Combi-targeting concept: an optimized single-molecule dual-targeting model for the treatment of chronic myelogenous leukemia

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Abstract

Blockade of Bcr-Abl by the inhibitor Imatinib has proven efficacious in the therapy of chronic myelogenous leukemia (CML). However resistance to the drug emerges at the advanced phases of the disease. Therefore, novel therapy models remained to be designed. We have developed a novel dual targeted agent termed “combi-molecule” designed to block Bcr-Abl but also damage DNA. ZRF1, the first optimized prototype of the approach, was “programmed” to degrade into another inhibitor ZRF0 plus a methyl diazonium species. It was ~2-fold stronger Abl tyrosine kinase inhibitor than Imatinib and a more potent DNA-damaging agent than Temodal. In the p53 wild-type Mo7p210 cells, the potency of ZRF1 was ~1,000-fold superior to that of the equieffective combinations of Imatinib plus Temodal. More importantly, its superior potency over Imatinib was more pronounced in Bcr-Abl-positive cells coexpressing wild-type p53. Studies to rationalize these results showed that, through its Bcr-Abl inhibitory function, it down-regulated p53. However, sufficient level of the latter protein was available for transactivating p21 and Bax, which are required for cell cycle arrest and apoptosis. The results suggest that, in p53 wild-type cells, apoptosis is induced not only through Bcr-Abl inhibition but also through the p53-controlled DNA-damaging pathway, leading to an additive effect that translates into enhanced cell death. The study conclusively showed that p53 is a major determinant for the cytotoxic advantages of the novel combi-molecular approach in CML, a disease in which 70% to 85% of all the cases express wild-type p53. [Mol Cancer Ther 2008; 7(5):1033–43]

Introduction

Chronic myelogenous leukemia (CML) stems from a chromosomal translocation leading to the Philadelphia chromosome (Ph) and expression of a fusion gene that encodes a chimeric protein Bcr-Abl associated with uncontrolled activity of tyrosine kinase (1–4). Blockade of the latter protein with the inhibitor Imatinib has proven efficient in the therapy of CML and Ph+ acute lymphoblastic leukemia with minimal toxicity (5–7). Imatinib has been approved by the Food and Drug Administration in 2001 and it is now the first-line therapy for newly diagnosed CML patients (8). However, despite its smashing success, its clinical use is associated with resistance and relapse during treatment is often imputed to mutations in the kinase domain of Abl that interfere with its binding (9–12). Recent development in CML therapy has led to Dasatinib, a compound that showed significant potency in imatinib-resistant patients. However, in phase II clinical trial, nearly all patients in the blast crisis phase relapsed within 6 months (13, 14). Therefore, alternative therapy still remains to be developed for patients in advanced phases of the disease. Recently, we designed a new approach that seeks to synthesize molecules termed “combi-molecules” capable of not only blocking the Abl kinase but also acting through a non-Abl-related mechanism of cell-killing (e.g., DNA damage; refs. 15–17). As outlined in Fig. 1A, the combi-molecule represented as TZ-I is designed to penetrate the cell by passive diffusion, bind to Bcr-Abl (see TZ-Bcr-Abl complex), or hydrolyze into I, another Bcr-Abl inhibitor (see I-Bcr-Abl complex), and TZ that can diffuse through the nucleus (see TZ-DNA). This type of combi-molecule that requires hydrolytic cleavage to generate the DNA-damaging potency is now known as type I combi-molecule. As outlined in Fig. 1, TZ-I, known as type II combi-molecules, can also be designed to bind to Bcr-Abl and to damage DNA without requirement for hydrolytic cleavage. ZRCM5 (Fig. 2B), the first prototype of type I combi-molecule, although capable of inducing high levels of DNA damage, exhibited only weak Bcr-Abl inhibitory activity and moderate antiproliferative effects against Bcr-Abl-expressing cells (17). The first prototype of type II combi-molecules AK04 (Fig. 2A) contained a pyrido-pyrimidinedione moiety designed to target Bcr-Abl and a nitrogen mustard designed to damage DNA (16). Although this compound...
has shown significant Abl tyrosine kinase inhibitory activity (IC\textsubscript{50} 0.22 μmol/L), it exhibited weak alkylating property and moderate levels of DNA damage in whole cells. Its antiproliferative activity was primarily attributed to its ability to block Abl tyrosine kinase (16). After several unsuccessful efforts to improve the Bcr-Abl tyrosine kinase inhibitory activity of AK04, all attempts to achieve a balanced Bcr-Abl-DNA targeting combi-molecule of type II were abandoned. Therefore, a refined Bcr-Abl/DNA optimized combi-molecule was yet to be synthesized. Recently, inspired by reports on the contribution of a trifluoromethyl group to the enhancement of the potency of Imatinib analogues (18), we designed ZRF1, a type I combi-molecule to contain a trifluoromethyl benzamide moiety for enhancing its Bcr-Abl potency and methyl-1,2,3 triazene moiety for generating high levels of DNA damage. This has led to the synthesis of ZRF1, a molecule with a 1.6-fold greater Abl tyrosine kinase inhibitory potency than Imatinib and 37-fold greater potency than ZRCM5. Molecular modeling studies confirmed the important interactions involved in the binding of ZRF1 with the ATP-binding site of Abl, one of which being the enhancement of hydrophobic interaction mediated by the CF3 group (Fig. 2C; ref. 19).
We have now in hand a combi-molecule with strong Abl tyrosine kinase inhibitory potency and a highly reactive DNA-damaging moiety. Here, we use it as a valid probe to study CML cell response to the tandem targeting of DNA and Bcr-Abl tyrosine kinase. Our standard of comparison in this study was the combination of Imatinib plus the clinical DNA methylating agent Temodal. It should be remembered here that, as outlined in Fig. 2A, Temodal is a cyclic triazene designed to generate a monoalkyltriazene that decomposes to a methylidiazonium species plus a 5-aminoimidazole-6-carboxamide species (20). Unlike 5-aminoimidazole-6-carboxamide, which is deprived of biological function, ZRF0, the amine released from ZRF1, possesses significant Abl tyrosine kinase inhibitory potency. Thus, as depicted in Fig. 2A, ZRF1 is a type I combi-molecule (TZ-I) designed to bind to the ATP-binding site of Bcr-Abl on its own and to degrade into a DNA-damaging species (TZ) and an inhibitor I (ZRF0) capable of sustaining the blockade of Bcr-Abl following the rapid degradation of the combi-molecule. Here, we report on the mechanism underlying CML cell response to this novel type of combi-molecule.

