

The Effect of Cyclosporin A Used Alone and in Combination with either 2-Chlorodeoxyadenosine or Fludarabine on Normal and Chronic Myelogenous Leukemia Progenitors *in Vitro*

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Abstract. We evaluated the influence of cyclosporin A (CsA) used alone or together with the new purine nucleoside analogues (PNAs) 2-chlorodeoxyadenosine (2-CdA) and fludarabine (F-ara-A) on the colony growth of normal and chronic myelogenous leukemia (CML) granulocyte-macrophage progenitor cells (CFU-GM) in cultures *in vitro*. The assay was based on the Iscove's method in our modification. Specimens of bone marrow were collected from 15 patients with CML in the chronic phase and from 10 hematologically normal patients. CsA at the concentrations of 1, 2 and 4 $\mu\text{g/ml}$ was used alone and, at the concentration of 4 $\mu\text{g/ml}$, was preincubated with mononuclear cells (MNCs) and, after 30 min PNAs were added to the culture medium. Concentrations of 5, 10, and 20 nM 2-CdA and 0.4, 0.8 and 1.6 μM F-ara-A were used. After 14 days of incubation, the colonies were scored under an inverted microscope. We observed that CsA used alone at all three concentrations inhibited the colony growth of CML CFU-GM to a statistically significant degree compared with the control ($p < 0.02$) and that it did not significantly influence normal colony growth. The IC_{50} for CsA was 3.9 $\mu\text{g/ml}$ in the case of normal CFU-GM and 2.7 $\mu\text{g/ml}$ in the case of CML CFU-GM. After the use of CsA in combination with either the highest concentrations of 2-CdA or F-ara-A, statistically significant differences compared with CsA used alone were observed ($p = 0.008$, $p = 0.03$ for CsA with 2-CdA, and $p = 0.0007$, $p = 0.005$ for CsA with F-ara-A, respectively, for normal and CML CFU-GM). However, there were no significant differences between the combinations of drugs and PNAs used alone. In the case of the combination of CsA with the highest concentrations of both PNAs, significant differences in colony growth inhibition between normal and CML CFU-GM were observed ($p = 0.002$ and $p = 0.005$, respectively, for 2-CdA and F-ara-A). In conclusion, at the concentrations of the drugs used, a subadditive action was observed either between CsA and 2-CdA or between CsA and F-ara-A.

Key words: cyclosporin A; purine nucleoside analogues; chronic myelogenous leukemia; CFU-GM; cultures *in vitro*.

Abbreviations used: CsA – cyclosporin A, 2-CdA – 2-chlorodeoxyadenosine, F-ara-A – fludarabine, PNAs – purine nucleoside analogues, CML – chronic myelogenous leukemia, AML – acute myeloid leukemia, NHL – non-Hodgkin's lymphoma, CLL – chronic lymphocytic leukemia, CFU-GM – colony-forming unit granulocyte-macrophage, BFU-E – burst-forming unit erythrocyte, MNCs – mononuclear cells, MDR – multidrug resistance, P-gp – P-glycoprotein, IDMEM – Iscove's modified Dulbecco minimum essential medium, HBSS – Hanks' balanced salt solution, GM-CSF – granulocyte-macrophage colony-stimulating factor, G-CSF – granulocyte colony-stimulating factor, IL-2 – interleukin 2, IC_{50} – concentration of agents that achieved 50% inhibition of colony formation, O/E – interaction index as a ratio between the observed and expected survival index values.

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Introduction

In the last few years many new anticancer agents have been synthesized and introduced to experimental as well as preclinical and clinical studies. New purine nucleoside analogues (PNAs), 2-chlorodeoxyadenosine (2-CdA) and fludarabine (F-ara-A), whose structures are similar to deoxyadenosine, have shown high efficacy in the treatment of hematological neoplasms, especially in lymphoid malignancies such as chronic lymphocytic leukemia (CLL) and low-grade non-Hodgkin's lymphoma (NHL)^{9, 24, 30}. Moreover, these drugs also have chemotherapeutic activity in the treatment of myeloid leukemias^{8, 23}. Although the role of PNAs in the treatment of chronic myelogenous leukemia (CML) is still controversial, there are studies suggesting therapeutic effects of 2-CdA and F-ara-A in CML patients^{5, 21}. Complete hematological remission was observed by SAVEN et al.²⁶ in chronic-phase Ph⁺ CML patients treated with 2-CdA. Moreover, in our previous *in vitro* studies, 2-CdA used alone and in combination with other agents significantly inhibited the colony growth of CML granulocyte-macrophage progenitor cells (CFU-GM), as compared with their normal counterparts^{15, 19, 25}.

One of the more important problems of anticancer therapy, besides myelotoxicity, is drug resistance. Several mechanisms of drug resistance are known, with multidrug resistance (MDR) being the best characterized. MDR can be due to enhanced expression of certain genes (MDR1, MRP or LRP), alterations in glutathione-S-transferase activity, and to a reduction of the amount or activity of topoisomerase II²². The induced expression of MDR-associated genes as a direct response of tumor cells to antineoplastic drugs could be an important factor affecting the success of cancer chemotherapy. P-glycoprotein (P-gp), a product of the MDR1 gene, transports hydrophobic agents, such as cytostatics, out of neoplastic cells and does not allow them to obtain their effective concentrations. Certain drugs, such as cyclosporin A (CsA) or varapamil, are well known as modulators of MDR1. They inhibit P-gp and permit greater intracellular retention of cytostatics²⁸. However, P-gp is expressed not only in neoplastic cells, but also in many normal bone marrow and peripheral blood cells, including CD34⁺, CD56⁺ and CD8⁺ cells⁶.

