Screening for Cytotoxic Compounds in Poor-prognostic Chronic Lymphocytic Leukemia

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Abstract. *Background/Aim: For chronic lymphocytic leukemia (CLL) patients with poor-prognostic genomic aberrations the therapeutic options are limited. We used the Spectrum Collection library to identify compounds with anti-leukemia activity in high-risk CLL. Materials and Methods: We identified substances with equal high cytotoxic activity in vitro in samples from poor-prognostic CLL (11q-/17p-, n=3) as compared to those from favourable-prognostic CLL (13q-, n=3). Cell survival was measured by fluorometric microculture cytotoxicity assay. Results: Out of 2,000 compounds, 65 had a similar effect in both prognostic groups. Fifteen compounds were selected for dose-response experiments in 16 additional CLL samples. Of these compounds, 12 continued to have similar cytotoxicity between prognostic subgroups. Additional experiments demonstrated that in CLL cells with 11q or 17p deletion, 5-azacytidine induced apoptosis in a dose-dependent manner and lipoprotein lipase expression was reduced following orlistat treatment. Conclusion: Using primary cultures of cells from high-risk CLL patients for compound screening is a feasible approach and that 5-azacytidine and orlistat exemplify substances that exhibit cytotoxicity in poor-risk CLL.*

Among a number of biological parameters that have emerged as independent prognostic markers in chronic lymphocytic leukemia (CLL), of particular importance are cytogenetic aberrations (1) and the mutational status of the immunoglobulin heavy variable (*IGHV*) genes (2, 3). Today, the marker portending the most adverse prognosis in CLL is deletion of chromosome 17p, covering the *TP53* gene, a key regulator of cell death following DNA damage (4). This deletion is found in approximately 5-10% of patients with CLL (1, 5, 6) and the median overall survival for patients within this group is approximately 2.5-4 years (1, 7, 8). CLL patients with 17p deletion usually have disease resistant to alkylating agents (9) and fludarabine (10); however, therapy with drugs such as steroids (11), lenalidomide (12) or alemtuzumab (13) may have an effect. Furthermore, deletion of 11q, found in 12-18% of patients with CLL (1, 5, 6) is also considered a marker of inferior prognosis and is associated with a lower response rate to purine analogs (14) and refractoriness to DNA-damaging chemotherapy (15). There is still a need for new anticancer agents that are active in the poor-prognostic CLL subgroups resistant to conventional therapy.

Today, most efforts to screen for new candidate drugs are based on compounds with known actions and molecular targets. This increase in biological knowledge has led to the development of high-throughput screening that allows identification of a large number of compounds that specifically interfere with the targets (16). However, there is a growing interest in screening systems based on substance-induced changes in a specific cellular phenotype using large and chemically diverse compound libraries (17). This approach may lead to the identification of compounds selectively affecting specific cellular subtypes. When screening for new treatments, cell lines are often the starting material of choice since they can provide initial information regarding antitumor efficiency and mechanisms of action (18). However, primary patient tumor cells would provide a more robust clinical validation, given that cell lines may lose many of the characteristics of the original cells.

Considering the need for new treatment strategies in poor-prognostic CLL, in particular for patients with del(17p), it would be of value to screen a large number of compounds on primary CLL cells from these patients. In this study, the Spectrum Collection compound library was utilized to

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identify substances that may have cytotoxic activity in CLL with poor-prognostic aberrations. This library consists of 2,000 compounds (1,000 pharmacological substances, 580 natural products and 420 additional compounds with varying functions *e.g.* receptor blockers and cellular toxins) with a wide range of biological activity and structural diversity (19). Since this procedure requires a large number of CLL cells (140×10^6), we initially screened primary tumor cells from three patients with CLL with poor-prognostic aberrations (*i.e.* 11q/17p deletion) and three patients with favorable prognostic aberrations (*i.e.* 13q deletion) as well as peripheral blood mononuclear cells (PBMCs) from two healthy donors.

Materials and Methods

Patient samples and cell preparation. Primary cell samples were obtained from 24 patients with CLL at Uppsala University Hospital, Uppsala, Sweden and G. Papanicolaou Hospital, Thessaloniki, Greece. Samples were collected mainly from peripheral blood but also from bone marrow. Patients were diagnosed according to recently revised criteria (20) and all samples contained ≥70% tumor cells. Genomic aberrations, immunoglobulin heavy variable (*IGHV*) gene mutational status and gender are detailed in Table I. Peripheral blood mononuclear cells (PBMC) from five healthy donors (screening n=2, dose-response experiment n=3) were used for comparison purposes. Informed consent was provided according to the Declaration of Helsinki, and the study was approved by the local Ethics Review Committee of each institution.

Lymphocytes were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density-gradient centrifugation (21). Cell preparations were cryopreserved and stored at –150˚C or in liquid nitrogen in fetal calf serum with 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich Co., St. Louis, MO, USA). Immediately before use, the cells were thawed and washed twice.

Compounds. All substances (n=2,000) from the Spectrum Collection compound library (MicroSource Discovery Systems Inc., Gaylordsville, CT, USA) were supplied at a concentration of 10 mM in DMSO and further diluted with phosphate buffered saline (PBS), transferred to 384-well microplates using the programmable pipetting robot Biomek2000 (Beckman Coulter Inc, Fullerton, CA, USA) and for the initial screening tested at a final concentration of 10 μM. For the dose-response analyses that followed, compounds were also purchased from MicroSource Discovery Systems Inc. with the exception of fludarabine and prednisolone, which were purchased from the local pharmacy, and brefeldin A and sodium lasalocid which were obtained from Sigma Aldrich. All compounds were tested in duplicate and at five concentrations, with five-fold serial dilutions starting at 250 μM for prednisolone and hexetidine; 50 μM for acetochlor, benserazide hydrochloride, colchicine, anisomycin, ethacrycin acid, thimerosal, 5-azacytidine, sodium lasalocid, orlistat, amitriptyline hydrochloride, clomipramine hydrochloride, podofilox and fludarabine; and 10 μM for carvedilol, oxyphenbutazone and brefeldin A. Substances were diluted and transferred to 384-well microplates as described above, and plates were stored at –70˚C until required.

