5-HT was reflected in the response to fenfluramine and—to a somewhat lesser extent—MDMA: one exception is the myeloma line KMS11, where a strong antiproliferative reaction to fenfluramine is not recaptured with 5-HT. This line was also unusual in being the only one where the response to the SSRI fluoxetine was greater than that to the TCA clomipramine. Overall, 8 of the lines showed a ≥50% antiproliferative response to 20 μM clomipramine with half of these responding similarly to equimolar fluoxetine. As with L3055 cells, none of the lines were substantially affected by cocaine, even at 1 mM. For three DLBCL lines—Oz, Karpas 422, and PR-1—we assessed whether the observed antiproliferative effects were accompanied by death and apoptosis. Whether measured by PI uptake or caspase activation, none of these lines displayed change in these parameters (relative to vehicle controls) on exposure to any of the drugs for 24 h (data not detailed).

Finally, we asked whether normal resting (tonsilar) B cells were adversely affected by the serotonergic compounds at concentrations impacting upon the malignant B cell lines. We had already shown they remain viable when exposed to 20 μM fluoxetine (9). Measuring viability by trypan blue dye exclusion after 24 h of exposure, the following results were obtained (mean cell viability as % of control±SEM of 3 separate experiments): clomipramine 20 μM = 98.9 ± 3.9; MDMA 250 μM = 99.3 ± 1.0, 500 μM = 100.5 ± 2.5; fenfluramine 250 μM = 100.3 ± 1.3, 500

μM = 94.6 ± 3.3; 5-HT 250 μM = 100.4 ± 0.67, 500 μM = 95.5 ± 1.5; cocaine 1 mM = 95.8 ± 8.1. Thus, none of the compounds appeared substantially deleterious to resting B cell viability, a finding confirmed by dual staining of cells with propidium iodide and the active caspase substrate PhiPhiLux (data not detailed).

DISCUSSION

We recently reviewed and posited why B cells should possess components of the serotonergic pathway (7). Subsequently, it has been documented in rhesus macaques that both CD3+ and CD20+ lymphocytes are proximal to 5-HT-containing enteroendocrine cells of the gut (26) and that even under nonpathological conditions, B cells cross the blood-brain barrier (27), where neural serotonin flow could potentially impact their function directly.

The present study establishes expression of the serotonin transporter as a phenotype common to neoplastic B cells of widely distinct origins. Levels of SERT were appreciably higher among the cells of malignant lines compared with normal B cell populations found in tonsil. This appeared not simply to reflect active cycling. Germinal center B cells—an approximately equal mix of out-of-cycle centrocytes and rapidly dividing centroblasts (20)—revealed low abundance (essentially, undetectable) SERT protein. Nevertheless, extrafollicular B cells, comprising resting naïve and memory subsets (28), were encouraged to express readily detectable SERT protein in response to mitogenic stimulation. Interestingly, both BDNF (brain-derived neurotrophic factor) and IL-4 down-regulate functional SERT in transformed B lymphoblasts, while glucocorticoids up-regulate, confirming that it is indeed a regulated phenotype (29–31). The finding by FACS that some lines displayed bimodal expression of SERT further points to its regulation here, possibly in relation to cell cycle progression. Indeed, a statistically significant (though not strict) correlation between SERT levels and proliferation index was noted among the lines studied ($P<0.001$).

Irrespective of tumor origin, each B cell line carried two major SERT components of ~60 and 70 kDa as revealed by Western blotting. Previous reports on the transporter's size indicate apparent molecular weights ranging from 43 to 105 kDa, depending on cell type/species studied (1, 8, 23, 32). Glycosylation accounts for some of the heterogeneity, with cellular background being one factor determining this modification (23, 33). That both components appear in HEK 293 cells transfected with full-length neuronal *sert* (albeit in amounts lower than the diffuse presumed heavily glycosylated 95kDa band), with immunoblotting efficiently blocked by antigenic peptide, indicates that lymphoid SERT is closely related to the analogous protein in brain. Moreover, for L3055 cells, we have detected SERT transcripts using primers designed against the neuronal sequence (our own unpublished data), while showing uptake characteristics for 5-HT compatible with the properties of the transporter from brain (8).

The drive for this study was the desire to identify novel therapeutic modalities for non-Hodgkin's lymphoma and related diseases. Despite the undoubted success of target-selective agents such as Rituximab, there remain subgroups of patients that either relapse or are refractory to even the most effective of the currently trialed regimens (34). There can also be "local" issues (as we have discussed previously in relation to endemic Burkitt's lymphoma) such as cost, availability, and/or deliverability of sophisticated treatments in certain geographical/socio-economic regions

of the world (35). Then there is the specific example of brain lymphoma (36). As discussed below, the approaches indicated here might have particular benefit.

The present study finds that additional serotonergic compounds can be effective in driving apoptosis in model BL cells, namely the widely used recreational drug "Ecstasy" (3,4-methylenedioxymethamphetamine) and the appetite suppressant formally marketed as "Pondimin," fenfluramine. The rationale for assessing the actions of these compounds was the fact that serotonin, though equally effective in vitro, is not a deliverable drug. Unfortunately, despite their efficacy in arresting BL cells, there remain strong reasons why they are unlikely to constitute a suitable therapy. First, as seen also with 5-HT, the durability of their actions are short-lived. Thus, by day 6 of culture with these drugs, the effects on BL population kinetics were little greater than observed at one day. This could reflect a short half-life for these compounds or, alternatively, that they work through a "one-hit" mechanism. Second, concentrations of the amphetamine derivatives needed to affect a response are appreciably above those associated with their in vivo safety. The sustained plasma concentration of the anorexic fenfluramine that "correlates with the best rate of weight loss" has been stated as 1 μM, while lethality can result once 20 μM is reached (37). Various estimates on MDMA concentrations in the circulation of users who find themselves in the emergency room as a result of an overdose is of the order of 10 μM or below (38, 39). A recent study where either drug was administered to rhesus macaques in doses associated with "neurotoxicity" reported peak plasma levels of 2.7 μM for Fen and 9-13 μM for MDMA (40). Though the concentrations of MDMA and FEN required to elicit an antitumor effect are high, their efficacy nonetheless indicates a potential for amphetamine analogs in this novel context: one perhaps reached by redesigning "designer drugs"? The in vitro concentrations of cocaine required to register an antilymphoma affect appears to discount it for such therapeutic application.