Materials and Methods

Drug Treatment

Temodal was provided by Shering-Plough. Detailed synthesis and rational design of ZRF1 and ZRF0 were described elsewhere (19). In all assays, drug was dissolved in DMSO and subsequently diluted in RPMI 1640 containing 10% fetal bovine serum (Wisent) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

Cell Culture

K562 cells (American Type Culture Collection) and U937 cells (generous gifts from Dr. Carlo Gambacorti-Passerini,...

Figure 2. A, proposed hydrolytic degradation pathways of the combi-molecule ZRF1 in comparison with those of Temodal. B, molecular structures of ZRCM5 and AK04. C, three-dimensional view of Imatinib and ZRF1 in the Abl pocket.
Division of Experimental Oncology D and Medical Oncology C, Tumour National Institute) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics as described previously. A K562-derived cell line, transfected with a wild-type p53 human cDNA into the pCMV-Neo-Bam vector containing the neomycin analogue (G418) resistance gene (provided by Dr. Maria Alessandra Santucci, Institute of Hematology and Medical Oncology), was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, G418 (500 µg/mL), and antibiotics. The human megakaryocytic cell lines Mo7e and Mo7p210 (generous gift from Dr. Brian J. Druker, Division of Hematology and Medical Oncology, Oregon Health Sciences), were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Mo7e cells were supplemented with 10 ng/mL granulocyte-macrophage colony-stimulating factor. All cells were maintained in an atmosphere of 5% CO₂.