Table I. *Molecular characteristics of patients with chronic lymphocytic leukemia included in the present study.*

Sample	Gender	IGHV mutational status[+]	FISH[‡]
1	M	M	del(13q)
2*	M	M	del(13q)
3	M	M	del(13q)
4	F	UM	del(17p), del(11q)
5	M	UM	del(11q)
6	M	UM	del(17p)
7	M	M	del(13q)
8	M	M	del(13q)
9	F	M	del(13q)
10	M	M	del(13q)
11	M	M	del(13q)
12	M	UM	del(17p)
13	M	UM	del(17p), del(11q)
14	F	UM	del(17p)
15	M	UM	del(11q)
16	F	UM	del(11q)
17	M	UM	del(11q)
18	M	No data	del(11q)
19	M	UM	del(11q)
20**	F	UM	del(17p)
21	M	UM	del(17p)
22	M	UM	del(11q)
23	M	UM	del(11q)
24	F	UM	del(17p)

From references [+](2, 3); [‡](1). Samples 1-6 Spectrum screening; 7-22 dose response experiments; 20, 22-24 apoptosis experiments. *Included in both the Spectrum screening and dose-response experiments;**included in both the dose-response and apoptosis experiments. IGHV: immunoglobulin heavy variable; FISH: fluorescence *in situ* hybridization. M: IGHV mutated. UM: IGHV unmutated.

Cytotoxicity assay. Cell survival was measured using the fluorometric microculture cytotoxicity assay (FMCA) (21, 22). In brief, FMCA is a total cell kill assay, where cells with an intact membrane convert non-fluorescent fluorescein diacetate to fluorescent fluorescein. For the cytotoxicity experiments, the required density for CLL cells was reached by adding approximately 50,000 cells to 45 μl cell suspension that was subsequently seeded in the compound prepared microplates using the programmable pipetting robot Precision 2000 (Bio-Tek Instruments, Inc., Winooski, VT, USA). This resulted in a total of 140×10^6 primary cells being required for each CLL sample used in the screening.

Tumor cells were incubated with compounds at 37˚C for 72 h and were thereafter placed in the automated Optimized Robot for Chemical Analysis (Orca, Beckman Coulter) using the software SAMI (Beckman Coulter). Fluorescence was measured by a Fluostar Optima instrument (BMG Technologies, Offenburg, Germany) at 485 nm/520 nm. Cytotoxic activity is presented as a survival index (SI), which is defined as the percentage of mean fluorescence in duplicate wells as compared to control wells once blank values were subtracted. Therefore, a low SI value indicates a high cytotoxic effect. A successful assay required a ratio of >5 between control wells and blank wells, a coefficient of variation

(CV) of <30% in control wells, and a tumor cell fraction exceeding 70% in the cell preparation.

Screening and hit validation. Screening of the Spectrum library was performed using cells from three patients harboring poor-prognostic aberrations (11q-, 17p-) and three patients with favorable genomic markers (13q deletion). The criteria for compounds to be considered for further analysis were: i) Substantial activity in poor-prognostic samples *i.e.* an SI value <50% in two out of the three samples carrying poor-prognostic aberrations (11q-, 17p-); ii) similar activity in poor- and favorable-prognostic samples *i.e.* an SI ratio of good/poor prognostic samples >0.5. Compounds with generally high toxicity (SI<20% in all samples tested) were excluded.

Fifteen of the screening hits were selected for further validation, together with fludarabine, prednisolone and brefeldin A. Fludarabine was included since it is known that CLL patients with 17p deletion have a poor response to fludarabine (23), whereas prednisolone and brefeldin A were included since they have been shown to function independently of *p53* status (11, 24). A dose-response study was subsequently performed using CLL samples carrying 13q deletion (n=6), 11q deletion (n=5), and 17p deletion (n=5), and PBMCs from healthy donors (n=3). Unlike the screening experiments where CLL samples with 17p- and 11q- were analyzed as a group, 11q- and 17p- samples included in the dose-response experiments were analyzed separately.

5-Azacytidine and orlistat treatment. Two interesting substances to emerge from both the screening and dose-response experiments were the methyl inhibitor 5-azacytidine and the lipoprotein lipase (*LPL*) inhibitor, orlistat. We therefore wanted to examine the effect of treatment with 5-azacytidine or orlistat on poor-prognostic CLL by analyzing the rate of apoptosis and *LPL* gene expression. CLL cells from patients with 17p- (n=2) and 11q- (n=2) were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 4 mM glutamine, 10% fetal bovine serum (FBS; Invitrogen), and 1× penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37˚C in an atmosphere with 5% CO_2 until confluent. Cultured cells were counted, checked for viability (generally above 98%) and split to contain 1×10^6 cells/ml. For 5-azacytidine treatment, the drug concentrations used were 0.2 and 10 μM for 48 h, and for orlistat treatment the drug concentration was 2 μM for 72 h.

The rate of apoptosis in CLL samples prior to and after 48 h of treatment with 5-azacytidine was detected using annexin V staining. Labeled annexin V for detection of phosphatidyl serines exposed on the plasma membrane was carried out according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA, USA). Apoptosis was calculated as the percentage of annexin V-positive cells, corrected for the percentage of spontaneous apoptosis observed in control wells.