The finding that Fen and MDMA closely follow the actions of 5-HT against BL cells does, however, support SERT being a functioning molecule in lymphocytes and transformed counterparts. We had previously excluded a role for 5-HT receptors in the serotonin-dependent apoptosis of BL cells (8). Likewise, the concentrations at which the two amphetamine analogs trigger a functional response are within the range attributable to them activating SERT in other cellular contexts, while being much higher than those required for the triggering of any receptor-mediated pathway. For example, d-Fen at 100 μM is mitogenic for Chinese hamster lung fibroblasts through the SERT-dependent formation of superoxide anion while generating in rat pulmonary artery smooth cells an increase in intracellular Ca^{2+} (5, 17). One report documents fenfluramine's proapoptotic activity against serotonergic human placental choriocarcinoma cells (16). More studies describe the cellular toxicity of MDMA, reflecting its abuse and link to neurodegeneration. The number that focus on apoptosis remains small and generally use much higher concentrations than those seen by us to elicit an apoptotic response in BL cells (14, 15, 41). We therefore suggest that BL lines could provide models for studying apoptotic pathways driven by amphetamine analogs in general. At least, regarding 5-HT, a completely novel pathway for SERT function has been described that may prove relevant within the context described here, namely the "serotonylation" of small GTPases via transported 5-HT (6). While MDMA and Fen would lack such activity directly, they could potentially stimulate this pathway indirectly through their capacity to release 5-HT from intracellular stores. SERT surface expression and function are, in turn, regulated by multiple intracellular signal transduction pathways, the manipulation of which could enhance transport activity in lymphoma cells to

therapeutic ends (42–44). Although the arguments above support the notion that Fen and MDMA exert their antilymphoma effect through impacting SERT we cannot at this stage rule out the possibility of other, as yet undiscovered, targets. Future experiments aim to address this; for example, by specific knockdown of SERT in lymphoma cells using RNA silencing or antisense approaches.

Although providing the rationale for its inclusion in the present study, we feel it unlikely that the antitumor actions of clomipramine are focused at SERT. A similar conclusion was reached regarding the proapoptotic effects of SSRIs, noting that fluoxetine, for example, has been reported to act on multiple nonserotonergic pathways (9). A SERT-independent mechanism for clomipramine's antiproliferative actions is indicated in part by the supra-saturating (at the serotonin transporter) concentrations required. Particularly compelling is the remarkable similarity it shares in this regard, not only with structurally disparate SSRIs but, as now shown here, also with maprotiline and GBR12909: selective uptake inhibitors at the transporters for norepinephrine and dopamine, respectively (24). A common aspect of the SSRI, TCA, maprotiline, and GBR12909 is that they are strong cationic amphiphiles (45, 46). This contributes to the potent lipophilicity of the drugs, with a correspondingly high partition to tissues through phospholipid binding and lysosomal trapping (46). Each of these properties could account for their capacity to influence cell dynamics (47). Alternatively, they make act via a completely distinct mechanism, such as directly impacting mitochondrial pathways, as was recently described for the antitumor actions of clomipramine against human glioma cells (48).

Why some clones should be sensitive and others resistant to these amphiphilic compounds is unclear. Although Bcl-2 protects from the apoptotic process activated by the antidepressants, it fails to offer blanket resistance to their actions: clones expressing high-level Bcl-2—including several t(14;18) DLBCL and BL lines overexpressing a *bcl*-2 transgene—still exhibited reductions in proliferation rate on exposure to clomipramine. Only two lines expressed detectable P-glycoprotein, and of these, one was sensitive to the antidepressants (also to the amphetamine analogs), the other resistant. This multidrug transporter is therefore unlikely to be a major factor determining outcome. That an antidepressant could inhibit high rate proliferation in more than half of the clones indicates a potentially broad application for this class of drug in aggressive B cell neoplasia. Combined with knockdown of *bcl*-2 (or other prosurvival) activity, the predominantly antiproliferative effect could be converted to an even more efficacious proapoptotic outcome in lymphomas other than Burkitt types (49).

The amphiphilic nature of the TCA and SSRI make them particularly suited to crossing the blood-brain barrier (50). Together with their high lipophilicity and a particularly phospholipid-rich environment means that these drugs concentrate strongly in the brain compared with the periphery (46, 51). We wish to suggest that—similar to current trials with clomipramine for brain tumors (12)—these properties make TCA and SSRI particularly exciting prospects for the treatment of CNS lymphoma. The evolution and origin of primary CNS lymphoma remains a mystery but like all non-Hodgkin's lymphomas, it continues to increase in frequency and is predominantly a B cell disease (36). It is highly aggressive, with a particularly poor prognosis, especially so in AIDS patients (52). Secondary CNS lymphoma also tends to be high-grade and is disproportionately frequent among Burkitt's patients (53).

In summary, we have shown the presence of the serotonin transporter to be a phenotype common to a nonselected panel of malignant lines representing B cell tumors of diverse origin. Its low to absent expression among normal B cells in their basal state indicates a degree of selectivity when considering it as a potential tumor target. The antiproliferative/proapoptotic actions of 5-HT and amphetamine analogs that act at the serotonin transporter indicate a potential in this respect, though given the concentrations required, it is unlikely that the actual drugs tested here would reach the clinic in this context. By contrast, antidepressants of either the SSRI or tricyclic class appear to offer greater promise even though their primary target is unlikely to be SERT itself. Notably, the concentrations at which they are effective in vitro—particularly on prolonged exposure—are comparable to steady-state levels achieved in the serum of individuals taking these drugs for depression and anxiety-related disorders. The lack of any conspicuous immune disturbance or leukopenia in this large, intensely studied patient cohort is particularly encouraging when considering these classes of antidepressants as adjuncts to current and/or failed therapeutic modalities in lymphoid malignancy.

