

tors such as toxicity and cost need to be considered.

With the current chelation regimen, the balance between iron accumulation and excretion is fine.^{1,6,10} In contrast to the iron balance achieved by monotherapy with either DFP or DFO, iron balance achieved with combined therapy was negative in the majority of patients.

In conclusion, combined therapy with DFO and DFP showed an additive and occasionally synergistic effect on UIE, which could reach levels higher than iron accumulation from transfusions, leading to a negative iron balance. Long-term studies are required to validate the efficacy and safety of combined therapy.

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References

- Olivieri NF, Brittenham GM. Iron-chelating therapy and the treatment of thalassaemia. *Blood* 1997;89:739-61.
- Al-Refaie FN, Wonke B, Hoffbrand AV, Wickens DG, Nortey P, Kontoghiorghes GJ. Efficacy and possible adverse effects of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) in thalassaemia major. *Blood* 1992;80:593-9.
- Breuer W, Ermers MJ, Pootrakul P, Abramov A, Hershko C, Cabantchik ZI. Desferrioxamine-chelatable iron, a component of serum non-transferrin-bound iron, used for assessing chelation therapy. *Blood* 2001;97:792-8.
- Link G, Konijn AM, Breuer W, Cabantchik ZI, Hershko C. Exploring the "iron shuttle" hypothesis in chelation therapy: effects of combined deferoxamine and deferiprone treatment in hypertransfused rats with labeled iron stores and in iron-loaded rat heart cells in culture. *J Lab Clin Med* 2001;138:130-8.
- Hershko C, Link G, Konijn AM, Huerta M, Rosenmann E, Reinus C. The iron-loaded gerbil model revisited: effects of deferoxamine and deferiprone treatment. *J Lab Clin Med* 2002;139:50-8.
- Wonke B, Wright C, Hoffbrand AV. Combined therapy with deferiprone and desferrioxamine. *Br J Haematol* 1998; 103: 361-4.
- de Franceschi L, Shalev O, Piga A, Collell M, Olivieri O, Corrocher R, et al. Deferiprone therapy in homozygous human beta-thalassaemia removes erythrocyte membrane free iron and reduces KCl cotransport activity. *J Lab Clin Med* 1999;133:64-9.
- Shalev O, Hileti D, Nortey P, Hebbel RP, Hoffbrand VA. Transport of ¹⁴C-deferiprone in normal, thalassaemic and sickle red blood cells. *Br J Haematol* 1999;105:1081-3.
- Giardina PJ, Grady RW. Chelation therapy in β -thalassaemia: an optimistic update. *Semin Hematol* 2001;38: 360-6.
- Collins AF, Fassos FF, Stobie S, Lewis N, Shaw D, Fry M, et al. Iron-balance and dose-response studies of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) in iron-loaded patients with sickle cell disease. *Blood* 1994; 83: 2329-33.

Lack of *Bcr-Abl* point mutations in chronic myeloid leukemia patients in chronic phase before imatinib treatment is not predictive of response

We describe the presence of *abl* point mutations detected using a highly sensitive technique in 5 out of 9 patients with chronic phase CML resistant to imatinib. These mutations were not detected in samples obtained before initiating therapy with imatinib. Unless more sensitive techniques are developed, the presence or absence of point mutations before starting imatinib therapy will not help in predicting responses to treatment.

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Despite the positive results of treatment with imatinib mesylate (IM) in patients with chronic myelogenous leukemia (CML)¹ a number of patients develop clinical resistance to this drug, resulting in progression of the disease at 18 months in 11% of interferon resistant/intolerant patients.² Most patients in blast crisis will eventually suffer disease progression despite continuous treatment with imatinib.^{3,4}

Among the different mechanisms of *in vivo* resistance to IM, the most frequently detected in patients with advanced phase (accelerated or blast crisis) CML is point mutations in the kinase domain of *Abl*.⁵⁻⁷ We studied the presence of *Bcr-Abl* mutations in a homogeneous group of CML patients in chronic phase with primary cytogenetic resistance to IM in order to determine the incidence of point mutations and whether the presence of these substitutions before treatment could predict resistance to IM therapy.

We studied a group of 89 patients with CML enrolled in an extended access trial of IM (chronic phase CML patients resistant to or intolerant of interferon- α). All patients had 100% Philadelphia positive metaphases. Patients with no cytogenetic response after at least 6 months of therapy were defined as having primary resistance to IM and analyzed for the presence of *Abl* mutations. Bone marrow mononuclear cells were obtained before initiating treatment with IM and every 3 months thereafter.

Total RNA was extracted using RNeasy[®]Mini Kit (Qiagen, Hilden, Germany) from frozen cells. Total RNA (1 μ g) was used for cDNA synthesis using SuperScript[™] II RNase H-RT (Invitrogen Life Technologies, Paisley, UK) with random hexamers. A BCR-ABL transcript of 1.3 kb was amplified by PCR using 4 μ L of cDNA and CM10 (5'-GAAGCTTCTCCCTGACATCCGT-3') and 3ABL2 (5'-CGGACTTGATGGAGAATTG-3') primers under the following conditions: 94°C for 10 min, 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 90 seconds, and a final elongation cycle at 72°C for 10 min. The *Abl* kinase domain was amplified in a second PCR using 1 μ L of the first PCR product and 5ABLKD (5'-GCGCAACAAGCCACTGTCTATGG-3') and 3ABLKD (5'-GCCAGGCTCTCGGTGCAGTCC-3') primers with the following conditions: 94°C for 10 min, 30 cycles at 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 30 seconds, followed by an elongation cycle at 72°C for 10 min. All PCR reactions were carried out in a total volume of 25 μ L, with 2.5 U of native PFU polymerase (Stratagene, Amsterdam, The Netherlands), 0.4 mM dNTPs and 20 pmol of each primer.

