Roscovitine in B-chronic lymphocytic leukemia cells: high apoptosis-inducing efficacy and synergism with alemtuzumab independent of the patients' pretreatment status

Roscovitine induced apoptosis in isolated B-chronic lymphocytic leukemia cells of 25 patients of whom nine with relapsed and seven with fludarabine-refractory disease. It was synergistic with alemtuzumab and restored sensitivity to alemtuzumab in initially alemtuzumab-resistant samples. Observed roscovitine-induced up-regulation of CD52 surface expression may be one of the underlying mechanisms for this synergism.

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Despite excellent remission rates now achieved with purine analogs as well as combination therapies, the vast majority of patients with B-cell chronic lymphocytic leukemia (B-CLL) relapses after primary treatment. Among these patients, resistance to purine analogs, such as fludarabine, is common and related to poor prognosis.[1] Since treatment options for such patients are still limited, additional therapeutic strategies including new drugs are mandatory.

Roscovitine, a small-molecule inhibitor of cyclin-dependent kinases (CDK), was shown to induce apoptosis in isolated B-CLL cells by caspase activation and modulation of bcl-2 family proteins.[2] To establish a role for roscovitine in the treatment of relapsed B-CLL patients we isolated B-CLL cells of 25 patients. Nine of them had relapsed after and seven were clinically refractory to fludarabine therapy (clinical characteristics are shown in Table 1). Peripheral blood mononuclear cells (PBMC) of six healthy donors were also collected. All B-CLL cells and PBMC were incubated with increasing concentrations of roscovitine for 72 hours. The percentage of apoptotic cells was determined by $DiOC_6$/propidium iodide-staining and FACS analysis.[2] The exact ED50 (effective dose to induce apoptosis in 50% of cells) and ED90 values for each patient sample were calculated by median effect plots using CalcuSyn® software (Biosoft, Cambridge, UK). We observed a potent apoptosis-inducing activity of roscovitine in B-CLL cells compared with PBMC of healthy donors, with similar ED50 and ED90 values in B-CLL cells isolated from fludarabine-refractory compared to fludarabine-sensitive and -naive patients (Figure 1A). The high efficacy of roscovitine in B-CLL cells irrespective of patients' pretreatment status might be explained by different mechanisms of action of these drugs. While fludarabine treatment results in p53-mediated cell death, apoptosis induced by roscovitine or its pure R-enantiomer CYC202 is independent of p53 activation[2] or defects in p53-dependent pathways.[3] The preferential apoptotic activity in B-CLL cells, which has also been observed by others,[2,3] seems to be a particular clinical benefit of roscovitine. Indeed, other CDK inhibitors, such as flavopiridol, which has already been tested in clinical studies in B-CLL patients,[4,5] do not show this phenomenon.

To study the potential synergism with drugs commonly used in patients with relapsed B-CLL we examined roscovitine in combination with mitoxantrone or alemtuzumab[6,7] applying the combination index (CI)

Table 1. Patients' clinical characteristics.

#	Age (years)	Gender	Binet stage	Months since diagnosis	FISH analysis	CD38	Previous therapies	Months since last treatment	Fludarabine status
1	81	f	C	47	neg	neg	1	8	naive
2	74	f	B	8	del17p	pos	1	3	refractory
3	76	m	B	214	del13q (2x)	pos	3	9	sensitive
4	71	f	C	13	del17p	pos	3	4	refractory
5	54	f	B	55	del13q	pos	4	26	sensitive
6	80	m	B	19	del11q, del13q	pos	0	NA	naive
7	79	f	A	52	del13q	neg	0	NA	naive
8	68	f	B	300	del13q	neg	1	100	naive
9	72	m	C	25	del13q	pos	2	19	sensitive
10	52	m	C	71	neg	pos	5	20	refractory
11	66	m	C	163	del11q	pos	7	4	refractory
12	83	f	C	101	+12	neg	1	37	naive
13	76	f	C	57	del11q	pos	0	NA	naive
14	57	f	C	93	del11q	pos	2	34	sensitive
15	69	m	B	121	neg	neg	1	51	naive
16	82	m	C	62	del13q (2x)	neg	2	42	sensitive
17	76	m	C	28	neg	pos	2	12	refractory
18	76	m	C	46	del13q(2x)	neg	1	9	sensitive
19	78	f	C	47	del13q	pos	1	27	sensitive
20	71	f	B	68	neg	pos	1	10	sensitive
21	64	f	A	54	+12	neg	1	35	sensitive
22	73	m	B	49	neg	pos	1	2	refractory
23	72	f	C	21	del11q, +12, del17p	pos	0	NA	naive
24	73	m	C	198	del13q	neg	4	14	refractory
25	76	f	B	22	del13q	pos	0	NA	naive