Abl Kinase Assay

This assay is similar to the previously described epidermal growth factor kinase tyrosine kinase assay, with minor modifications (21). Briefly, Nunc Maxisorp 96-well plates were incubated overnight at 37°C with 100 µL/well of 0.25 mg/mL PGT in PBS. Excess PGT was removed and the plate was washed three times with wash buffer Tween 20 (0.1%) in PBS. The kinase reaction was done by using 1.0 ng/well c-Abl (Bioscience). Following drug administration, phosphorylation was initiated by the addition of ATP (50 µmol/L). After 10 min at room temperature with constant shaking, the reaction was terminated by the addition of 50 µL of 250 mmol/L EDTA before aspiration and by rinsing the plate four times with wash buffer. Phosphorylated PGT was detected following a 25-min incubation with 50 µL/well horseradish peroxidase–conjugated PY20 anti-phosphotyrosine antibody (Santa Cruz Biotechnology). It was subsequently incubated with horse radish peroxidase-goat anti-mouse antibody (Bio-Rad Laboratories). Protein concentrations of the compounds for 24 h. Expression of protein levels were detected by Western blot analysis using the following primary antibodies: anti-p53 (D-11-sc-17846; Santa Cruz Biotechnology), anti-p21 (sc-817; Santa Cruz Biotechnology), and anti-Bax (clone 6A7; BD Biosciences). Membranes were stripped of antibody and reprobed with anti-tubulin (clone D11A; Medicorp).

Bcr-Abl Inhibition of Autophosphorylation

Inhibition of Bcr-Abl autophosphorylation in viable cells was determined by anti-phosphotyrosine Western blots. K562 and Mo7p210 cells were plated at a density of 2 × 10⁶ per well and incubated with the compounds for 2 h. Thereafter, they were washed with PBS and resuspended in cold lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP-40, 1 mmol/L EDTA, 5 mmol/L NaF, 1 mmol/L Na₃PO₄, protease inhibitor tablet (Roche Biochemicals)]. The lysates were kept on ice for 30 min and collected by centrifugation at 10,000 rpm for 20 min at 4°C. Protein concentrations were determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories). Equal amounts of protein from each cell lysate were added to an 8% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). Nonspecific binding on the membrane was minimized with a blocking buffer containing nonfat dry milk (3%) in PBS. The membrane was blotted for 1 h with anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology) or anti-Abl antibodies (Upstate Biotechnology). It was subsequently incubated with horseradish peroxidase-goat anti-mouse antibody (Bio-Rad Laboratories) or with horseradish peroxidase-goat anti-rabbit (Upstate Biotechnology) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Band intensities were measured using the SynGene GeneTools software package.

Analysis of p53, p21, and Bax

Mo7p210 and K562/p53 cells were plated at a concentration of 2 × 10⁶ per well and incubated with varying concentrations of the compounds for 24 h. Expression of protein levels were detected by Western blot analysis using the following primary antibodies: anti-p53 (D-11-sc-17846; Santa Cruz Biotechnology), anti-p21 (sc-817; Santa Cruz Biotechnology), and anti-Bax (clone 6A7; BD Biosciences). Membranes were stripped of antibody and reprobed with anti-tubulin (clone D11A; Medicorp).

3-(4,5-Dimethylthiazolo-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The cytotoxic effects of our compounds were evaluated using the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide assay (22) with minor modification. Briefly, cells were grown in 12-well plates and subsequently exposed to compounds for 96 h. 3-(4,5-Dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide (50 µL; 5 mg/mL in sterile PBS) was added to 1 mL medium and the plates were incubated for 2 to 3 h at 37°C. The resulting colored residue was dissolved in DMSO and absorbance was analyzed in each well at 570 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicates.

Alkaline Comet Assay for Quantitation of DNA Damage

The alkaline comet assay was done as described previously (23). The cells were exposed to drugs for 30 min, the drug was washed off, and the cells were resuspended in PBS. Cell suspensions were diluted to ~10⁶ cells and mixed with agarose (1%) in PBS at 37°C in a 1:10 dilution. The cell/agarose mixture was then cast onto Gelbond strips (Mandel Scientific) using gel casting chambers and immediately placed into a lysis buffer [2.5 mol/L NaCl, 0.1 mol/L tetrasodium EDTA, 10 mmol/L Tris, and 1% (v/v) Triton X-100 (pH 10.0)]. After being kept on ice for 30 min, the gels were gently rinsed with distilled water and immersed in a second lysis buffer (2.5 mol/L NaCl, 0.1 mol/L tetrasodium EDTA, 10 mmol/L Tris) containing proteinase K (1 mg/mL) for 60 min at 37°C. Thereafter, the gels were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and then electrophoresed at 1 V for 20 min. After
rinsing with distilled water, they were placed in 1 mol/L ammonium acetate for 30 min. Thereafter, they were soaked in 100% ethanol for 2 h, dried overnight, and stained with SYBR Gold (1:10,000 dilution of stock supplied from Molecular Probes) for 20 min. Comets were visualized at ×330 magnification and DNA damage was quantitated using the Tail Moment variable (that is, the distance between the barycenter of the head and the tail of the comet × the percentage of DNA within the tail of the comet). A minimum of 50 cell comets were analyzed for each sample using ALKOMET version 3.1 image analysis software.