The second PCR product (597 bp) was subcloned into pCR[®] 4-

Table 1. BCR-ABL kinase domain mutations detected in 5 CML patients.

	Nucleotide	Nucleotide substitution	Amino acid substitution	Mutant clones
CML 1	749	G→A	G250E (Gl→Glu)	8/10
CML 2	749	G→A	G250E (Gly→Glu)	10/10
CML 3	730	A→G	M244V (Met→Val)	4/10
	1052	T→C	M351T (Met→Thr)	5/10
CML 4	1075	T→G	F359V (Phe→Val)	10/10
CML 5	1075	T→G	F359V (Phe→Val)	10/10

TOPO® plasmid using TOPO TA Cloning® Kit for Sequencing (Invitrogen Life Technologies, Paisley, UK). Ten colonies with recombinant plasmid containing the 597 bp PCR product were sequenced using T7 and T3 universal forward and reverse primers. Only substitutions present in at least 2 clones were considered as mutations in accordance with Shah *et al*.⁶

Nine of 89 patients showed complete cytogenetic resistance to IM (100% Ph positive metaphases after more than 6 months of treatment) despite having a complete hematologic response. No *BCR-ABL* gene amplification was detected by fluorescent *in situ* hybridization analysis at the time of resistance in any of our patients (*data not shown*) suggesting that this mechanism is rarely the cause of resistance in patients with CML in chronic phase.

Mutations of the *Abl* kinase domain were identified in 5 patients after treatment with IM (Table 1). The detected substitutions correspond to previously described mutations that conferred resistance to IM. The G250E and M244V mutations, located in the ATP phosphate binding loop (P loop), and M351T, located in the C-terminal loop of the kinase, confer resistance due to changes in the conformation of the kinase domain which prevent it from binding IM. The F359V mutation is located at sites that are in direct contact with IM, impairing binding of the drug without affecting binding of ATP.

The current hypothesis is that mutations within the *Abl* gene are induced throughout the course of the disease and that treatment with IM selects for these clones. Our study was initially designed to try to determine whether detection of point mutations in patients with chronic phase CML could predict response to IM. Unlike other studies^{6,8} we were not able to detect mutations in samples obtained before treatment with IM. Different techniques for detecting point mutations have different sensitivities. Direct sequencing can only detect mutations when most of the clones harbor the substitution. By subcloning and sequencing multiple independent clones we can detect mutant clones when these represent about 20% of the total *Bcr-Abl* positive cell population.⁶ Allele-specific oligonucleotide PCR may detect mutations even when less than 20% of the cells are mutated, however it can only be applied to specific substitutions and requires different primers to be designed for each mutation.⁸ The fact that we analyzed a group of patients in chronic phase, who are less likely to harbor mutations than patients in advanced phase, may also explain differences between our results and those of previous studies.^{6,8}

In a small group of patients, samples were analyzed in duplicate using 2 different Taq polymerases with or without proofreading activity. Interestingly, the use of a Taq polymerase without proofreading activity detected multiple substitutions present in single clones, some of which have been described as mutations in patients with resistance to IM. None of these substitutions was found when Taq polymerase with proofreading activity was employed suggesting that these were artifacts introduced in the PCR reaction (*data not shown*).

In 4 patients, the 700 bp region 5' to the kinase domain was sequenced using the same approach. No evidence of additional mutations could be found in these patients either before or after treatment with IM. These findings are in agreement with those of previous studies⁶ and suggest that there is no global increase in mutation frequency and further support the hypothesis that only mutations associated with an IM-resistant phenotype would be selected.

In conclusion, by subcloning and sequencing multiple clones we detected point mutations in the kinase domain of *Bcr-Abl* in more than 50% of chronic phase CML patients with primary cytogenetic resistance to IM indicating for the first time that this mechanism of resistance to IM is highly prevalent also in chronic phase CML. The fact that no mutations were found in samples obtained before IM treatment suggest that unless more sensitive techniques can be developed, the presence or absence of mutations will not help to predict resistance to IM.

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References

- O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. The IRIS Investigators. *N Engl J Med* 2003;348:994-1004.
- Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. International STI571 CML Study Group. *N Engl J Med* 2002;346:645-52.
- Sawyers CL, Hochhaus A, Feldman E, Goldman JM, Miller CB, Ottmann OG, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* 2002;99: 3530-9.
- Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001;344: 1031-7.
- Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 2001;293:876-80.
- Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2002;2:117-25.
- Gambacorti-Passerini C, Barni R, le Coutre P, Zucchetti M, Cabrita G, Cleris L, et al. Role of α 1 acid glycoprotein in the *in vivo* resistance of human BCR-ABL(+) leukemic cells to the abl inhibitor STI571. *J Natl Cancer Inst* 2000;92:1641-50.
- Roche-Lestienne C, Soenen-Cornu V, Grardel-Duflos N, et al. Several types of mutations of the *Abl* gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. *Blood* 2002;100:1014-8.