

## ZRF4, a combi-molecule with increased efficacy as compared with the individual components in chronic lymphocytic leukemia lymphocytes *in vitro*

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B-cell chronic lymphocytic leukemia (CLL) is characterized by actively dividing B-lymphocytes in the lymph nodes and bone marrow, as well as the accumulation of quiescent lymphocytes in the peripheral blood of affected patients. During treatment, the enzyme-mediated repair of DNA damage can induce resistance to chemotherapeutic drugs.<sup>1</sup> Even if combinations of chemotherapy with immunotherapy have shown higher response rates and longer duration of responses, they can be associated with poor tolerability characterized by deterioration of immune functions leading to infections.<sup>2</sup> More recently, bendamustine, a hybrid agent with a nitrogen mustard moiety and a purine analog, has been tested in clinical trials in CLL, demonstrating that hybrid molecules can result in increased therapeutic efficacy as compared with the nitrogen mustard analog alone.<sup>3</sup> We have previously demonstrated that imatinib, an inhibitor of c-abl (c-abl is involved in homologous recombination repair), sensitizes B-CLL lymphocytes to chlorambucil (CLB) *in vitro*,<sup>4</sup> an investigation which led to a phase I clinical trial in CLL patients in which the combination of CLB and imatinib resulted in a 45% response rate in a heavily pretreated population with minimal toxicity.<sup>5</sup> These results encouraged us to develop the combi-molecule ZRF4, composed of a bifunctional alkylating moiety with an imatinib-like moiety, designed to degrade to an inhibitor of c-abl tyrosine kinase plus DNA-damaging species.

The synthesis of ZRF4 proceeded according to an adaptation of a strategy developed for the synthesis of ZRF1 (Supplementary Figure).<sup>6</sup> The cytotoxic effect of ZRF4 was compared with CLB alone or CLB in combination with a non-toxic and clinically achievable concentration of imatinib (5  $\mu$ M) in B-lymphocytes isolated from the B-CLL patients enrolled in this study (Supplementary Table) utilizing the MTT assay. As previously reported,<sup>4</sup> 5  $\mu$ M imatinib sensitized most of the 22 CLL patients' lymphocytes to CLB

### Conflict of interest

The authors declare no conflict of interest.

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(mean  $IC_{50}$  (the drug concentration resulting in 50% of control) CLB=11.5  $\mu$ M vs mean  $IC_{50}$  of CLB plus 5  $\mu$ M imatinib=9.0  $\mu$ M;  $P=0.044$ ). ZRF4 is more cytotoxic than CLB alone (mean  $IC_{50}$  of CLB=11.5  $\mu$ M vs mean  $IC_{50}$  of ZRF4=5.3  $\mu$ M;  $P<0.001$ ) or in combination with 5  $\mu$ M imatinib in 88% of the CLL samples (mean  $IC_{50}$  of CLB plus 5  $\mu$ M imatinib=9.0  $\mu$ M vs mean  $IC_{50}$  of ZRF4=5.3  $\mu$ M;  $P=0.002$ ) (Table 1a, Figure 1a). To confirm the higher cytotoxic effect of ZRF4, we treated B-CLL lymphocytes from six patients with the  $IC_{50}$  of ZRF4 in each CLL sample and equimolar concentrations of CLB and imatinib alone or in combination. At these drug concentrations, imatinib, CLB and the combination were not toxic in CLL lymphocytes. In contrast, consistent with the results described above, ZRF4 was significantly more cytotoxic than the individual components ( $P<0.001$ ) (Figure 1b). Similar results were obtained when the drugs were tested in B-cells from two patients diagnosed with del 17p13.1 and the p53-deficient B-CLL cell line, MEC2 (Tables 1b and c, Figure 1c). It is noteworthy that the  $IC_{50}$  values for the two patients with del 17p13.1 are higher compared with the non-mutated patient samples tested, resulting in a reduced apparent advantage of ZRF4 compared with the separate drugs.