**Annexin V Binding Assay**

Cells were grown in six-well plates and then incubated with the compounds for 24 h. The cells were then collected, washed twice with PBS, and centrifuged. Cells (10^6) were then treated with Annexin V-FITC and propidium iodide using the apoptosis detection kit (BD Bioscience PharMin-gen). Annexin V-FITC and propidium iodide labeling were analyzed by flow cytometry. Data were collected using logarithmic amplification of both FL1 (FITC) and FL2 (propidium iodide) channels. Quadrant analysis of coordinate dot plots was done using CellQuest software.

**Flow Cytometry**

Cells (K562 and K562/p53) were grown in six-well plates until confluence and then incubated with Imatinib, ZRF1, and Temodal for 24 h. Thereafter, they were collected by centrifugation, washed twice with PBS, fixed with ethanol (70%), and stained with propidium iodide. Flow cytometric analysis was done on a Becton Dickinson Flow Cytometer and cell cycle analysis with the FlowJo software package.

**Results**

**Bcr-Abl Targeting**

Although ZRF1 has been shown to block Abl tyrosine kinase in an ELISA assay (19), the demonstration of its Abl tyrosine kinase specificity remained to be achieved. Thus, its Abl tyrosine kinase inhibitory activity was compared with its potency against other tyrosine kinases including

![Figure 3](http://mct.aacrjournals.org/)

**Figure 3.** Binary Bcr-Abl/DNA targeting properties of ZRF1. K562 cells (A–C) and Moe7p210 cells (D–F) were incubated for 2 h with the indicated concentrations of Imatinib, ZRF1, and ZRF0 and inhibition of Bcr-Abl phosphorylation determined by Western blotting as described in Materials and Methods. G, reversibility of Bcr-Abl autophosphorylation by Imatinib and ZRF1 in the K562 cells. Cells were incubated for 2 h with the indicated concentrations of Imatinib and ZRF1. The cells were washed free of the compounds with drug-free media, incubated for 2 h, washed again, incubated for another 2 h, and kept for a further 2 h after a subsequent wash. Equal amounts of lysate were analyzed by immunoblotting with antiphosphotyrosine or anti-Abl antibodies. H, DNA damage induced by ZRF1, ZRF0, and Temodal in the K562 cell line as determined by the comet assay. Tail moment was used as a variable for the detection of DNA damage in K562 cells exposed to the drugs for 30 min.
lymphocytes (Mo7e and its transfectant Mo7p210). Interestingly, in contrast to the alkylator Temodal (deprived of Bcr-Abl selectivity using the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide assay), Cytotoxic activity was analyzed in the isogenic pair of megakaryocytic cell lines Mo7e and its transfectant Mo7p210. Interestingly, in contrast to the alkylator Temodal (deprived of Bcr-Abl selectivity using the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide assay), Cytotoxic activity was analyzed in the isogenic pair of megakaryocytic cell lines Mo7e and its transfectant Mo7p210. Interestingly, in contrast to the alkylator Temodal (deprived of Bcr-Abl selectivity using the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide assay), Cytotoxic activity was analyzed in the isogenic pair of megakaryocytic cell lines Mo7e and its transfectant Mo7p210. Interestingly, in contrast to the alkylator Temodal (deprived of Bcr-Abl selectivity using the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide assay), Cytotoxic activity was analyzed in the isogenic pair of megakaryocytic cell lines Mo7e and its transfectant Mo7p210. Interestingly, in contrast to the alkylator Temodal (deprived of Bcr-Abl selectivity using the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide assay), Cytotoxic activity was analyzed in the isogenic pair of megakaryocytic cell lines Mo7e and its transfectant Mo7p210.