Clinical characteristics, time since diagnosis, number of previous chemotherapy regimens, time since last cycle of chemotherapy, fludarabine status as well as results of FISH analysis including the most frequent cytogenetic aberrations found in B-CLL (del11q23, +12, del13q14 and del17p13) and data on CD38 expression are shown. Patient samples were considered CD38 positive when the expression was found in >20% CD5+/CD19+ cells. Patients were considered fludarabine naive when they had not received fludarabine therapy prior to cell isolation. Fludarabine sensitive patients had responded to therapy but eventually relapsed prior to cell isolation. Patients who progressed while on treatment or within 6 months of the last dose of fludarabine were considered fludarabine refractory.[1]

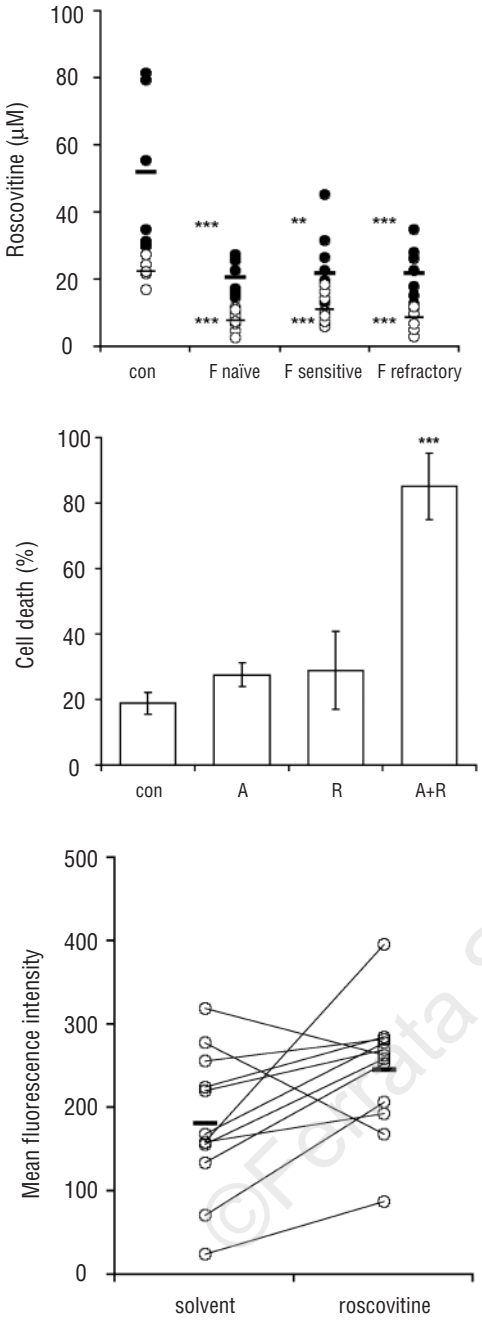

Figure 1 (left). A. ED50 (white dots) and ED90 values (black dots) for roscovitine in B-CLL-cells of patients naive, sensitive or refractory to fludarabine (F) as well as PBMC of healthy subjects. While patient fludarabine status did not influence ED50 and ED90 values for roscovitine in B-CLL cells, PBMC of healthy controls displayed significantly higher ED50 and ED90 values compared with B-CLL cells (**p=0.002 and *** p<0.001 vs. PBMC) as calculated by a one-way ANOVA with Tukey Test for post-hoc analysis. **B.** Roscovitine sensitized alemtuzumab-hyporesponsive/resistant B-CLL cells to alemtuzumab-induced complement-mediated cytotoxicity. Nine B-CLL cell samples displaying an ED50 for alemtuzumab >20 μg/mL were incubated with 10 μM roscovitine (R) for 48 hours followed by the addition of 20 μg/mL alemtuzumab (A) with complement for another 24 hours or either drug alone and assayed for cell death. Mean percentages of $DiOC_{low}$ cells ± SEM are shown. A two-way repeated ANOVA evaluation followed by Tukey post-hoc analysis revealed that alemtuzumab and roscovitine alone were without significant effect, but the combination of both resulted in a significant (***p<0.001) increase in cell death compared with each treatment alone or solvent. **C.** Roscovitine induced up-regulation of CD52 surface expression in 10 out of 12 B-CLL samples analyzed. A paired Student's t-test confirmed that the difference in mean fluorescence intensity (MFI) between roscovitine and solvent treated B-CLL cells was significant (p<0.05).