ZRF4 is a combi-molecule designed to target c-abl (through an imatinib moiety) and to induce DNA damage (through a nitrogen mustard-like moiety). The effects of the  $IC_{50}$  of ZRF4 as compared with equimolar concentrations of CLB alone or in combination with imatinib were examined at 24 h after *in vitro* treatment in nine available CLL lymphocyte samples. Our results indicated that CLB induced c-abl phosphorylation in the CLL lymphocytes tested ( $P<0.001$ ) (Figure 1d). Moreover, imatinib has no significant effect on the basal c-abl phosphorylation (data not shown) but inhibited CLB-induced c-abl phosphorylation ( $P<0.001$ ). The combi-molecule ZRF4 completely inhibits c-abl phosphorylation. ZRF4 inhibition of c-abl is significantly different from the basal ( $P<0.001$ ) and CLB-stimulated ( $P<0.001$ ) c-abl phosphorylation. Most importantly, ZRF4 is a more potent inhibitor of c-abl phosphorylation than imatinib in combination with CLB ( $P<0.05$ ) (Figure 1d). Some combi-molecules are more potent inhibitors of the tyrosine kinase than the individual component inhibitor.<sup>7</sup> We speculate that this property implies that after treatment of the B-CLL cells with ZRF4, c-abl was inhibited by two different molecules (the intact ZRF4 and the metabolite, which is an imatinib-like molecule) (Supplementary Figure). The effect of these two different molecules is an increase in the inhibition of c-abl as compared with imatinib plus CLB.

Chemotherapeutic agents that produce DNA damage cause H2AX phosphorylation on serine 139 ( $\gamma$ H2AX) within damaged cells.<sup>8</sup> In order to verify that the cytotoxic effect of ZRF4 was at least partially mediated throughout its nitrogen mustard-like moiety, we analyzed by flow cytometry, the  $\gamma$ H2AX status as a marker of drug-induced DNA damage in B-CLL lymphocytes. ZRF4 produced a 30% increase in  $\gamma$ H2AX, whereas CLB alone or CLB in combination with imatinib showed minimal changes in  $\gamma$ H2AX (Figure 2a). To confirm the results obtained above, we utilized a more direct assay of DNA damage, the alkaline comet assay. The results demonstrate that the CLL lymphocytes treated with ZRF4 showed more DNA damage than those CLL lymphocytes treated with CLB and imatinib ( $P=0.011$ ) (Figure 2b).

The tumor suppressor protein p53 accumulates in cells treated with drugs that create DNA damage and provides an important link between DNA damage and apoptosis. Before assessing the effect of drug treatments on apoptosis, we investigated the p53 protein expression in B-CLL lymphocytes at 24 h after treatment. B-CLL lymphocytes treated with ZRF4, CLB alone or CLB in combination with imatinib resulted in an increase in p53 protein expression (Figure 2c). We then investigated the cleavage of poly (ADP-ribose) polymerase by western blot analysis and caspase-3 by flow cytometry analysis as markers of apoptosis to determine the effect of ZRF4-induced cell death in primary B-CLL cells. Results demonstrated that ZRF4 induced a small, but significant increase in poly (ADP-ribose) polymerase cleavage and a more significant increase in caspase-3 cleavage than CLB or CLB in combination with imatinib (Figures 2d–f).

Taken together, these data sustain the idea that the higher potency of ZRF4 is mediated by a greater inhibition of c-abl along with its stronger DNA-damaging potential. The greater inhibition of c-abl in the presence of DNA damage results in more apoptosis and thus, cytotoxicity in B-CLL lymphocytes. This should translate into a more efficient treatment of CLL.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