We further studied the sustainability of the Abl tyrosine kinase inhibitory potency of these drugs. Thus, we designed an experiment wherein the drugs were given at high concentrations to K562 cells for 2 h followed by multiple washouts over 6 h and analyzed their Bcr-Abl phosphorylation status. Interestingly, even at concentrations as high as 10 to 20 μmol/L, Imatinib lost almost 100% of its Bcr-Abl inhibitory activity. In contrast, ZRF1 only lost ~50% of its inhibitory activity at the same doses. Thus, despite having Bcr-Abl inhibitory activities in the same range, the combi-molecule appeared to induce a more sustained inhibition than Imatinib (Fig. 3G).

DNA Damage
One of the conditions for the novel combi-molecule to be considered truly optimized was its ability not only to induce strong Bcr-Abl blockade but also to generate high levels of DNA damage. Thus, we analyzed and compared its DNA-damaging potential with that of Temodal in K562 cells. The results showed that ZRF1 was capable of producing 2.5-fold higher levels of DNA damage than Temodal in the cells. As expected, ZRF0 showed no detectable levels of DNA damage (Fig. 3H).

Antiproliferative Activity
Selective Cytotoxicity against Bcr-Abl-Expressing CML Cells. Having shown that the combi-molecule is a strong DNA-damaging agent and a potent Abl targeting combi-molecule, we analyzed the translation of its binary targeting principle into antiproliferative potency and Bcr-Abl selectivity using the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide assay. Cytotoxic activity was analyzed in the isogenic pair of megakaryocytic cell lines Mo7e and its transfectant Mo7p210. Interestingly, in contrast to the alkylator Temodal (deprived of Bcr-Abl potency), ZRF1 was ~700-fold selective for the Bcr-Abl transfectant Mo7p210 (Table 1). Imatinib and ZRF0 showed ~500- and 200-fold selectivity for the latter transfectant, respectively. These data indicate that the cytotoxic effect of ZRF1 is specific for cells expressing the Bcr-Abl oncogene.

Comparison between the Potency of ZRF1 and Classic or Nonclassic Combinations of Brc-Abl Inhibitors plus Temozolomide (a Methylating Agent). The principal objective of our approach being to synthesize single molecules designed to behave as targeted combinations of individual molecules, we compared the effect of ZRF1 with that of a molar combination of Imatinib plus a

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<th>PDGFR*</th>
<th>Lyn*</th>
<th>Src</th>
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<td>ZRF0 + Temodal</td>
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<td>Imatinib + Temodal</td>
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*Mean of two independent experiments run in duplicate.
†Data are as above but obtained after a 96-h drug exposure.
‡Mean ± SE of IC50 values for antiproliferative effects obtained from two independent experiments after a 24-h drug exposure.

Table 1. Tyrosine kinase inhibition by ZRF1 and its growth inhibitory effects against isogenic or established cell lines with various levels of expression of bcr-abl
methyldiazonium generator (e.g., Temodal) and with an equieffective combination of the latter two drugs. More importantly, ZRF1 being designed to generate ZRF0 plus a DNA-damaging species, we compared its potency with that of the combination of ZRF0 plus Temodal. The results showed that, in the p53 wild-type Mo7p210 cells, ZRF1 was 1,000- and 1200-fold superior to that of equieffective combinations of Imatinib plus Temodal and ZRF0 plus Temodal respectively (Table 1). In a nonclassic combination wherein the two drugs were combined at equimolar concentrations, ZRF1 was 5-fold more potent than Imatinib plus Temodal in the p55 wild-type Mo7e cells. In contrast, in the p53-null K562 cells, the activity of ZRF1 when compared with equimolar combinations was in the same range.