LPL gene expression analysis using RQ-PCR was performed on cDNA from a sample with 17p- and another with 11q-, both samples treated with orlistat. Total RNA was isolated from cells using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA integrity and quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and cDNA was synthesized from 400 ng total RNA using the M-MLV RT kit (Invitrogen AB, Sweden). The *LPL* gene expression assay was purchased from Applied Biosystems (Applied Biosystems, Applera Sweden, Applera Europe BV). Gene expression data was normalized against internal beta-2 microglobulin (*B2M*) expression levels using the comparative Ct method and all reactions were run on a Stratagene Mx 3005 instrument (La Jolla, CA, USA).

Statistical analysis. The 50% inhibitory concentration (IC_{50}) was determined by non-linear curve fit to Hills equation using GraphPad Prism5 software (GraphPad Software, Inc., San Diego, CA, USA). Mean IC_{50}s of the compounds were compared between the two prognostic subgroups. For some of the substances, flat concentration-effect curves did not allow proper determination of the IC_{50}, and in such instances, the SI at the concentration where the mean SI was as close to 50% as possible was used (acetochlor, sodium lasalocid 50 μM, amitriptyline hydrochloride, prednisolone 10 μM, orlistat, thimerosal 0.4 μM and anisomycin, colchicine, podofilox 0.08 μM) to compare the prognostic subgroups. Data were analyzed using non-parametric statistics: Mann-Whitney test was applied to compare medians between groups ($p\leq0.05$ significant).

Results

Screening of the spectrum collection compound library. Having screened all substances from the Spectrum Collection compound library at a concentration of 10 μM on six CLL samples, 129/2000 (6.5%) compounds had an SI value <50% in two out of the three samples with poor-prognostic aberrations. However, of these 129 compounds, 28 had an SI value <20% in all six CLL samples as well as in the healthy PBMCs. These substances were therefore considered to be too toxic/non-selective and were excluded from further analysis. Among the remaining 101 compounds, an SI ratio of good/poor prognostic samples >0.5 was observed for 65 substances. Since we have previously suggested corticosteroids to be effective in poor-prognostic CLL with unmutated *IGHV* genes, we also excluded the steroids (n=13) (25). The 52 remaining compounds represented many different bioactivities *e.g.* anti-neoplastic, anti-mitotic and anti-depressant (Table II). To further reduce the number of substances to a practical level, factors such as their effect on healthy PBMCs, as well as solubility and known characteristics of the compounds were considered. The chosen 15 substances represented a broad range of bioactivities (Table III). The workflow to identify the compounds within the library displaying high cytotoxic activity in poor-prognostic CLL samples is illustrated in Figure 1.

Hit validation. As described above, 15 compounds, in addition to fludarabine, prednisolone and brefeldin A, were further selected for dose response experiments in additional CLL samples (17p-, n=5, 11q-, n=5, 13q-, n=6). Due to flat dose-response curves for some substances, the IC_{50} could only be calculated for nine compounds (Figure 2a), whereas for the remaining nine compounds, the SI value at a set concentration close to the IC_{50} was used for the comparison between prognostic groups (Figure 2b). A statistically significantly higher sensitivity was observed in 13q-deleted cases than in 17p-deleted samples for podofilox (Figure 2b). Additionally, a trend for higher sensitivity (arbitrarily defined

Table II. *Results for the 52 screening hits from the Spectrum Collection compound library. Mean survival index (SI) for del(13q) (n=3), del(17p)/del(11q) (n=3) and peripheral blood mononuclear cells (n=2).*

Compound	13q-	17p-,11q-	Ratio 13q-/17p-,11q-	PBMC	Bioactivity
Methiothepin maleate	NA	10	NA	22	5HT1&2 receptor antagonist
Tegaserod maleate	44	50	0.9	79	5HT4 receptor agonist, peristaltic stimulant
Disulfiram	4	5	0.8	4	Alcohol antagonist
Sodium monensin	11	9	1.2	18	Antibacterial
Sodium lasalocid	26	28	0.9	67	Antibacterial
Sodium salinomycin,	5	3	1.4	29	Antibacterial
Cloxyquin	19	33	0.6	56	Antibacterial, antifungal
Zinc pyrithione	4	3	1.4	3	Antibacterial, antifungal, antiseborrheic
Amitriptyline hydrochloride	30	38	0.8	45	Antidepressant
Alaproclate	47	63	0.8	74	Antidepressant
Fluoxetine	5	3	1.5	24	Antidepressant
Clomipramine hydrochloride	23	20	1.1	51	Antidepressant
Sertraline hydrochloride	2	2	1.0	2	Antidepressant, 5HT uptake inhibitor
Amoxepine	31	43	0.7	51	Antidepressant, inhibits norepinephrine uptake
Prochlorperazine edisylate	27	39	0.7	44	Antiemetic, antipsychotic, treatment of vertigo
Cyclopirox olamine	22	33	0.7	33	Antifungal
Hexetidine	61	36	1.7	44	Antifungal
Phenylmercuric acetate	3	4	0.6	24	Antifungal
Thiram	6	5	1.2	3	Antifungal
Chloroacetoxyquinoline	6	10	0.6	33	Antifungal
clemastine	28	33	0.8	45	Antihistaminic
Benzethonium chloride	7	12	0.6	8	Antiinfective (topical)
Benzalkonium chloride	5	8	0.6	4	Antiinfective (topical)
Thimerosal	30	15	2.0	18	Antiinfective, preservative
Oxyphenbutazone	21	38	0.5	53	Antiinflammatory
Colchicine	26	29	0.9	89	Antimitotic, antigout agent
Colchiceine	10	8	1.1	38	Antimitotic, antigout agent
Aclacinomycin a1	NA	2	NA	3	Antineoplastic
Mitoxanthrone hydrochloride	7	6	1.1	3	Antineoplastic
4'-Demethylepipodophyllotoxin	8	9	0.9	40	Antineoplastic
Puromycin hydrochloride	4	8	0.6	8	Antineoplastic, antiprotozoal
Podofilox	21	26	0.8	62	Antineoplastic, inhibits microtubule assembly, and human DNA topoisomerase II; antimitotic agent
5-Azacytidine	34	34	1.0	94	Antineoplastic, pyrimidine antimetabolite
Vinblastine sulfate	7	11	0.7	18	Antineoplastic, spindle poison
Hydroquinone	6	3	1.7	5	Antioxidant
Anisomycin	34	16	2.1	18	Antiprotozoal, antifungal, protein synthesis inhibitor
Thioridazine hydrochloride	24	40	0.6	49	Antipsychotic
Chlorprothixene hydrochloride	36	57	0.6	45	Antipsychotic
Carvedilol	19	9	2.0	30	Betaadrenergic blocker
Benserazide hydrochloride	42	36	1.2	65	Decarboxylase inhibitor
Ethacrynic acid	23	17	1.4	66	Diuretic
Astemizole	4	1	3.1	2	H1 antihistamine (nonsedating)
Propachlor	3	2	1.9	5	Herbicide
Acetochlor	26	31	0.8	76	Herbicide, cell division inhibitor
Triptonide	22	17	1.2	17	Immunosuppressant, antineoplastic
Cyclobenzaprine hydrochloride	25	31	0.8	33	Musle relaxant (skeletal)
Orlistat	38	52	0.7	83	Reversible lipase inhibitor, antiobesity
2-Methyl gramine	10	8	1.3	30	NA
Totarol-19-carboxylic acid	24	45	0.5	57	NA
Kinetin riboside	5	6	0.8	13	NA
18-Aminoabieta-8,11,13-triene sulfate	23	21	1.1	44	NA
Podophyllotoxin acetate	7	10	0.7	25	NA

