Anti-Cancer Drugs 2002, **13,** pp. 1055–1060

Preclinical report

DNA damage and repair in BCR/ABL-expressing cells after combined action of idarubicin, STI571 and amifostine

Janusz Blasiak,[1] Ewa Gloc,[1] Tomasz Pertyński[2] and Józef Drzewoski[3]
[1]Department of Molecular Genetics University of Lodz, 90-237 Lodz, Poland. [2]Polish Mother's Memorial Hospital, 93-335 Lodz, Poland. [3]Department of Clinical Pharmacology, Medical University of Lodz, 94-214 Lodz, Poland.

STI571 is a specific ABL family tyrosine kinases inhibitor approved for treatment of leukemias. It can differentially modulate the action of other antileukemic drugs. We have recently shown that deregulation of the mechanisms of DNA damage and repair in BCR/ABL-positive cells may be involved in drug resistance of these cells, and thus determine the response of cancer cells to therapy. In the present work we investigated DNA damage and repair induced by idarubicin in the presence of STI571 and amifostine, a normal cell protector, in the BCR/ABL fusion tyrosine kinase-expressing cell line. Amifostine increased the viability of both kinds of cells in the absence of STI571, but had no effect in the presence of the inhibitor. STI571 did not change the response of both BCR/ABL-expressing cells and their control counterparts to idarubicin in terms of DNA damage and repair. However, the presence of amifostine modulated the response of the cells. In the absence of STI571 amifostine decreased the DNA-damaging effect of idarubicin in normal cells and increased it in BCR/ABL-positive cells. STI571 at 2 μM abolished the protective effect of amifostine against idarubicin in normal cells and diminished the magnitude of the amifostine-induce increase in cancer cells. These results suggest that amifostine should be applied with special caution in idarubicin-based chemotherapies of BCR/ABL-positive leukemias involving STI571 inhibitor. [© 2002 Lippincott Williams & Wilkins.]

Key words: Amifostine, BCR/ABL kinase, DNA damage, DNA repair, idarubicin, STI571.

Introduction

Anticancer drugs can kill cancer cells, inhibit their proliferation or target specific molecular abbreviations in them. The last feature is of special significance, because cytotoxic or cytostatic actions of a drug are rarely limited to cancer cells and more often also affect their normal counterparts. The discovery of oncogenic properties of some tyrosine kinases led to the synthesis of their inhibitors, among them Herceptin, directed against Her-2/*neu*, and STI571 (imatinib mesylate, Gleevec, CGP57148B, Figure 1), targeting the ABL family of tyrosine kinases, are the most prominent.[1] STI571 focuses interest on the therapy of leukemias with phenotypic traits of the fusion oncogenic tyrosine kinase BCR/ABL, which results from the fusion of exons 2–11 of the c-*ABL* gene from chromosome 9 to N-terminal exons of t!he c-*BCR* gene on chromosome 22.[2] The fusion chromosome is often refereed as the Philadelphia (Ph) chromosome. BCR/ABL is found in 95% of cases of chronic myelogenous leukemia (CML) and in 25–30% of cases of adult acute lymphoblastic leukemia (ALL), but its role in the etiology of these cancers remains unknown. Thus, the expression of the *BCR/ABL* fusion gene can be considered as a specific abnormality in the cells of some leukemias and therefore regarded as a potential target in the therapy of these diseases. Druker *et al.* showed that 2-phenylaminopyrimidine (STI571) selectively inhibited the growth of BCR/ABL-expressing cells derived from CML patients.[3]

STI571 can be used singly or in combination with other drugs. It is reported to produce additive, antagonistic or synergistic effects with classical antileukemic drugs depending on the cell line employed and particular drug.[4–6] Anthracycline antibiotics target DNA topoisomerase II leading to DNA fragmentation, especially in dividing cells, and subsequent cell death, and are widely used in the chemotherapy of leukemias. Idarubicin (Figure 1) is an new-generation anthracycline which can be

This work was supported by grant 505/431 from the University of Lodz.