Molecular Events Underlying Cellular Response to the Dual Action of the Combi-Molecules

Having demonstrated that the combi-molecule showed superior potency when compared with its free inhibitor, Imatinib alone and in combination with Temodal, we sought to determine the mechanism underlying such a potency. Bcr-Abl being known to be involved in the activation of the apoptotic pathways, we surmised that its down-regulation by the combi-molecule should sensitize the cells to the effect of the induced DNA damage, thereby triggering them to undergo apoptosis. Thus, we compare the levels of apoptosis induced by the combi-molecule with those by Imatinib and the combination of Imatinib plus Temodal using the Annexin V binding assay in a panel of cells that included the p53-proficient Mo7e wild-type and its transfectant Mo7p210 or the p53-deficient K562 cells. The results showed that although ZRF1 induced the same levels of apoptosis as Imatinib in the K562 cells (Fig. 4, i), it induced significantly higher levels of apoptosis than Imatinib in its p53 transfectant K562/p53 cells (Fig. 4, ii). Similarly, when apoptosis was studied under conditions where the Mo7p210 cells were exposed to the drugs for a short period (2 h), the proportion of cells entering the early stage of apoptosis was significantly higher in those treated with ZRF1 than those with Imatinib or Imatinib plus Temodal (Fig. 4, iii). This indicates that ZRF1 induced apoptosis at a much faster rate than Imatinib in the Mo7p210 cells.

DNA Damage Response Pathway

It should first be noted that in the most temozolomide-sensitive cell line of the panel (non-Bcr-Abl-expressing Mo7e), ZRF1 is almost equipotent with temozolomide (Table 1), indicating that the absence of Bcr-Abl the effect of the DNA-damaging potency of the latter two drugs is

Figure 4. Comparison of levels of early apoptosis induced by ZRF1 and Imatinib in the p53-deficient parental K562 cell line (i) and in the p53 transfectant (ii) after a 24-h drug treatment. Quantitation of proportions of cells entering the early apoptotic stage on treatment with ZRF1, Imatinib, and their corresponding combination Imatinib plus Temodal in the Mo7p210 cells after a 2-h drug exposure (iii). Levels of apoptotic cells were determined using Annexin V-FITC staining. Statistical analysis was done with a f test. *, P < 0.05, statistical significance (n = 3). Comparison between levels of p53 accumulation and corresponding transactivated proteins, p21 and Bax, in the Mo7p210 cells. Levels of p53 (A), p21 (B), and Bax (C) on exposure to ZRF1, Temodal, ZRF0, and Imatinib alone and to imatinib plus Temodal (D–F) after a 24-h drug exposure. Levels of p53, p21, and Bax were analyzed by Western blot using anti-p53, anti-p21, anti-Bax, and anti-tubulin antibodies.
equivalent. More importantly, the observation that, in contrast to the p53-proficient Mo7p210 and K562/p53 cells, no significant difference in levels of apoptosis and cell killing were induced by ZRF1 and Imatinib in the p53-deficient K562 cells has stimulated our interest in determining the role of p53 in the response to the dual action of the combi-molecule. Having shown that ZRF1 is capable of significantly damaging DNA in whole cells, it was critical to analyze the role of p53 in cell sensitivity to its mixed mechanism of action. It has already been shown previously that 24 h after treatment with Temodal, p53 accumulation reaches its maximum (24, 25). Therefore, we determined p53 levels in the cells 24 h after treatment with ZRF1 and compared its effect with those of Temodal, Imatinib, and combination of Imatinib plus Temodal. Doses were chosen as a function of survival after a 24-h exposure at ~ 1 to 2 × IC50 concentration range (see IC50 values in Table 1). The results showed that in the Bcr-Abl-transfected Mo7p210 cells, p53 levels were dramatically increased following exposure to Temodal alone (Fig. 4A). Surprisingly, p53 accumulation was markedly less pronounced in cells treated with ZRF1, Imatinib, and ZRF0 (Fig. 4A) or a combination of Imatinib with Temodal (Fig. 4E), indicating that Bcr-Abl inhibition tend to down-regulate p53 in these cells. Expression of p21, a protein transactivated by p53, followed the same trend, being down-regulated by ZRF1, ZRF0, and Imatinib alone or its combination with Temodal (Fig. 4A and B). Bax expression was clearly down-regulated by ZRF0 or Imatinib and up-regulated by Temodal. In cells treated with ZRF1, its levels remained lower than those induced by Temodal and slightly higher than those induced by ZRF0 or Imatinib, an effect that reflects its combined DNA-damaging and Bcr-Abl inhibitory properties.