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Table 1

Description of cell lines used in this study

Line	Patient diagnosis	Cytogenetics (where known)	Bcl-2 levels % pos (MFI)*
L3055	Burkitt's lymphoma (FAB L3)	t(8;14)	28 ± 1.5(1.5±0.3)
L3055/Bcl-2	*transfectant of above*	t(8;14)	97 ± 1.2(16±4.8)
KHM-2B	Burkitt's lymphoma	t(8;14);(14;18)	97 ± 0.7(6.6±2.2)
LILA	B-cell precursor ALL		92 ± 2.4(5.1±0.5)
JVM-2	B-cell prolymphocytic leukemia	t(11;14)	88 ± 5.6(4.9±1.4)
Rec-1	Mantle cell lymphoma	13q31-q32 amplification	71 ± 12(1.8±0.3)
NCEB-1	Mantle cell lymphoma	t(11;14)	40 ± 17(4.5±1.9)
Karpas 1106	Primary mediastinal BCL	complex	12 ± 8.8(2.5±1.1)
OZ	Diffuse large B cell lymphoma	t(14;18)	95 ± 1.6(6.6±1.0)
Karpas 422	Diffuse large B cell lymphoma	t(14;18)	98 ± 0.4(4.6±2.1)
PR-1	Diffuse large B cell lymphoma		93 ± 2.7(5.7±1.1)
CTB1	Diffuse large B cell lymphoma	t(14;22)	14 ± 7.6(2.0±0.2)
RC-K8	Diffuse large B cell lymphoma	complex	17 ± 6.3(2.0±0.2)
DoHH2	Diffuse large B cell lymphoma	t(14;18)	74 ± 9.7(3.7±0.6)
MD901	Diffuse large B cell lymphoma	t(3;14)	89 ± 7.5(4.6±0.5)
RL	Diffuse large B cell lymphoma	t(14;18)	81 ± 9.1(4.7±0.4)
KMS11	Multiple myeloma	t(4;14)	97 ± 0.4(5.0±1.6)
H929	Multiple myeloma	t(4;14)	90 ± 4.1(3.7±0.3)

*Results are given as means ± SEM of 3 separate experiments MFI, mean fluorescence intensity.

Table 2

DNA synthesis as % control at 24 h

Compound:	Clomipramine		Fluoxetine		MDMA		Fenfluramine		5-HT	Cocaine
μM present:	20	10	20	10	500	250	500	250	250	1000
Burkitt's lymphoma										
L3055	2.0±0.0	17±5.2	11±4.7	48±11	7.7±4.7	11±3.9	13±6.1	34±3.5	6.3±4.3	61±14
KHM-2B	26±5.0	60±11	47±6.9	68±4.7	47±13	95±39	19±5.5	42±2.2	65±4.5	57±21
Pre-B Acute lymphoblastic leukemia										
LILA	49±2.9	77±0.7	54±6.5	80±2.2	57±4.4	75±5.2	35±3.9	59±8.5	65±11	89±6.6
Prolymphocytic leukemia										
JVM-2	50±3.6	78±4.1	65±13	82±7.2	63±22	93±36	24±6.7	59±9.4	63±4.4	80±12
Mantle cell lymphoma										
Rec-1	28±8.4	66±7.7	48±16	74±7.6	46±21	60±20	11±5.8	43±5.9	11±8.1	76±13
NCEB-1	113±8.5	103±9.0	98±5.7	101±2.2	87±7.0	102±5.7	64±14	89±4.5	87±2.2	66±33
Primary mediastinal B-cell lymphoma										
Karpas 1106	56±7.5	75±23	76±1.7	116±26	58±11	82±8.8	35±15	64±7.3	36±11	67±8.0
Diffuse large B cell lymphoma										
OZ	32±7.7	75±10	56±9.0	85±4.3	13±4.1	47±8.3	6.0±3.2	17±7.8	0.4±0.2	63±19
Karpas 422	41±7.8	81±9.5	65±25	88±13	43±13	46±13	26±4.2	48±4.8	5.0±2.3	89±1.5
PR-1	12±2.3	67±19	32±13	78±24	53±12	67±12	14±2.6	41±5.5	44±9.4	80±7.0
CTB1	70±7.9	83±5.4	74±17	87±17	61±15	82±16	46±8.0	77±7.9	55±3.2	53±13
RC-K8	89±1.0	116±2.4	95±2.3	106±0.3	91±8.6	100±19	73±14	94±8.6	50±16	74±8.9
DoHH2	71±12	81±5.9	98±6.6	103±6.5	58±20	90±12	57±22	84±10	86±6.3	108±12
MD901	58±15	89±7.5	89±6.9	101±4.7	55±28	70±12	55±23	81±17	82±15	88±10
RL	87±14	103±6.3	85±5.6	99±9.0	87±14	98±11	62±11	92±4.0	86±5.6	139±30
Multiple Myeloma										
KMS11	69±14	80±4.3	24±11	44±18	54±4.3	71±5.6	9.0±4.9	39±12	70±3.2	112±46
H929	105±22	105±23	126±36	133±21	117±28	116±19	110±13	110±7.3	83±4.0	88±17

Results are given as means ± SEM of 3 separate experiments; Boxed figures = ≥50% inhibition compared with vehicle controls. MDMA, 3,4-methylenedioxymethamphetamine; 5-HT, 5-hydroxytryptamine.