Correspondence to J Blasiak, Department of Molecular Genetics, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland.
Tel: (+48) 42 635 43 34; Fax: (+48) 42 635 44 84;
E-mail: januszb@biol.uni.lodz.pl

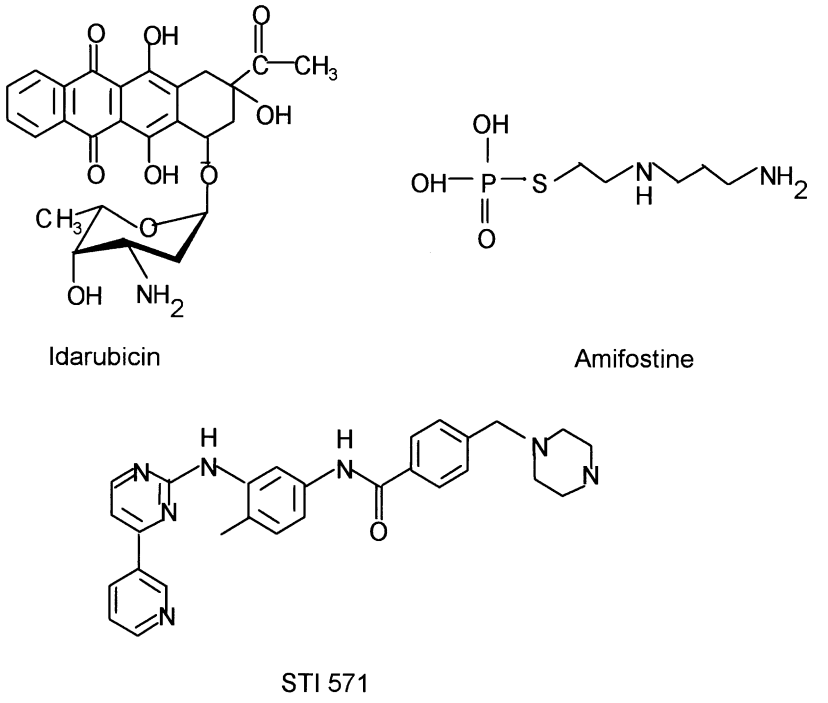

Figure 1. Structure of idarubicin, STI571 and amifostine.

applied orally, and we have recently shown that in addition to its interaction with topoisomerase II it can damage DNA by producing free radicals in both normal and cancer cells including those expressing BCR/ABL.[7] In order to diminish unwanted side effects of anticancer drugs in normal cells a variety of cell protectors can be applied. Amifostine (Ethyol, WR 2721, Figure 1), an analog of cysteamine, is a phosphorylated aminothiol prodrug that is dephosporylated in the tissue by the membrane-bound alkaline phosphatase to its active metabolite, WR-1065. Amifostine can selectively protect normal cells against anticancer drugs and radiation by free radical scavenging, donating hydrogen ions to free radicals, depleting oxygen and binding to active derivatives of antineoplastic compounds.[8] Recently, we have shown that amifostine may decrease the DNA-damaging effect of idarubicin in normal human lymphocytes and increase this effect in BCR/ABL-positive cells.[9]

In the present work we investigate the survival and the DNA-damaging effects of the combined action of idarubicin, STI571 and amifostine in murine pro-B lymphoid BaF3 cells transformed with *BCR/ABL* oncogene. These cells are characterized by the stable expression of BCR/ABL oncogenic tyrosine kinase and can be considered as model cells of human acute myelogenous leukemia.[10] Alkaline single-cell gel electrophoresis (comet assay), which is a sensitive method for studying DNA damage and repair, was applied to asses the DNA-damaging potential of the combination of drugs.

Materials and methods

Chemicals

Idarubicin was obtained from Pharmacia & Upjohn (Milan, Italy). Amifostine [*S*-2(3-aminopropylamino)ethyl phosphothioic acid], Tris, RPMI 1640 medium, agarose, low-melting-point agarose, phosphate-buffered saline (PBS), DAPI, fetal bovine serum (FBS) and MTT were obtained from Sigma (St Louis, USA). STI571 was kindly provided by Novartis Pharma (Basel, Switzerland). WEHI 3B medium was obtained from Dr Jovani Rovera (Wistar Institute, University of Pennsylvania).

Cells

Murine growth factor-dependent pro-B lymphoid cell line BaF3 and BaF3-*BCR/ABL* transformed clone were obtained from Dr Richard VanEtten (Harvard Medical School). Cell lines were maintained in RPMI 1640

supplemented with 10% FBS and 15% WEHI-conditioned medium (the growth medium). The viability of the cells was measured by Trypan blue exclusion staining and was about 99%. The viability of the cells was checked concurrently in all further experiments in the appropriate drugs concentration range and was never below 80%. The final concentration of the cells was adjusted to $1–3 \times 10^5$ cells/ml by adding the growth medium to the single-cell suspension.