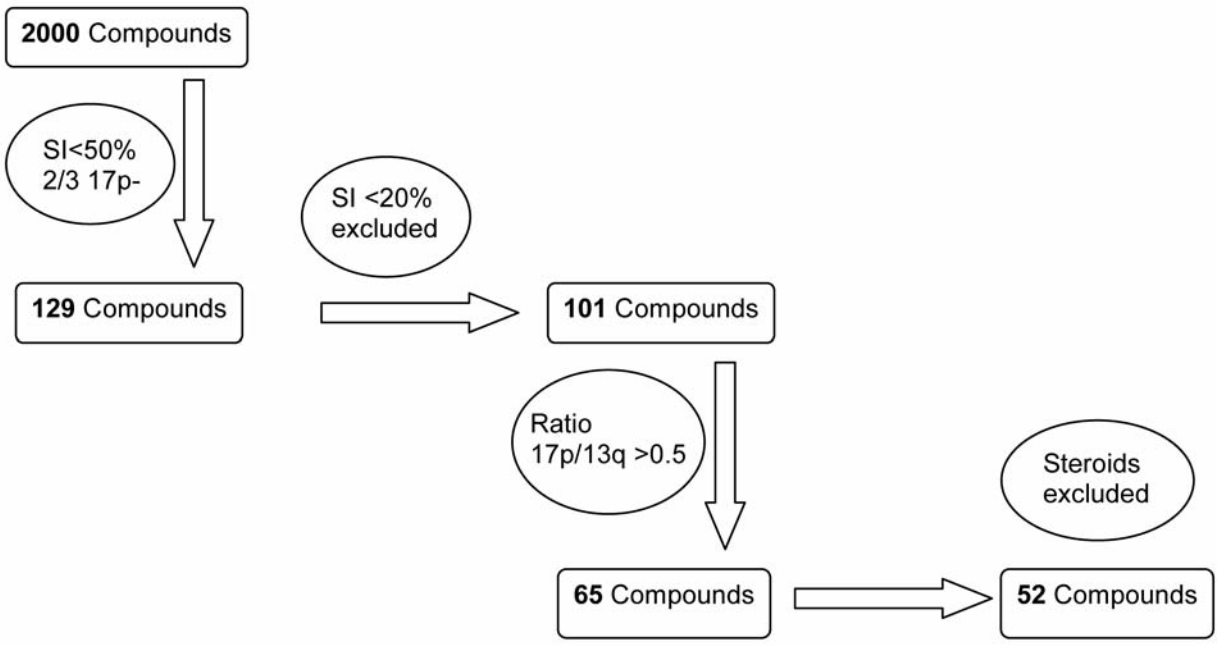

Figure 1. *Flowchart outlining the selection of compounds for dose-response experiments following library screening.*

Table III. *Details of the 15 compounds selected from the Spectrum Collection drug library for dose-response analysis. Mean survival index (SI) at 10 μM for del(13q) (n=3), del(17p)/del(11q) (n=3) and PBMCs (n=2) are shown.*

Compound	13q-	17p-, 11q-	Ratio 13q-/17p-,11q-	PBMC	Bioactivity
5-Azacytidine	34	34	1.0	94	Antineoplastic, pyrimidine antimetabolite
Acetochlor	26	31	0.9	76	Herbicide, cell division inhibitor
Amitriptyline hydrochloride	30	38	0.8	45	Antidepressant
Anisomycin	34	16	2.1	18	Antiprotozoal, antifungal, protein synthesis inhibitor
Benserazide hydrochloride	42	36	1.2	65	Decarboxylase inhibitor
Carvedilol	19	9	2.0	30	Beta adrenergic blocker
Clomipramine hydrochloride	23	20	1.2	51	Antidepressant
Colchicine	26	29	0.9	89	Antimitotic, antigout agent
Ethacrynic acid	23	17	1.4	66	Diuretic
Hexetidine	61	36	1.7	44	Antifungal
Sodium lasalocid	26	28	0.9	67	Antibacterial
Orlistat	38	52	0.7	83	Reversible lipase inhibitor, antiobesity
Oxyphenbutazone	21	38	0.6	53	Anti inflammatory
Podofilox	21	26	0.8	62	Antineoplastic, inhibits microtubule assembly, and human DNA topoisomerase II; antimitotic agent
Thimerosal	30	15	2.0	18	Anti infective, preservative

as ≥2× IC_{50} or ≥20% difference in median SI values) in 13q-deleted samples compared to poor-prognostic samples was observed for colchicine and sodium lasalocid (Figure 2b). The remaining 12 compounds chosen following the screening process retained their activity in 17p-deleted cells. As expected, there was a trend for lower sensitivity in 17p-deleted cases *versus* 13q-deleted cases for fludarabine (Figure 2a) but not for prednisolone (Figure 2b). The mean dose-response curves for all compounds in each prognostic subgroup are provided in Figure 3.