To validate these results, the experiments were repeated in K562 cells transfected with p53 to create a unique context in which the effects of our combi-molecule can be analyzed in two isogenic Bcr-Abl-expressing cells: one of

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Figure 5. Cell cycle perturbations induced by Imatinib, ZRF1, and Temodal in (A) K562 and (B) K562/p53 cells. The cells were treated for 24 h and fixed in 70% ethanol and cell cycle profile was analyzed by flow cytometry.
which being p53 proficient and the other deficient. The analysis done in the K562/p53 cells led to the same results as those observed in Mo7p210 (data not shown). Interestingly, in the Bcr-Abl isogenic cells, ZRF1 induced 2-fold greater cytotoxicity in the K562/p53 transfectant than in its parental K562 (Table 1), suggesting that p53 may play a significant role in the enhancement of the potency of the combi-molecule.

**Cell Cycle Effects**

To determine whether p53 accumulation would translate into G2-M arrest following DNA damage, it was critical to analyze the cell cycle following exposure to the various drugs. Here, we chose to study the K562 cell line and its transfectant, which only differed from its parental line by expression of p53. The results showed that in the parental K562 cells ZRF1 was capable of arresting the cell cycle in S and Temodal induced a slight arrest in the G2-M phase. However, for Imatinib, only G1 arrests were observed (Fig. 5). Importantly, the cell cycle profile was markedly different in the p53-transfected K562 cells when compared with its parental line. In contrast to Temodal and Imatinib, ZRF1 induced significant cell cycle arrest in G2-M (at 25 μmol/L, 55% increase), indicating that its mechanism of action is unique and distinct from that of the other drugs (Fig. 5). Importantly, at the highest 25 μmol/L dose, the proportion of cells in G1 in ZRF1-treated K562/p53 cells (24.5%) was 2-fold lower than those exposed to Imatinib (48.4%) and 2.2-fold higher than those treated with Temodal (11.2%), a result that again reflects the balanced mixed Bcr-Abl DNA-damaging property of the combi-molecule.

**Discussion**

The combi-molecular approach is based on the premise that cancer cells result from a complex array of interrelated molecular events from which they inherit multiple mechanisms and pathways to evade the cytotoxic action of antitumor drugs (15–17, 21, 23). To circumvent this problem, drugs need to be designed that block multiple targets in the cells. CML cells being characterized by expression of a target (Bcr-Abl) involved in multiple events, including antiapoptotic signaling and stimulation of DNA repair proteins, are typical examples of tumor cells with robust resistance to cytotoxic drugs (26–29). In previous studies, we showed that CML cells are resistant to temozolomide and chlorambucil, two of the most cytotoxic alkylating agents used in the clinical management of refractory tumors and we suggested that dual targeting compounds termed “combi-molecules” with refined Bcr-Abl inhibitory and DNA-damaging properties could exert enhanced cytotoxicity against these cells (16, 17). In previous models, we synthesized ZRCM5 and AK04, which, while capable of killing Bcr-Abl cells, were lacking one arm of their binary component. ZRCM5 was a strong DNA-damaging agent but a poor Bcr-Abl inhibitor, whereas AK04 was a rather strong Bcr-Abl tyrosine kinase inhibitor but a weak DNA-damaging agent. In contrast, as shown herein, ZRF1 is as good a Bcr-Abl inhibitor as Imatinib and a more potent DNA-damaging agent than the clinical agent Temodal. More importantly, it was ~500-fold more potent than an equieffective combination of Imatinib plus Temodal in the K562 cell line. Thus, it can be considered a refined probe for the demonstration of the advantages and the mechanisms involved in cell response to this novel mixed targeted approach using Imatinib as a reference.

Surprisingly, the first analysis in K562 cells revealed that ZRF1 only induced 1.6-fold greater antiproliferative activity than Imatinib, a minor difference in potency that could be attributed to their almost equivalent Bcr-Abl potency. It should be remembered that ZRF1 is a 1.7-fold stronger Abl tyrosine kinase inhibitor than Imatinib, but in whole-cell assays their Bcr-Abl phosphorylation inhibitory activities were in the same range. Thus, it appears that continuous exposure or continuous inhibition of Bcr-Abl induced cytotoxic effects that could not be further enhanced by DNA damage in these cells. Indeed inhibition of Bcr-Abl in these cells suffices to trigger the apoptotic event in the absence of DNA damage. Our results are in agreement with those by Goldberg et al. (30) who showed that exposure of K562 cells to Imatinib plus ionizing radiation (a condition that induces high levels of DNA damage) does not induce levels of apoptosis and cell death beyond that evoked by Imatinib alone. The authors suggested that the apoptotic pathways in these cells are constitutively activated but countered by Bcr-Abl signaling. Thus, inhibition of the latter will commit the cells to apoptosis regardless of any exogenous stress.