Fig. 1

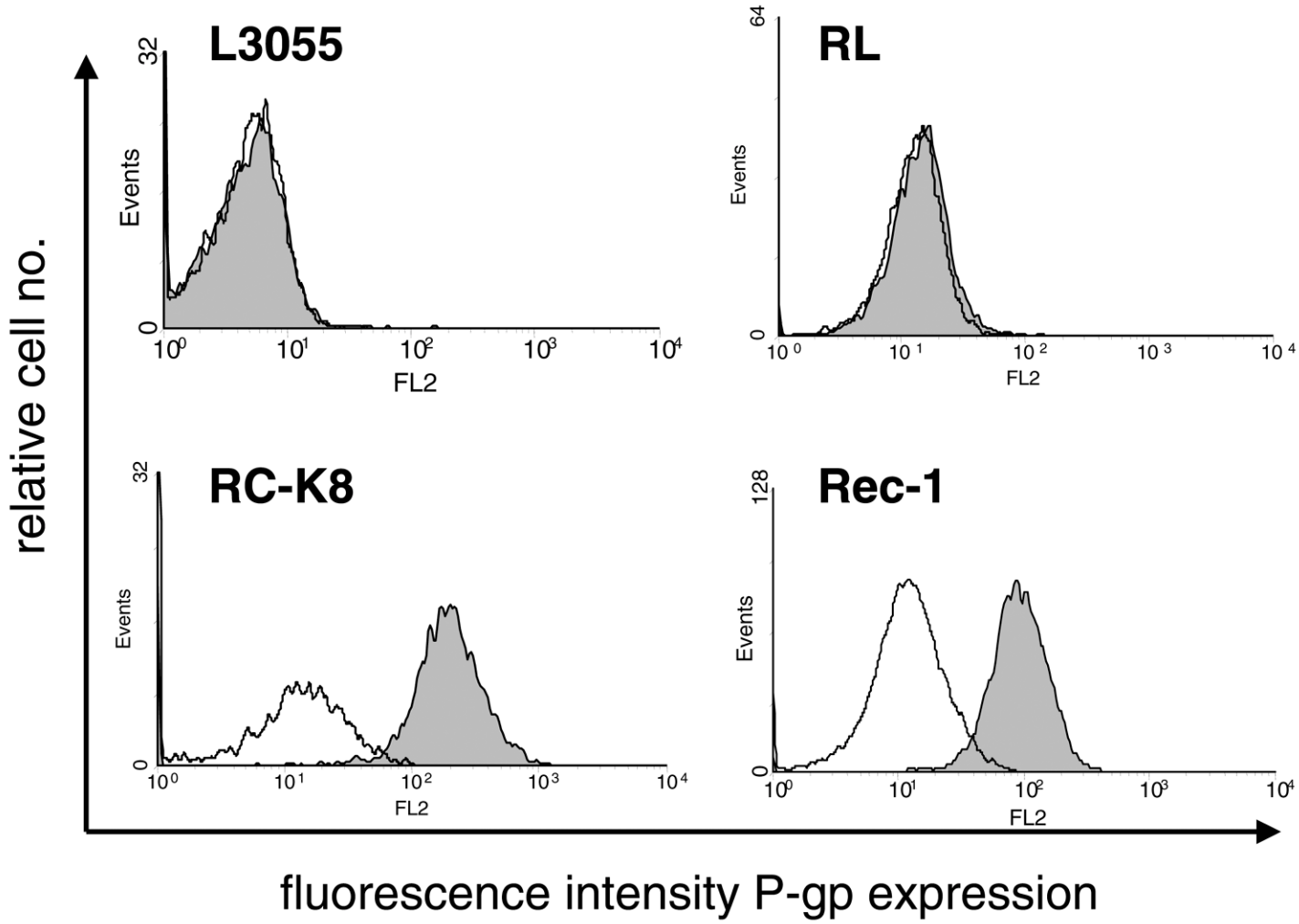

Figure 1. P-gp expression on malignant B cell lines. Cells stained for Pg-P as detailed in Materials and Methods: open histogram = control stain; solid histogram = specific Pg-p stain. Plots representative of 3 separate experiments.