Many compounds demonstrated higher cytotoxic activity in CLL cells compared to healthy PBMCs. Eight substances, 5-azacytidine, carvedilol, colchicine, hexetidine, orlistat, podofilox, oxyphenbutazone, and clomipramine hydrochloride,

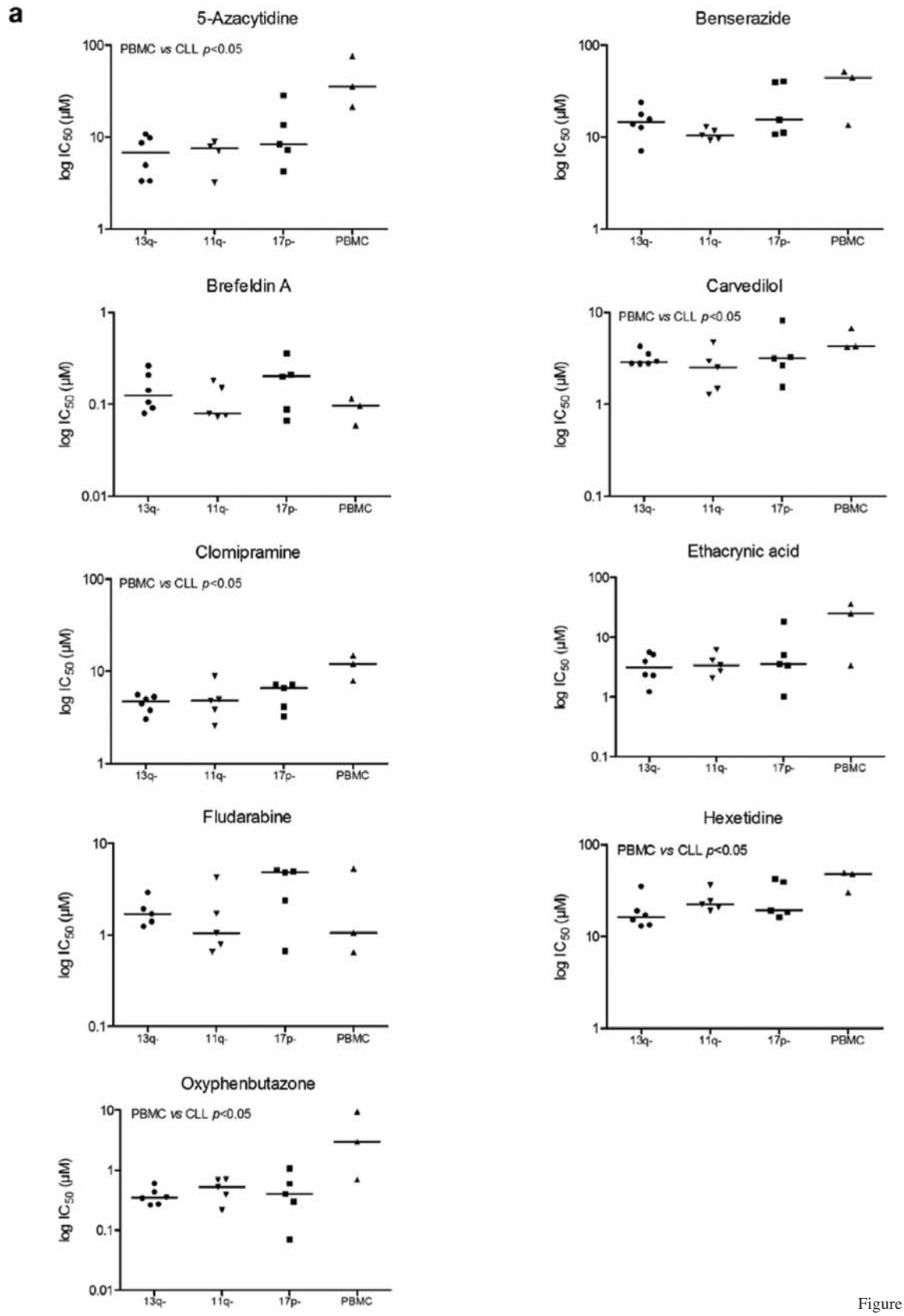

Figure 2. *Continued*

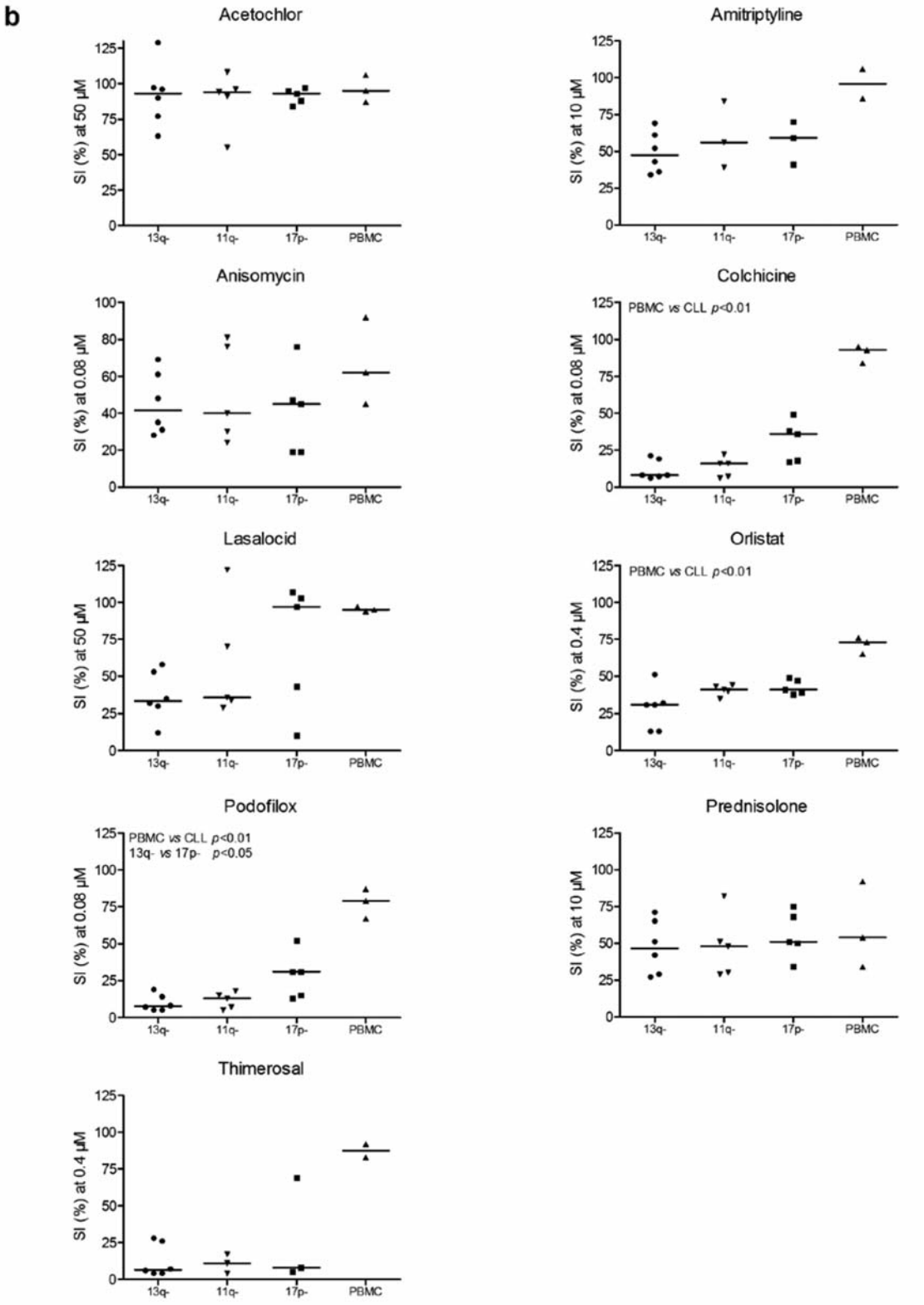

Figure 2. *In vitro activity of the 15 screening hits and three reference compounds in primary tumor cells from patients with chronic lymphocytic leukemia with 13q- (n=6), 11q- (n=5), 17p- (n=5) and healthy PBMC (n=3). The effect is expressed as a: IC_{50} and b: survival index (SI%) at 50 µM, acetochlor, sodium lasalocid; 10 µM, amitriptyline hydrochlrode, prednisolone; 0.4 µM, orlistat, thimerosal; and 0.08 µM, anisomycin, colchicine, podofilox. Each dot represents one sample and the line represents the median value of the samples.*

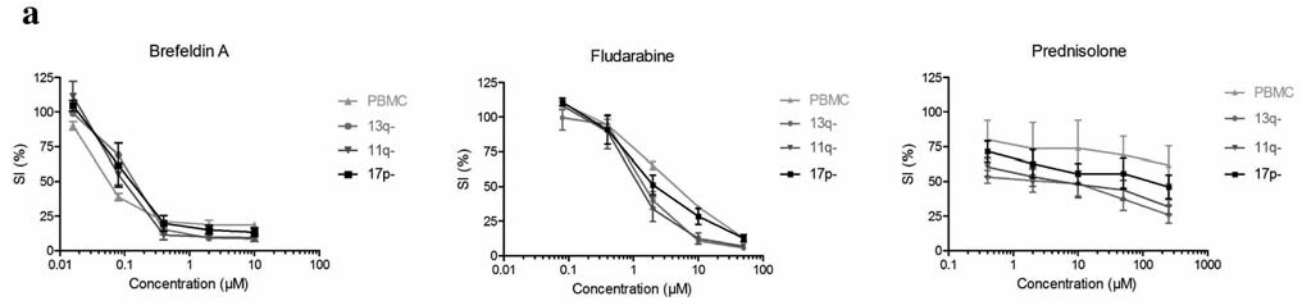

Figure 3. *Continued*

had significantly higher IC_{50} or SI values for healthy PBMCs compared to CLL samples (Figures 2 and 3).

Treatment with 5-azacytidine may induce apoptosis. In order to examine the apoptotic effect of 5-azacytidine, annexin V experiments were performed on four poor-prognostic CLL samples, where two had 17p deletion and two 11q deletion. An increase in apoptosis was observed for three out of four samples after 48 h treatment with increasing concentrations of 5-azacytidine (Figure 4), whereas for the remaining sample with 17p deletion, no effect was observed. The initial viability of the samples analyzed was ≥90%, which was determined by the trypan blue exclusion test.

Orlistat treatment may lead to down-regulation of LPL. We then performed gene expression analysis to examine whether treatment with the *LPL* inhibitor orlistat can effect *LPL* expression in poor-prognostic CLL (one 11q and one 17p deleted sample), as measured by RQ-PCR following 72 h of incubation with orlistat. Expression of *LPL* clearly decreased after treatment with orlistat (Figure 5).