Importantly, the potency of ZRF1 in the Bcr-Abl-expressing cells harboring wild-type p53 was consistently 2- to 3-fold stronger than that of Imatinib, indicating that DNA damage in these cells has conferred cytotoxic advantage. Indeed, the Bcr-Abl transfectant Mo7p210 cell line was the most sensitive to ZRF1 of the whole panel of a established cell lines and transfection of K562 cells with p53 wild-type enhanced sensitivity to ZRF1 by 2-fold, indicating that p53 plays a role in translating the effects of the DNA-damaging arm of the combi-molecule into additional cytotoxic effects in these cells. Despite the cytotoxic advantage conferred by p53, its down-regulation through Bcr-Abl inhibition was apparent in all treatment that included a Bcr-Abl inhibitory function. A similar result has already been reported previously by Goldberg et al. (30) who showed that Bcr-Abl can activate Chk1, which in turn phosphorylates serine 20 of p53, thereby protecting it against the action of the Mdm2 protein. Other groups reported that transfection of p53 wild-type cells with Bcr-Abl increases the levels of p53 (31). Thus, inhibition of Bcr-Abl decreases serine 20 phosphorylation and the subsequent stability of p53. Our results showed that despite the concomitant down-regulatory effect of Bcr-Abl inhibition, sufficient levels of the p53-transactivated proteins, p21 and Bax, were still observed in these cells, indicating that the p53-mediated DNA-damaging response pathway was still operative in these cells. It should be noted that Bax,
a proapoptotic protein whose action is countered by the Bcr-Abl-mediated release of Bel-xL or Bel-2, was only moderately down-regulated by Bcr-Abl inhibition. Therefore, the combi-molecule being able to induce a tandem down-regulation of Bcr-Abl and DNA damage could gain a greater cytotoxic advantage in these cells than Imatinib. It is also important to mention that at supratheral 50 μmol/L concentration, significant accumulation of p53 levels was observed and the down-regulatory effect was overridden by the excessive levels of DNA damage. Indeed, at cytotoxic doses, as early as 24 h post-treatment, in contrast to Imatinib that only induced 50% kill at this concentration, ZRF1 killed ~90% of the p53-proficient cells. Thus, these results indicate that at lethal concentrations the Bcr-Abl antiapoptotic signaling is shut down and mechanisms programmed to induce apoptosis in the presence of DNA damage are activated. Indeed, ZRF1 could induce p21 for cell cycle arrest and Bax for apoptosis. More importantly, in contrast to the other drugs, it induced strong G2-M arrest in the p53 transfected K562/p53 cells. Thus, although the role of p53 in cell sensitivity to Imatinib is still debatable, this study clearly showed that it should be pointed out as a major determinant for the cytotoxic advantage of the combi-molecular approach in CML cells.

In summary, the mechanism underlying the potency of the combi-molecule is proposed as depicted in Fig. 1B. Briefly, the combi-molecule (TZ-I) penetrates the cells and bind to the ATP site of Bcr-Abl or degrade to generate ZRF0 (I) and a methyldiazonium species (TZ) that damages DNA. As already shown previously, Bcr-Abl inhibits apoptosis through the phosphatidylinositol 3-kinase/Akt pathway; therefore, its blockade alleviates antiapoptotic effects (29). DNA alkylation and damage detected by nuclear sensors leads to activation of p53 that transactivates genes required for cell cycle arrest (e.g., p21) and apoptosis (e.g., Bax). Apoptosis is induced through Bcr-Abl inhibition and further enhanced through the p53-controlled DNA-damaging pathway. Thus, ZRF1 is the first ever multitargeted combi-molecule exerting a tandem targeting of Bcr-Abl mediated antiapoptotic signaling and activation of the DNA damage response pathway.

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