Fig. 2

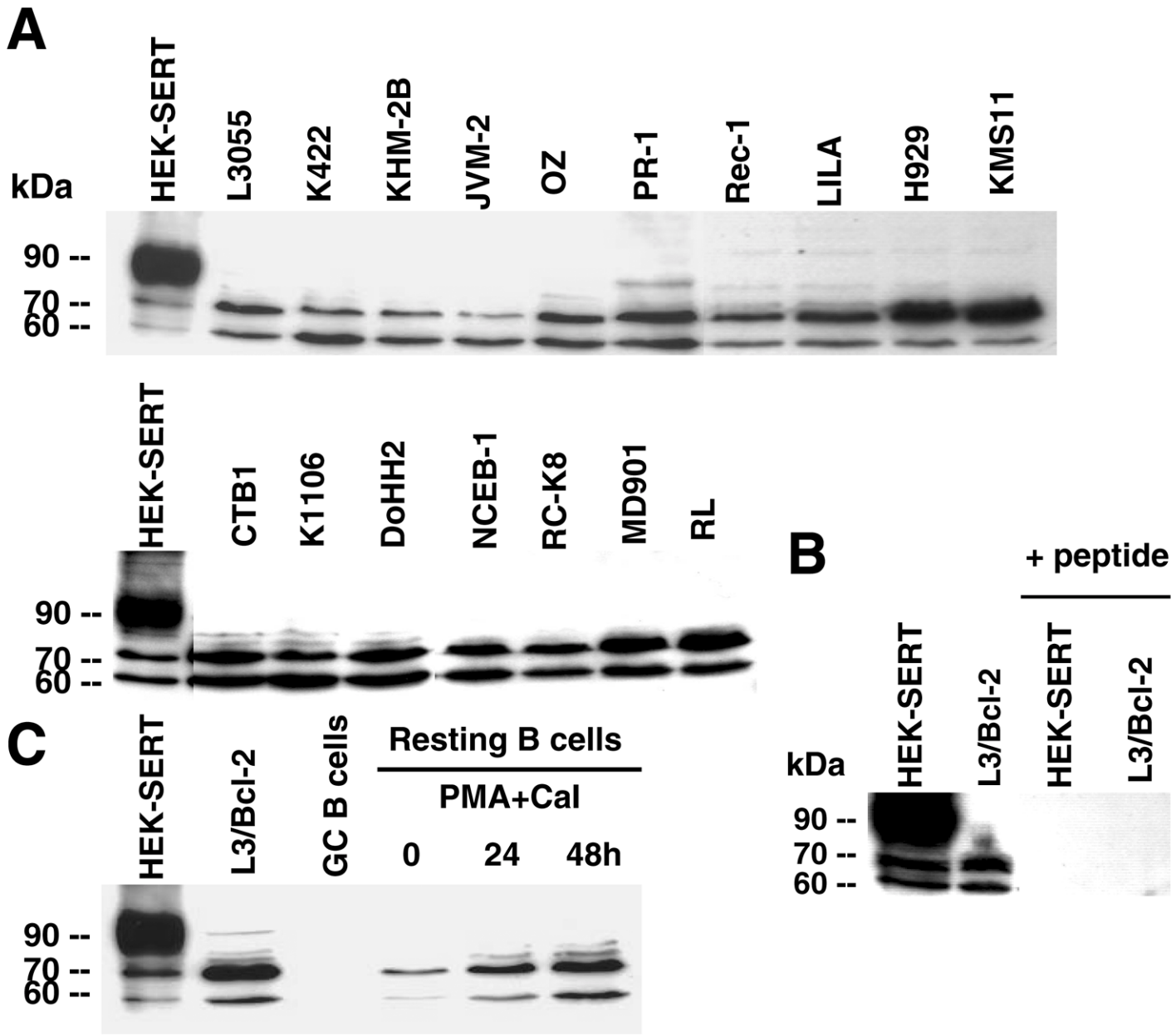

Figure 2. Western blot for SERT in malignant and normal B cells. In each individual blot 10 μg of protein from HEK293 cells transfected with full-length neuronal SERT (HEK-SERT) was resolved on the gels to provide comparison with 50 μg protein from B cells and B cell lines. ***A*)** Lysates prepared from panel of cell lines used in this study; ***B*)** Samples from HEK-SERT and L3055/Bcl-2 transfectants (L3/Bcl-2) blotted with anti-SERT in absence or presence of blocking peptide as indicated; ***C*)** Lysates form germinal center (GC) B cells or resting B cells stimulated with PMA (5 ng/ml) and calcium ionophore (CaI; 1 μg/ml) ionomycin for times shown. Blots are representative of 3 similar experiments.

Fig. 3

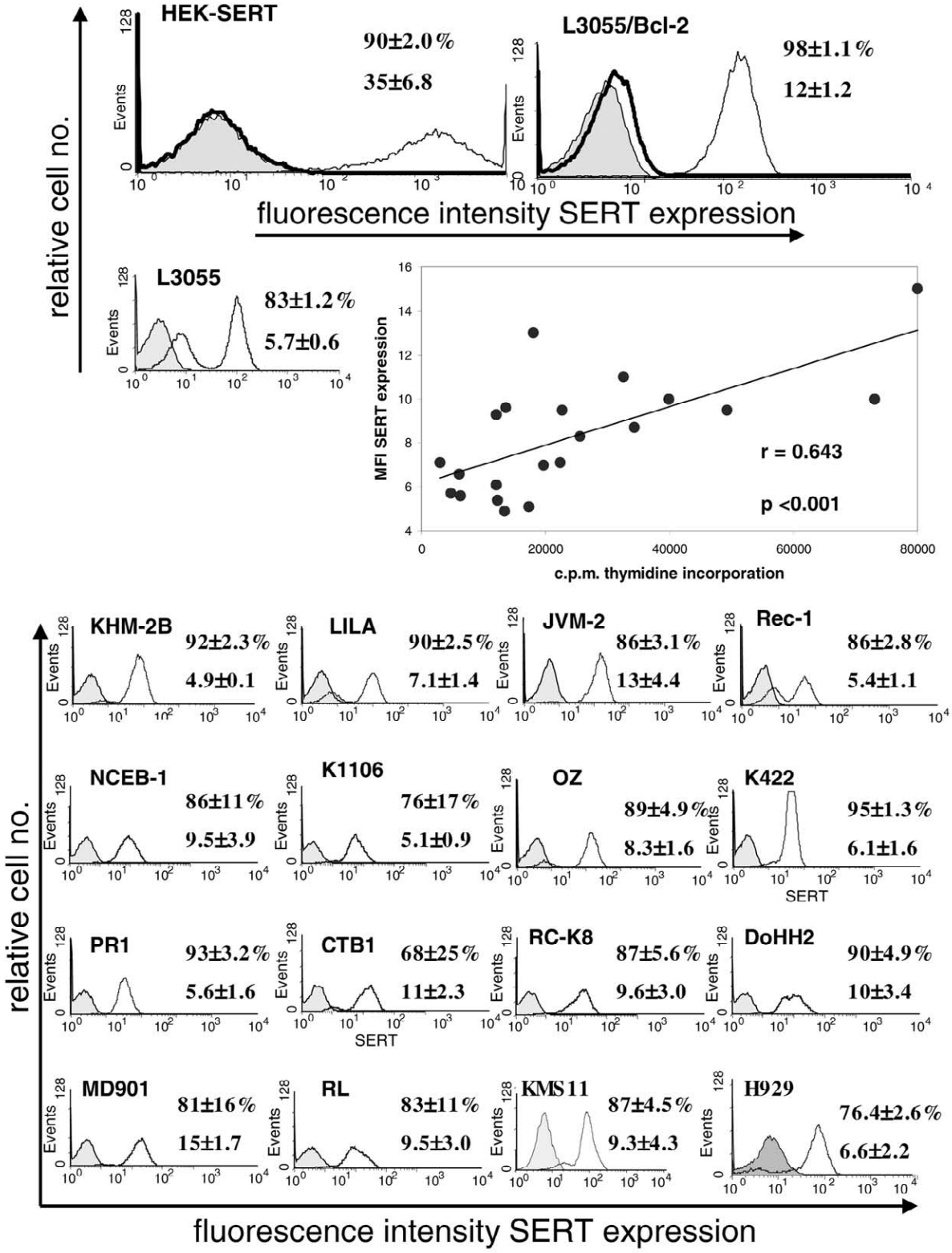

Figure 3. FACS-based analysis of SERT in B cell lines. Cells stained for total cellular SERT as described in Materials and Methods. Histograms shown as control stain (gray shaded) and specific SERT stain (open) with numbers representing % cells positive (***upper***) and mean fluorescence intensity (MFI) of stain (***lower***) as means ± SEM of 3 separate experiments. Top 2 histograms additionally indicate staining in the presence of blocking SERT peptide, shown as heavy line. Linear regression analysis plots mean counts per minute (cpm) ^{3}[H]Tdr incorporation in control cultures for each line against mean MFI. SERT expression from 3 independent experiments.