Discussion

Despite recent advances in the treatment of CLL, there is still a need for new treatment strategies for CLL patients with poor-prognostic genomic aberrations. In our current effort to identify interesting compounds for these patients, 65 out of the 2,000 compounds screened fulfilled our criteria, and 15 were subsequently chosen for further analysis, including antidepressants, antineoplastics and antifungals. Notably, 12/15 substances selected for further studies were found to retain their activity in CLL cells with poor-prognostic genomic aberrations, and hence may be of interest for future investigations.

Many of the selected compounds had a greater effect on CLL cells compared to healthy PBMCs, where the most striking CLL selectivity was observed for the tubulin inhibitors colchicine ($p<0.01$) and podofilox ($p<0.01$). However, patients with 17p deletion may be unresponsive to this group of compounds, since in our current study, podofilox was the only compound that had a significantly lower effect on 17p-deleted samples than 13q-deleted samples ($p<0.05$). Furthermore, a trend for lower sensitivity in 17p-deleted samples was also observed for both colchicine and sodium lasalocid. Taken together, these results may indicate a therapeutic value for colchicine and podifilox in CLL, however, not for patients with 17p deletion.

Fludarabine was included in the study since it has long been established that CLL patients harboring 17p deletion are known to exhibit clinical resistance to fludarabine. This inferior activity in CLL cells harboring 17p deletion has also been detected *in vitro* in previous studies from our laboratory (25), but did not reach statistical significance, possibly due to the low number of samples analyzed. Prednisolone was included because of its beneficial effect on CLL patients with 17p deletion, and the high activity of prednisolone in poor-prognostic CLL samples observed in our previous study (25) was verified by the current findings. Brefeldin A targets the endoplasmatic reticulum (ER), a notable fact since CLL cells appear to host a more extensive ER network than do normal B cells (24). Brefeldin A has also been suggested to induce apoptosis in CLL cells independently of *p53* status (24). This compound was therefore also included in our study and, as with prednisolone, retained its activity in 17p-deleted samples.

The methyl inhibitor 5-azacytidine was selected for further analysis based on the current interest in methylation in CLL. One of the reasons for the considerable interest in DNA methylation is due to the fact that this process can be reversed by agents which inhibit DNA methyltransferase activity (26, 27). The hypomethylating agents azacytidine and decitabine have shown clinical activity in patients with myelodysplastic syndromes (MDS) and AML (28-30). Recently, a study analyzing the global methylation pattern in CLL demonstrated differential methylation patterns in *IGHV*-mutated and unmutated cases (31). In another study of CLL, where genome-wide analysis of aberrant DNA methylation was performed, 280 potential targets of aberrant DNA methylation were identified (26). However, *in vivo*,