MTT assay

Idarubicin at final concentrations of 0.01–10 μM was added to the cells (1.5×10^6/ml) in the growth medium. When STI571 was applied, the addition of idarubicin was preceded by 24 h incubation with the inhibitor at 2 μM; the final concentration of amifostine was 14 mM and it was added just prior to idarubicin when necessary. Four days later the viability of cells was evaluated by the MTT assay.[14] Briefly, cells were plated onto 96-well plates in 200 μl of growth medium and 20 μl of 10 mg/ml MTT was added to each well. After incubation at 37°C for 4 h, the supernatant was removed and 200 μl of a solution containing 10% SDS and 0.04 M HCl was added to dissolve the water-insoluble formazan salt. One hour later, the difference $OD_{650\,nm} - OD_{570\,nm}$ was measured with an ELISA microplate reader (Bio-Rad, Hercules, CA).

DNA damage

To examine DNA damage, the cells were incubated for 1 h at 37°C with idarubicin with or without 24-h pre-incubation with STI571. The incubation with idarubicin was in the presence or in the absence of amifostine at 14 mM. Each experiment included a positive control, which was H_2O_2 at 10 μM. H_2O_2 produced pronounced DNA damage, which resulted in tail DNA of 30–40%.

DNA repair

To examine DNA repair, after the treatment with drugs the cells as well as control samples were washed and resuspended in fresh, drug-free RPMI 1640 medium preheated to 37°C. Aliquots of the suspension were taken immediately, and 15, 30, 60 and 120 min later. Placing the samples in an ice bath stopped the repair incubation.

Comet assay

The comet assay was performed under alkaline conditions essentially according to the procedure of Singh *et al.*[11] with some modifications[12] as described previously.[13] Freshly prepared cell suspensions in 0.75% low-melting-point agarose dissolved in PBS were placed onto microscope slides pre-coated with 0.5% normal melting agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100 and 10 mM Tris, pH 10. After lysis, the slides were placed in an electrophoresis unit and DNA was allowed to unwind for 40 min in an electrophoretic solution consisting of 300 mM NaOH and 1 mM EDTA, pH > 13. Electrophoresis was conducted at 4°C (the temperature of the running buffer not exceeding 12°C) for 30 min at an electric field strength 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 μg/ml DAPI and covered with cover slips. To prevent additional DNA damage all steps were conducted under a dimmed light or in the dark.

Comet analysis

The slides were examined at × 200 magnification in a Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, San Diego, CA) equipped with a UV filter block containing an excitation filter (359 nm) and barrier filter (461 nm), and connected to a personal computer-based image analysis system, Lucia-Comet version 4.51. (Laboratory Imaging, Prague, Czech Republic). Fifty images were randomly selected from each sample and the comet tail moment (a product of the fraction of DNA in tail and tail length) was measured. Two parallel tests with aliquots of the same sample of cells were performed for a total of 100 cells and the mean percentage of tail DNA was calculated. The tail DNA is positively correlated with the level of DNA breakage in a cell.[11] A mean value of tail DNA in a particular sample was taken as an indicator of DNA damage in this sample.

Statistical analysis

All the values in this study were expressed as means ± SEM from two separate experiments. If no significant differences between variations were found, as assessed by the Snedecor–Fisher test, the differences between means were evaluated by

applying Student's *t*-test. Otherwise, the Cochran–Cox test was used. The data were analyzed using the STATISTICA (StatSoft, Tulsa, OK) statistical package.

Results

Viability

The results of the MTT assays of the survival times of BaF3 cells and their BCR/ABL-transformed counterparts after treatment with idarubicin singly or in combination with amifostine in the presence or absence of the STI571 inhibitor are shown in Figure 2. The viability of the BCR/ABL-transformed cells was significantly lower than control cells and decreased with time. Amifostine increased the viability of both kinds of cells in the absence of STI571, but had no effect in the presence of the inhibitor.

DNA damage and repair

Figure 3 shows the mean level of DNA damage for the BaF3 cells and their transformed counterparts exposed to idarubicin at different concentrations in the presence or absence of 14 mM amifostine with or without 24 h incubation with STI571. Idarubicin significantly increased DNA damage in all cases in a dose-dependent manner. In the absence of STI571, amifostine reduced the level of DNA damage induced by idarubicin in BaF3 control cells, but it increased idarubicin-induced DNA damage in the transformed cells. In the presence of STI571, amifostine had no effect on DNA damage evoked by idarubicin in non-transformed cells and increased the damage in transformed cells. The magnitude of this increase was, however, much smaller than in the absence of the inhibitor.