Fig. 4

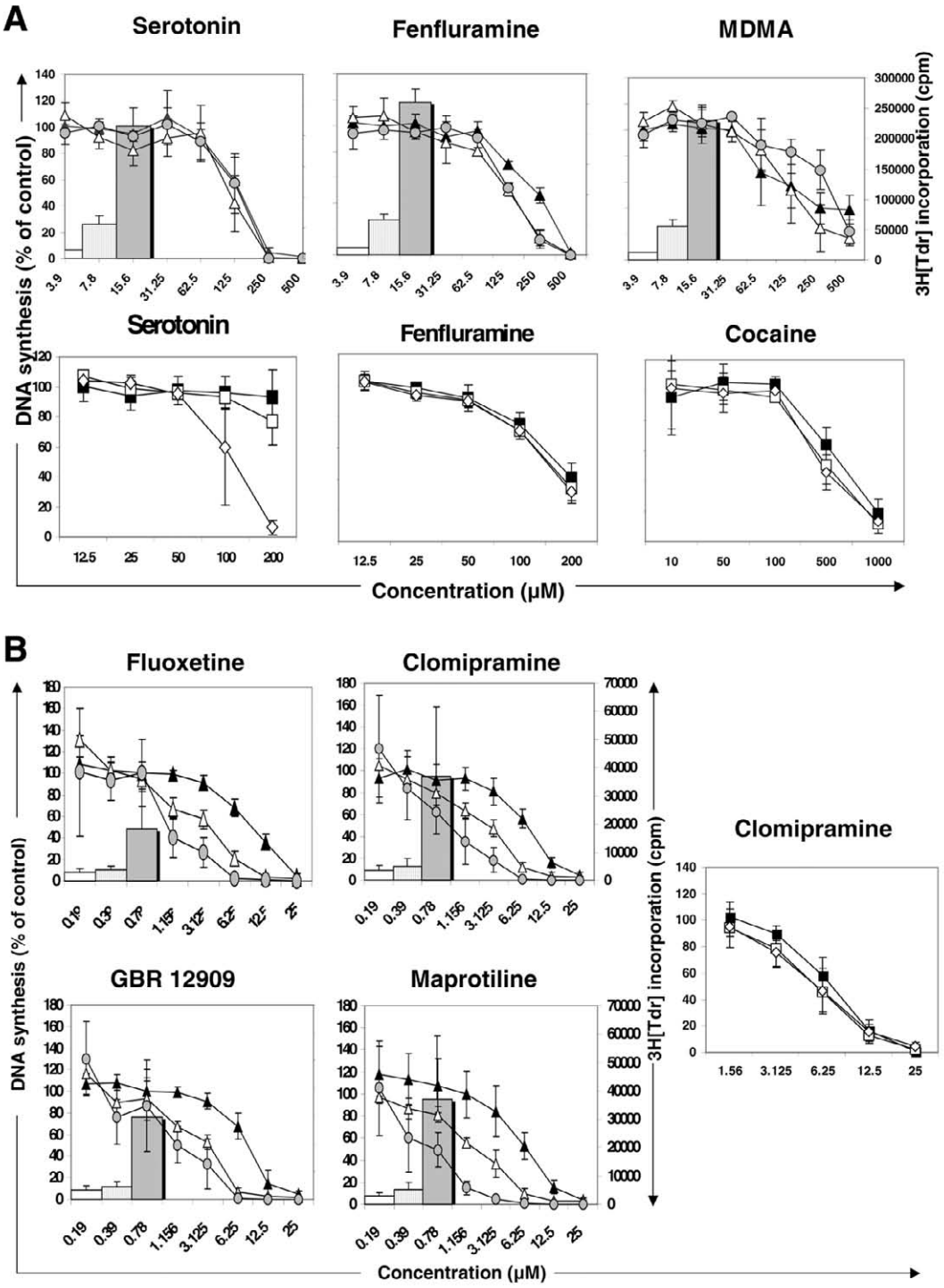

Figure 4. Time-course and concentration-dependent actions of 5-hydroxytryptamine (5-HT), amphetamines, and cocaine on DNA synthesis in L3055 cells. ***A, upper panel***) L3055 cells were seeded at an initial density of 2.5×10^4/ml in quadruplicate wells. Specifically, 100 μl of cells were added to 100 μl drug per well. Cells were incubated for 1 (solid triangles), 3 (open triangles) or 6 days (gray circles) then pulsed with 0.5 μCi/well of ^{3}[H]Tdr for the final 4h. Graphs show cpm for untreated cells (bars) and DNA synthesis as a % of control (untreated) for each time (lines) expressed as means ± SEM of 3 separate experiments. ***A, lower panel)*** As above except cells plated at three different cell densities: 2.5×10^4/ml (open diamonds), 5×10^4/ml (open squares) or 1×10^5/ml (solid squares) and incubated for 24 h before pulsing with ^{3}[H]Tdr for a final 4 h. ***B)*** In the set of 4 similar experiments, L3055 cells were cultured exactly as for (***A***) ***upper panel*** (seeded at an initial density of 2.5×10^4/ml in quadruplicate wells) with the drugs indicated. For the experiment shown top right with clomipramine, L3055 cells were cultured for 24 h at the different densities as detailed in (***A, lower panel***).