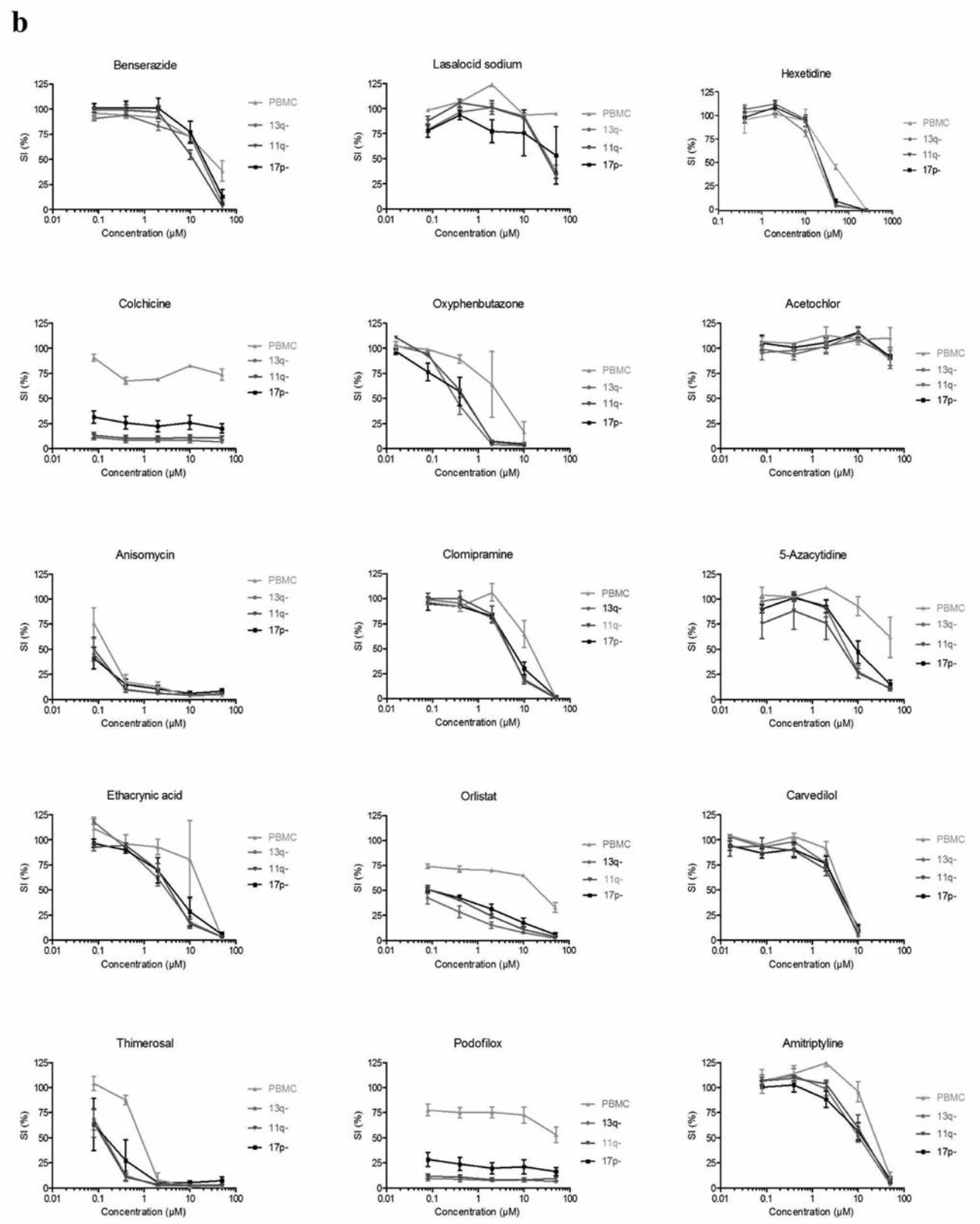

Figure 3. *In vitro activity of a: control substances and b: 15 substances selected from screening. Mean survival index (SI) of PBMCs (n=3), del(13q) (n=6), del(11q) (n=5) and del(17p) (n=5) is shown with error bars indicating standard error of mean (SEM).*

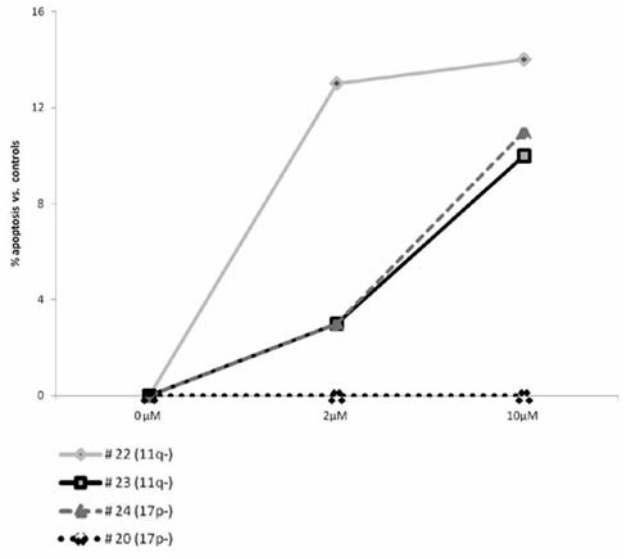

Figure 4. *Increase in apoptosis after 48 h incubation with 0 μM, 2 μM and 10 μM 5-azacytidine in two chronic lymphocytic leukemia samples with del(11q) and two with del(17p).*

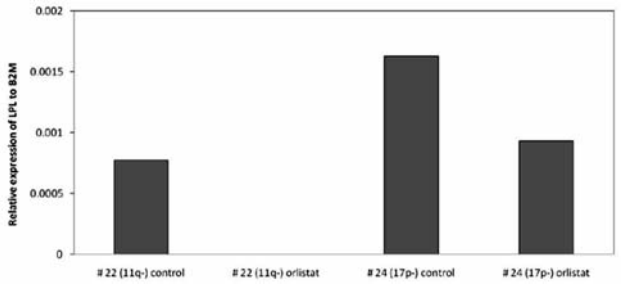

Figure 5. *LPL expression after 72 h of incubation with or without orlistat using RQ-PCR analysis for one chronic lymphocytic leukemia sample with del(11q) and one with del(17p).*

hypomethylating treatment strategies for CLL have not been examined extensively. In a recent study, 20 patients were enrolled in two phase I trials to determine the minimum effective pharmacological dose of decitabine in patients with relapsed/refractory CLL. Unfortunately, dose-limiting myelosuppression and infectious complications prevented dose escalation of decitabine to levels associated with changes in global methylation or gene re-expression (32). Considering our results with the retained sensitivity of CLL cells with poor-risk aberrations to 5-azacytidine, as well as our preliminary data on the ability of this compound to induce apoptosis in 17p-/11q-deleted CLL cells (Figure 4), 5-azacytidine remains an interesting agent for CLL, with potential interest also for the poor-prognostic group, and early clinical studies with 5-azacytidine in CLL are ongoing.

Orlistat is an *LPL* inhibitor (33) originally used to treat obesity by inhibiting gastrointestinal lipases (34). Expression of *LPL* has been shown by us and others to be of prognostic significance in CLL, where patients with unmutated *IGHV*-genes have higher expression of *LPL* compared to patients with mutated *IGHV*-genes (35-38). Accordingly, high levels of *LPL* mRNA expression are associated with shorter progression-free survival and time to treatment (39-41, 38). In a study by Pallasch *et al*. an *LPL* inhibitor was shown to induce apoptosis in primary CLL cells, irrespective of *IGHV*-mutational status (42). Sensitivity towards orlistat treatment was analyzed in three CLL samples harboring 17p deletion, indicating susceptibility to orlistat also in the *p53*-deleted subgroup (42). This susceptibility was confirmed in our dose response study, where samples with 17p deletion had similar SI values at 0.4 μM (the concentration closest to IC_{50} for orlistat) compared to samples with 13q deletion (Figure 2). We also demonstrated that treatment with orlistat can reduce *LPL* expression in poor-prognostic CLL cells and hence we believe that substances which target lipid metabolism may be possible candidates for treatment in CLL, considering the potential, yet still unclear, involvement of *LPL* in disease pathogenesis. Finally, the observation that CLL cells were more sensitive to orlistat than healthy PBMCs lends further support to its role in CLL therapy.

In conclusion, the present study indicates that using primary cultures of CLL cells to screen for compounds with cytotoxic activity represents a useful and clinically relevant alternative to the use of cell lines in the search for new treatment options in poor-prognostic CLL. As a result of the current screening effort, we have identified a dozen compounds from various biological classes that would be interesting for further studies in poor-prognostic CLL; among them we corroborated the potential of the methyl inhibitor 5-azacytidine and the *LPL* inhibitor, orlistat.

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