method.[8,9] Roscovitine and alemtuzumab revealed a significant synergistic activity in 22 B-CLL samples (mean CI (±SEM) at 50%, 75% and 90% apoptotic cells: 0.92±0.05, 0.68±0.04 and 0.63±0.05, while a CI <0.9 indicates synergism, between 0.9 and 1.1 an additive effect and >1.1 antagonism). Three B-CLL samples had to be excluded from the analysis, since an ED50 for alemtuzumab due to resistance to alemtuzumab-induced complement-mediated cytotoxicity could not be established. By contrast, roscovitine plus mitoxantrone was only additive in the eleven B-CLL samples tested (CI50% 0.88±0.09, CI75% 0.96±0.07 and CI90% 1.17±0.08). These findings point to a specific mechanism of roscovitine to induce synergistic activity only with selected drugs. Interestingly, CYC202 exhibited synergistic activity with bortezomib and doxorubicin in a multiple myeloma cell line,[10] but was not even additive with fludarabine in B-CLL cells.[3]

In our cohort there were three B-CLL samples resistant and six B-CLL samples hyporesponsive to alemtuzumab, i.e. with an ED50 for alemtuzumab >20 μg/mL compared with 4,85 μg/mL in alemtuzumab-responsive B-CLL samples. To test whether roscovitine can restore sensitivity to alemtuzumab in these samples they were incubated with 10 μM roscovitine for 48 hours followed by addition of 20 μg/mL alemtuzumab with complement for another 24 hours.[6,7] Roscovitine was able to restore alemtuzumab-induced complement mediated cytotoxicity in the initially hyporesponsive/resistant B-CLL tumors (Figure 1B).

FACS analysis showed an up-regulation of CD52 on the surface of B-CLL cells after 24 hours of incubation with 10 μm roscovitine in 10 out of 12 patient samples analyzed (Figure 1C). Expression of CD20, CD23 and CD24, another GPI-anchored protein found on the surface of B-CLL cells, remained unchanged (*data not shown*). However, since synergistic CI values were found in one of the two B-CLL samples, which did not show roscovitine-induced up-regulation of CD52, additional mechanisms may be involved in the synergistic activity of roscovitine and alemtuzumab.

In conclusion, we describe a potent apoptosis-inducing effect of roscovitine in isolated B-CLL cells irrespective of the patients' pretreatment status. We also demonstrate a synergistic activity with alemtuzumab in these B-CLL tumors involving up-regulation of CD52 expression. In addition, roscovitine could restore sensitivity to alemtuzumab-induced complement-mediated cytotoxicity in initially alemtuzumab-resistant/hyporesponsive B-CLL tumors. These results suggest roscovitine is a promising candidate drug for clinical tests alone and in combination with alemtuzumab in relapsed B-CLL patients.

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