All treated cells were able to repair damage to their DNA in a 120-min period; there were no differences in kinetics of the repair between drug-exposed and control cells (data not shown).

Discussion

The expression of the BCR/ABL tyrosine kinase is responsible for the malignant phenotype of the BCR/ABL-expressing CML and ALL blasts. Leukemic blasts expressing BCR/ABL display arrested differentiation

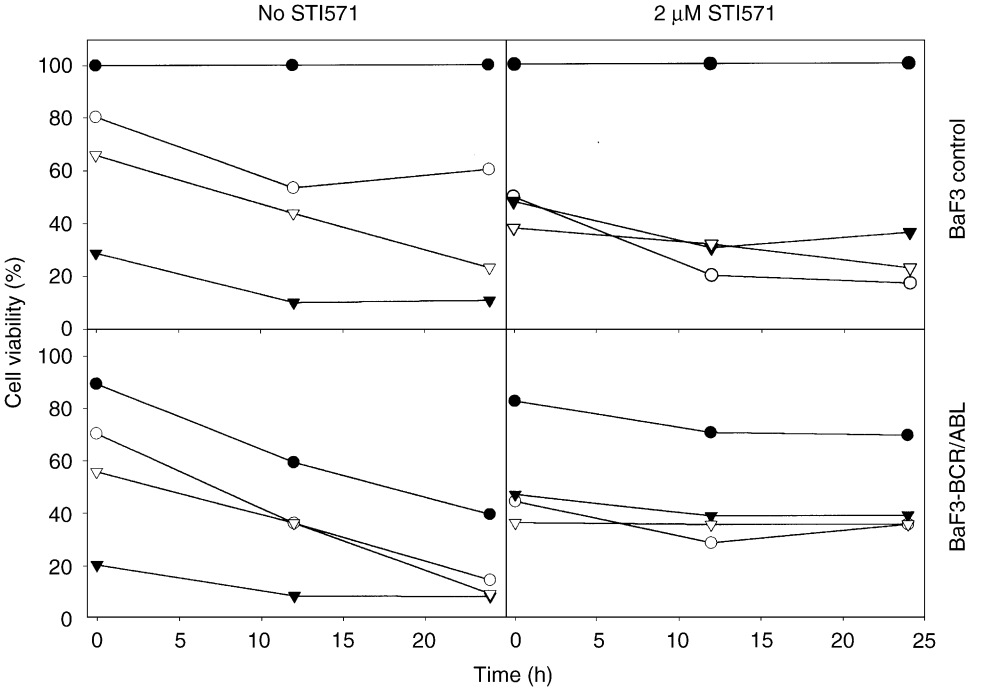

Figure 2. The survival of murine growth factor-dependent pro-B lymphoid cells BaF3 and their BCR/ABL-transformed counterparts in the presence of 0.5 μM idarubicin (solid triangles), 14 mM amifostine (circles) or 0.5 μM idarubicin combined with 14 mM amifostine (open triangles) with or without 24 h pre-incubation with STI57 as evaluated by the MTT assay. Results represent the means of three independent experiments; error bars are smaller than the radius of the symbols.