Fig. 5

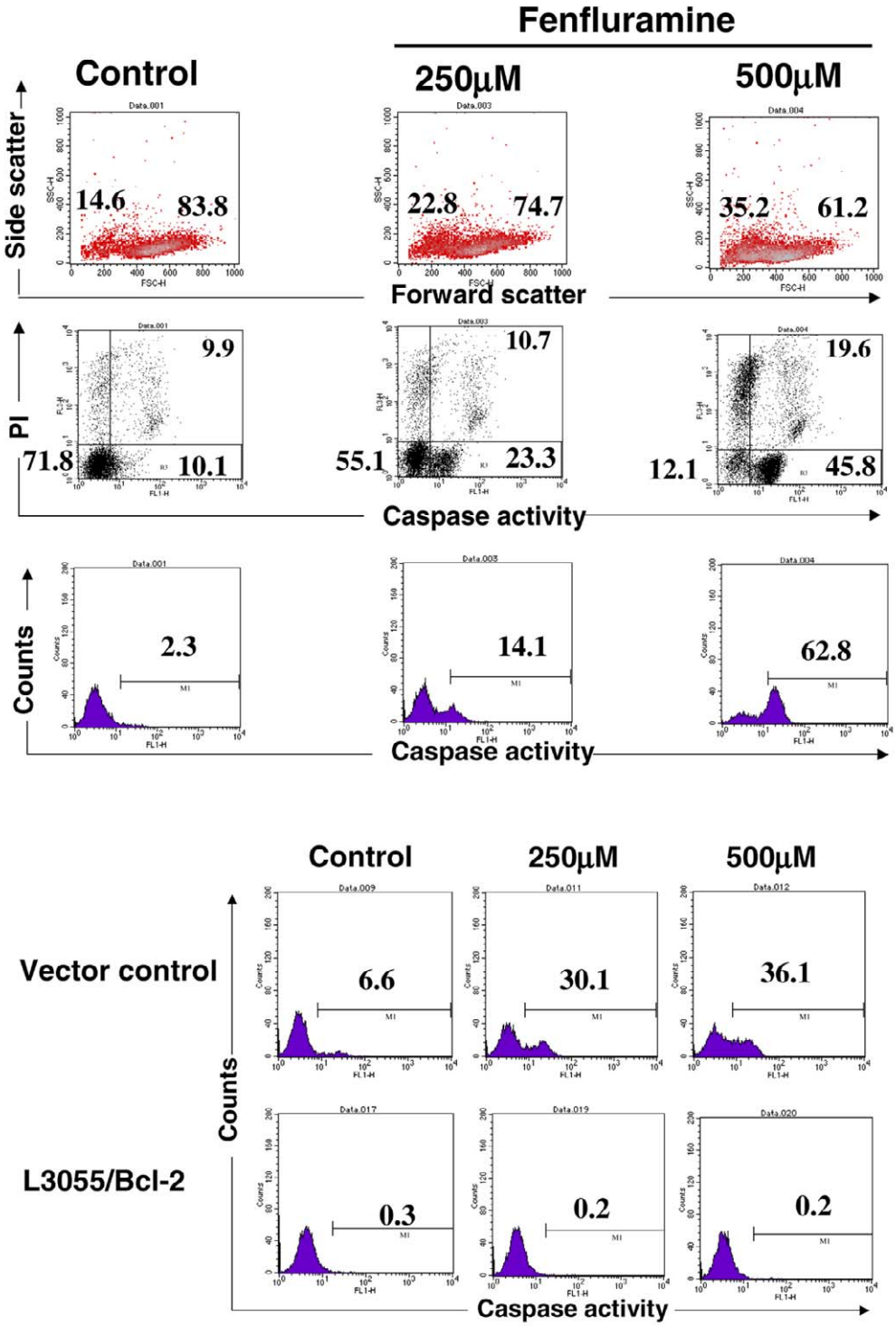

Figure 5. Cell death and apoptosis promoted in L3055 cells by fenfluramine. L3055 cells (or their transfected variants where indicated) were seeded at an initial density of 2.5×10^5/ml and treated for 6 h with or without (+/−)-fenfluramine at concentrations indicated then stained with PI and PhiPhiLux (for active caspases) as in Materials and Methods. Forward/Side scatterplots of total events indicate % cells falling into viable (right) and dead (left) gates. PI/caspase dot plots similarly show % of total events falling into viable (bottom left – $PI^{-ve}/casapse^{-ve}$), early apoptotic (bottom right –$PI^{-ve}/casapse^{+ve}$) and late apoptotic (top right –$PI^{+ve}/casapse^{+ve}$) gates. Histograms indicate % early apoptotic $caspase^{+ve}$ cells gated on the PI^{low} population. Data shown are representative of 3 independent experiments.