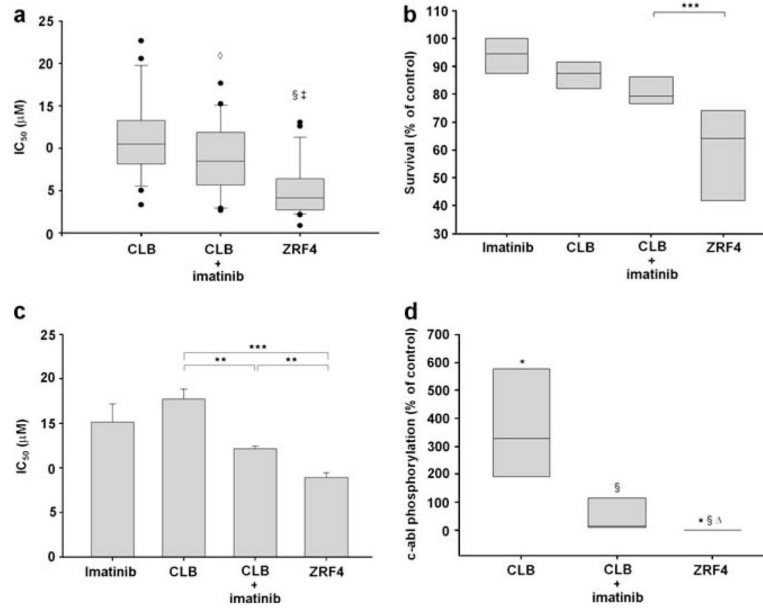
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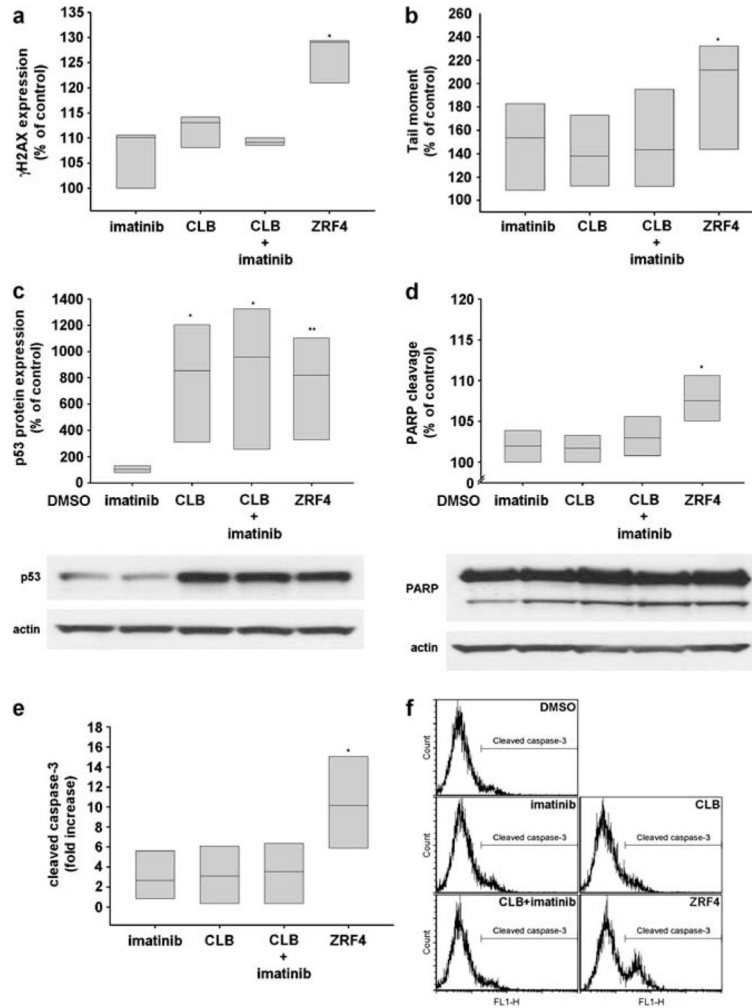
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**Figure 1.**