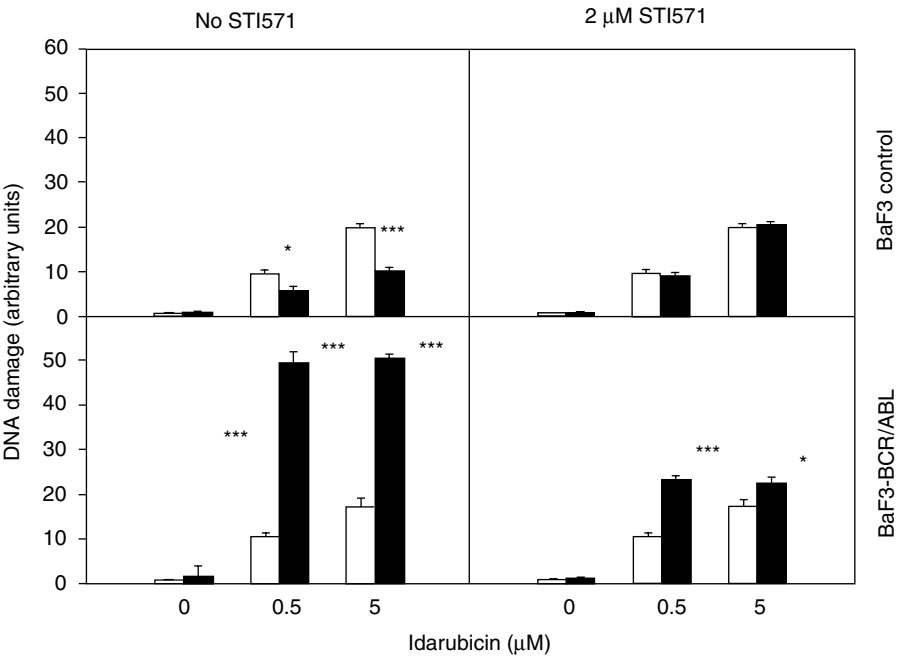

Figure 3. DNA damage measured by the comet assay as percentage of tail DNA in murine growth factor-dependent pro-B lymphoid cells BaF3 and their BCR/ABL-transformed counterparts in the presence of idarubicin singly (empty bars) or idarubicin combined with 14 mM amifostine (filled bars) with or without 24 h pre-incubation with STI57. One hundred cells were analyzed per point. Results are the mean of three independent experiments; error bars represent SEM; $*p < 0.05$, $***p < 0.001$.

and resistance to apoptosis even at high doses of antileukemic drugs.[15,16] Therefore, there is a need to undertake a special strategy of chemotherapy in patients with blasts expressing BCR/ABL. Our recent studies revealed a novel mechanism of resistance in BCR/ABL-positive cells: stimulation of DNA double-strand break repair by homologous recombination.[17,18] This phenomenon is dependent on *BCR/ABL*-induced elevation of the level of RAD51,[17] the mammalian homolog of *Escherichia coli* RecA protein, which plays an essential role in homologous recombination. We suggest that RAD51 in conjunction with Bcl-x_L increase and G_2/M arrest might be responsible for the drug resistance in BCR/ABL-positive cells.[18] In our previous work we demonstrated for the first time that deregulation of DNA repair mechanisms could be involved in drug resistance in BCR/ABL-positive leukemias.[19] Moreover, BCR/ABL is thought to contribute to the genetic instability responsible for leukemia progression.[20] Consequently, the mechanisms of DNA damage and repair may determine the sensitivity of BCR/ABL-expressing cells to anticancer drugs and radiation, and thus the effectiveness of cancer therapy. That is why we investigated DNA damage and repair in BCR/ABL-positive cells in the presence of STI571 inhibitor.

We showed that STI571, an ABL tyrosine kinase inhibitor, did not change the response of both BCR/ABL-expressing cells and their control counterparts to idarubicin in terms of DNA damage and repair. However, the presence of the cell protector amifostine modulated the response of the cells. In the absence of STI571 amifostine increased the viability of the cells (Figure 2), and it decreased the DNA-damaging effect of idarubicin in normal cells and increased it in BCR/ABL-positive cells (Figure 3). These results are in agreement with our previous findings.[9] STI571 at 2 μM abolished the protective effect of amifostine against the idarubicin-evoked DNA-damaging effect in normal cells and diminished the magnitude of the amifostine-evoked increase in cancer cells. Amifostine had no effect on the viability of the cells in the presence of STI571. These results clearly suggest that amifostine should be applied with caution in idarubicin-based chemotherapy of BCR/ABL-positive leukemias when STI571 inhibitor is employed.

The use of STI571 in conjunction with other anticancer drugs is a consequence of expected and observed resistance to single agents. Thus a favorable approach would be to combine STI571 with other compounds either to prevent the emergence of

resistant clones or to enhance the eradication of the leukemic clone. STI571 was reported to modulate differentially the action of specific antileukemic agents. It showed additive or synergistic effects in combination with interferon-α, daunorubicin, doxorubicin, homoharringtonine, vincristine, cytosine arabinoside and etoposide, and antagonistic effects when combined with hydroxyurea or methotrexate.[4,6,21] On the other hand, hydroxyurea was shown to induce an additive effect with STI571,[6] so the action of STI571 is determined not only by the presence or absence of a TEL tyrosine kinase, but also by other components and mechanisms.

In conclusion, the present study showed that amifostine might not be advantageous for the DNA-damaging effect of idarubicin in BCR/ABL-positive cells in the presence of STI571, although it might not affect the viability of these cells, and this may be of importance in the design of STI571-based chemotherapy in BCR/ABL-positive leukemias.