Fig. 6

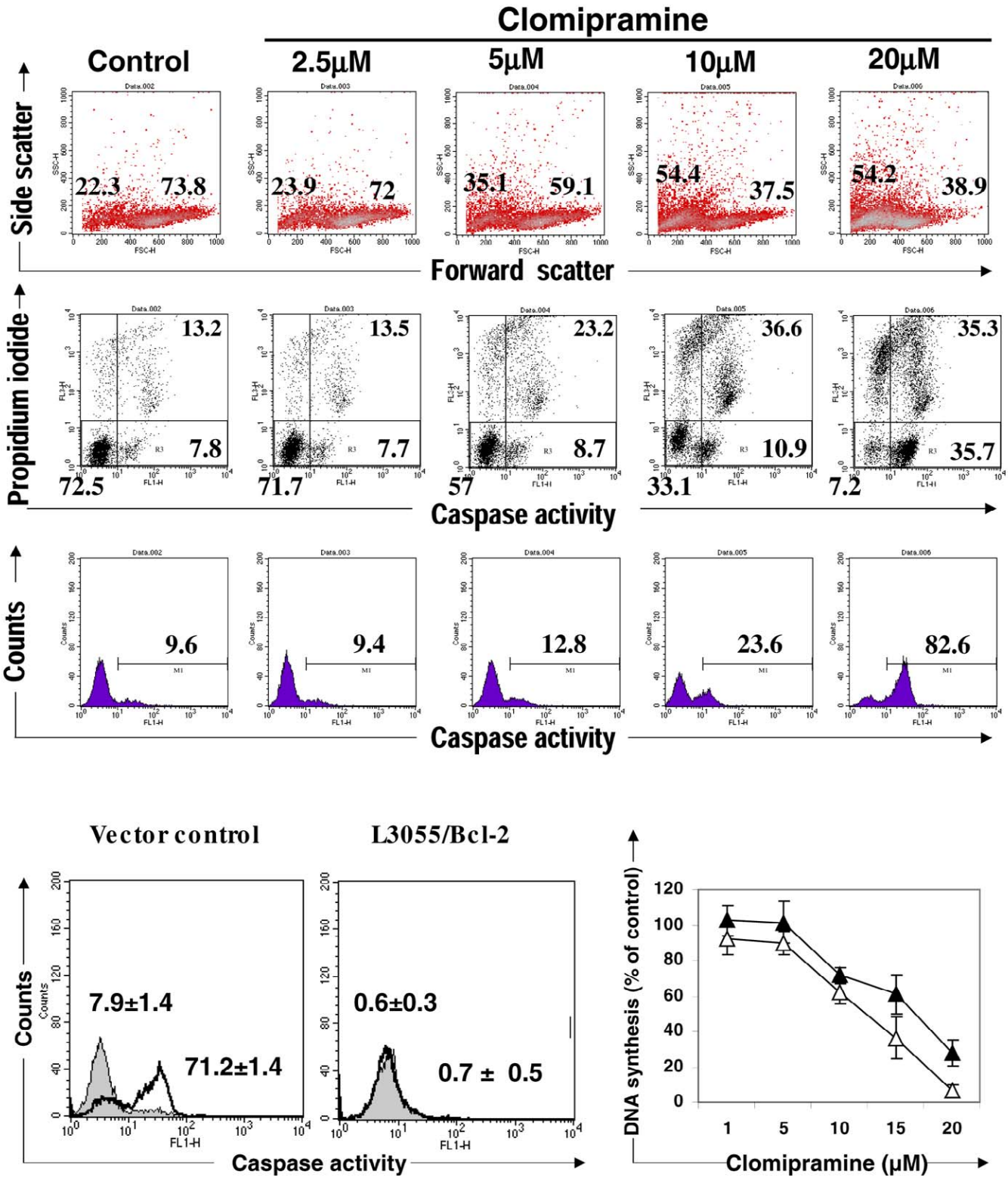

Figure 6. Cell death and apoptosis promoted in L3055 cells by clomipramine. Exactly as for **Fig. 5**, but this time with concentrations of clomipramine as indicated. ***Bottom*)** Histograms show levels of caspase activation in PI^{low} cells in untreated (shaded) vs. treatment with 20 µM clomipramine (open). ***Bottom right*)** showing DNA synthesis as in **Fig. 4** but using L3055/Vector controls (open triangles) and L3055/Bcl-2 transfectants (closed triangles). FACS data are representative or means ± SEM of 3 independent experiments; DNA synthesis expressed as means ± SEM of 3 separate experiments.

Fig. 7

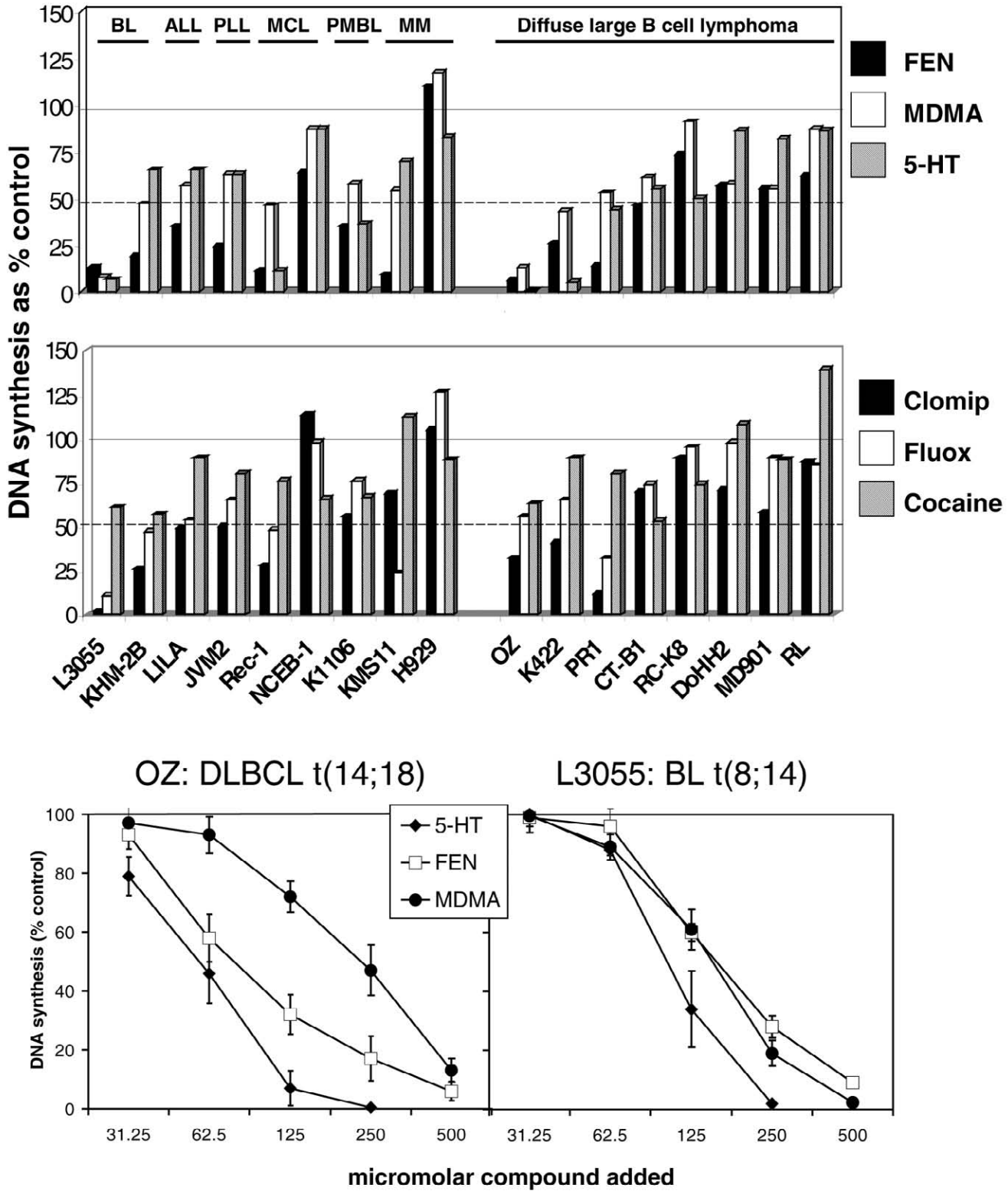

Figure 7. Antiproliferative actions of serotonergic compounds against B cell lines. Results shown as DNA synthesis (mean of 3 experiments) in response to compound as % of control (untreated) cultures. ***Top*)** Fen and 3,4-methylenedioxymethamphetamine (MDMA) at 500 μM; 5-HT, 250 μM; clomipramine and fluoxetine at 20 μM; cocaine, 1 mM. ***Bottom*)** as for **Fig. 4** but here comparing the OZ DLBCL line with L3055 BL cells.