(a) The MTT assay was utilized to determine the cytotoxicity of ZRF4, CLB alone and CLB plus 5  $\mu$ M imatinib in cells from 22 B-CLL patients. (b) Equimolar concentrations ( $IC_{50}$  concentration of ZRF4 in each CLL lymphocyte sample) of the drugs were used for treatment of B-cells. (c) Cytotoxic effect of the drugs in the p53 deficient B-CLL cell line MEC2. (d) An enzyme-linked immunosorbent assay was used to determine the c-abl phosphorylation status in six CLL samples at 24 h after incubation with equimolar concentrations of the drugs. Symbols indicate significantly different from: \*DMSO ( $P < 0.001$ ), §CLB ( $P < 0.001$ ), ◇CLB ( $P < 0.05$ ), ‡CLB+imatinib ( $P < 0.005$ ), ΔCLB+imatinib ( $P < 0.05$ ), \*\* $P < 0.05$ , \*\*\* $P < 0.001$ .



**Figure 2.** The effects of equimolar concentrations ( $IC_{50}$  of ZRF4 in each CLL sample) of the drugs were evaluated at 24 h after treatments by flow cytometry analysis of (a) H2AX phosphorylation and (e and f) caspase-3 cleavage, western blot analysis of (c) p53 protein expression and (d) Poly (ADP-ribose) polymerase cleavage and by (b) alkaline comet assay. \* $P < 0.05$ , \*\* $P < 0.005$ .

Table 1

Drugs cytotoxicity in primary B-CLL lymphocytes and B-CLL cell line

Patient	Clinical treatment	IC <sub>50</sub> (µM)				
		Imatinib	CLB	ZRF4	CLB+5 µM imatinib	
<i>(A) p53-proficient CLL lymphocytes</i>						
1	Untreated	34.5	17.6	9.4		14.8
2	Untreated	28.5	10.3	5.4		8.5
3	Untreated	42.0	10.1	4.8		10.2
4	Untreated	33.0	11.0	2.7		7.7
5	Untreated	35.4	10.0	2.7		5.7
6	Untreated	41.9	13.3	2.4		6.4
7	Untreated	25.8	9.2	0.9		8.7
8	Untreated	42.0	12.1	5.6		15.2
9	Untreated	40.7	8.1	2.2		6.5
10	Untreated	31.0	10.5	12.6		8.6
11	Untreated	20.0	8.1	3.7		9.5
12	CLB	18.1	11.9	13.1		13.0
13	CLB	36.3	3.3	3.7		4.3
14	CLB	23.1	22.6	6.4		17.7
15	CLB	25.6	8.8	3.7		11.5
16	CLB	31.4	6.3	2.4		2.7
17	CLB	22.8	10.5	6.1		7.6
18	Flu/cytosoxan	34.7	18.6	9.0		13.0
19	Flu	8.0	5.0	3.9		2.9
20	CLB/Flu	33.8	7.5	4.2		5.5
21	CLB	29.1	20.6	6.4		11.9
22	CLB	17.6	17.3	5.6		6.5
Average	Untreated	34.1	10.9	4.8		9.3
	Treated	25.5	12.0	5.9		8.8
	Total	29.8	11.5	5.3		9.0
Median	Untreated	34.5	10.3	3.7		8.6

Patient	Clinical treatment	IC <sub>50</sub> (μM)			
		Imatinib	CLB	ZRF4	CLB+5 μM imatinib
	Treated	25.6	10.5	5.6	7.6
	Total	31.2	10.4	4.5	8.6
<i>(B) Patients diagnosed with del 17p13.1</i>					
23	Untreated	28.6	15.5	9.3	11.1
24	Flu	33.1	14.9	9.4	9.9
<i>(C) p53-deficient B-CLL cell line MEC2</i>					
MEC2		16.7±4	17.8±2.8	8.9±1.5	12.2±0.7

Abbreviations: IC<sub>50</sub>, the drug concentration resulting in 50% of control; CLB, chlorambucil; CLL, chronic lymphocytic leukemia; Flu, fludarabine. IC<sub>50</sub> values were determined by the MTT assay at 72 h after exposure of primary CLL lymphocytes to CLB, imatinib, ZRF4 or CLB plus 5 μM imatinib.

Clinical treatment: the chemotherapeutics agents the patients were previously treated with at the clinic.