References

1. Sawyers CL. Rational therapeutic intervention in cancer: kinases as drug targets. *Curr Opin Genet Dev* 2002; **12**: 111–5.
2. Kurzrock R, Gutterman N, Talpaz M. The molecular genetics of Philadelphia chromosome-positive leukemias. *N Eng J Med* 1988; **319**: 990–8.
3. Druker BJ, Tamura S, Buchdunger E, *et al.* Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr–Abl positive cells. *Nat Med* 1996; **2**: 561–6.
4. Thiesing JT, Ohno-Jones S, Kolibaba KS, Druker BJ. Efficacy of STI571, an abl tyrosine kinase inhibitor, in conjunction with other antileukemic agents against bcr–abl-positive cells. *Blood* 2000; **96**: 3195–9.
5. Fang G, Kim CN, Perkins CL, *et al.* CGP57148B (STI-571) induces differentiation and apoptosis and sensitizes Bcr–Abl-positive human leukemia cells to apoptosis due to antileukemic drugs. *Blood* 2000; **96**: 2246–53.
6. Kano Y, Akutsu M, Tsunoda S, *et al. In vitro* cytotoxic effects of a tyrosine kinase inhibitor STI571 in combination with commonly used antileukemic agents. *Blood* 2001; **97**: 19999–2007.
7. Blasiak J, Gloc E, Woniak K, *et al.* Genotoxicity of idarubicin and its modulation by vitamins C and E and amifostine. *Chem-Biol Interact* 2002; **140**: 1–18.
8. Capizzi RL, Oster W. Chemoprotective and radioprotective effects of amifostine: an update of clinical trials. *Int J Hematol* 2000; **72**: 425–35.
9. Blasiak J, Gloc E, Młynarski W, Drzewoski J, Skorski T. Amifostine differentially modulates DNA damage evoked by idarubicin in normal and leukemic cells. *Leuk Res* 2002; **1538**: 1–4.
10. Deninger MWN, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. *Blood* 2000; **96**: 3343–56.
11. Singh NP, McCoy T, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988; **175**: 184–92.
12. Klaude M, Eriksson S, Nygren J, Ahnstrom G. The comet assay: mechanisms and technical considerations. *Mutat Res* 1996; **12**: 89–96.
13. Blasiak J, Kowalik J. A comparison of the *in vitro* genotoxicity of tri- and hexavalent chromium. *Mutat Res* 2000; **469**: 135–45.
14. Siu WY, Arooz T, Poon RYC. Differential responses of proliferating versus quiescent cells to adriamycin. *Exp Cell Res* 1999; **250**: 131–41.
15. McGahon A, Bissonete R, Schmit M, Cotter KM, Green DR, Cotter TG. BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. *Blood* 1994; **83**: 1179–87.
16. Amarante-Mendes GP, McGahon AJ, Nishioka WK, Afar DE, Witte ON, Green DR. Bcl-2-independent Bcr–Abl-mediated resistance to apoptosis: protection is correlated with up regulation of Bcl-x_L. *Oncogene* 1998; **16**: 1383–90.
17. Slupianek A, Schmutte C, Tombline G, *et al.* BCR/ABL regulates mammalian RecA homologs resulting in drug resistance. *Mol Cell* 2001; **8**: 795–806.
18. Slupianek A, Hoser G, Majsterek I, *et al.* Fusion tyrosine kinases induce therapeutic drug resistance by stimulation of homology-dependent recombination repair, prolongation of G_2/M phase and protection from apoptosis. *Mol Cell Biol* 2002; **22**: 4189–201.
19. Majsterek I, Blasiak J, Młynarski W, Hoser G, Skorski T. Does BCR/ABL-mediated increase in the efficacy of DNA repair play a role in drug resistance in cancer cells? *Cell Biol Int* 2002; **26**: 363–70.
20. Laneuville P, Sun G, Timm M, Vakemans M. Clonal evolution in a myeloid cell line transformed to interleukin-3 independent growth by retroviral transduction and expression of $p210^{bcr/abl}$. *Blood* 1992; **80**: 1788–97.
21. Liu WM, Stimson LA, Joel SP. The *in vitro* activity of the tyrosine kinase inhibitor STI571 in BCR–ABL positive CML cells: synergistic interactions with antileukaemia agents. *Br J Cancer* 2002; **86**: 1472–8.

(Received 6 August 2002; accepted 19 August 2002)