The aim of our present study was to evaluate the influence of CsA used alone or in combination with 2-CdA or F-ara-A on the colony growth of normal and CML CFU-GM progenitors *in vitro* and discover whether CsA influences the antiproliferative effect of PNAs.

Materials and Methods

Patients studied. Specimens of bone marrow were collected from 15 patients with CML in the chronic phase after informed consent. The mean age of patients was 44.8 years (range 29–74). All the patients were newly diagnosed, the diagnoses being based on standard clinical, morphological, cytochemical and cytogenetic criteria, and they were chromosome Ph⁺ and BCR-ABL⁺. None of the patients had received cytotoxic drugs before the collection of bone marrow for the study.

Normal bone marrow specimens were obtained from 10 hematologically normal patients. The mean age of these patients was 42.6 years (range 19–66). All specimens were collected with the consent of the donor.

Therapeutics. CsA (Sandimmun) was kindly provided by Sandoz (Switzerland) at the concentration of 50 mg/ml. 2-CdA was synthesized according to the method described by KAZIMIERCZUK et al.¹⁴ and was provided by the Foundation for the Development of Diagnostics and Therapy (Warsaw, Poland) at a concentration of 1 mg/ml. Fludarabine phosphate (Fludara) was kindly supplied by Schering AG (Germany) as a dry powder containing 50 mg fludarabine phosphate, which corresponds to 39.05 mg F-ara-A.

Assay for normal and CML CFU-GM. The assay for CFU-GM was based on the method described by ISCOVE et al.¹² in our modification¹⁶. Briefly, bone marrow samples were collected in preservative-free heparin (Sigma, England). The mononuclear cells (MNCs) were obtained from the marrow by layering over lymphoprep (Histopaque 1077, Sigma, England) and washed twice in Hanks' balanced salt solution (HBSS).

The MNCs were suspended in 20% fetal calf serum (FCS; Gibco Ltd, Scotland) at the concentration of 5×10^5 cells/ml and afterwards 20% Iscove's modified Dulbecco minimum essential medium (IDMEM; Gibco Ltd, Scotland), 55% methylcellulose (Fluorochem, Switzerland) and 5% growth factors: 20 ng/ml G-CSF (Neupogen, Roche, Switzerland), 25 ng/ml GM-CSF (Leucomax, Novartis, Switzerland) and 40 ng/ml IL-3 (Sigma, England), were added.

In the first part of our study, MNCs were plated separately either with CsA at concentrations of 1, 2, and 4 μ g/ml, or with 2-CdA at concentrations of 5, 10 and 20 nM, or with F-ara-A at concentrations 0.4, 0.8 and 1.6 μ M. The concentrations were chosen according to the references and modified by us^{6, 19, 28}. The control cultures were plated without any agent.

In the second part of the study, MNCs were preincubated with 4 μ g/ml of CsA for 30 min at 37°C and

then the following concentrations of PNAs were added to the culture medium: 5, 10 and 20 nM of 2-CdA, or 0.4, 0.8 or 1.6 μ M of F-ara-A.

All cultures were incubated for 14 days at 37°C in an atmosphere of 5% CO₂ in air and examined with an inverted microscope. Then, aggregates containing 40 or more cells were scored as colonies. The colonies were separated from the cultures and, after centrifuging, the cells were stained according to the May-Grunwald-Giemsa method for confirmation of their morphology.

Statistical analysis. Statistical differences between the experimental and control cultures were evaluated utilizing the Student's *t*-test. A value of $p < 0.05$ was the level for statistical significance.

Interactions. IC₅₀ values were defined as the concentrations at which agents achieved a 50% inhibition of colony formation. The interaction index, as the ratio between the observed and expected survival index values (O/E), was used to estimate subadditive, additive or synergistic interaction. An observed/expected ratio > 1.2 was defined as a subadditive, 0.8–1.2 as an additive, and < 0.8 as a synergistic effect¹⁸.

Results and Discussion

CsA is a cyclic polypeptide with high immunosuppressive activity. It is used in the prophylaxis of allograft rejection and in the treatment of many autoimmune disorders^{1, 7}. Recently, it has also been used in

oncology and hematology as a modulator of MDR1 resistance to enhance the cytotoxicity of drugs^{1, 17}. Promising results have been obtained especially in the treatment of acute myeloid leukemia (AML), childhood acute lymphoblastic leukemia (ALL), as well as in the blastic crisis of CML^{6, 13, 20}. It has also been shown that CsA used together with cytostatics, such as daunorubicin or idarubicin, can be useful in the prevention of MDR1 and allow more efficacious and less toxic treatment of neoplastic disorders^{3, 29}.

In the first part of our study we investigated the influence of CsA as well as new PNAs (2-CdA and F-ara-A) used alone on normal and leukemic progenitor cells *in vitro*. We have shown that CsA at all three concentrations statistically significantly inhibited the colony growth of CML CFU-GM, as compared with the control ($p < 0.02$). In contrast, the drug did not influence to a significant degree normal colony growth ($p > 0.05$; Fig. 1, 2, Table 1, 2). The IC₅₀ for CsA was 3.9 μ g/ml for normal CFU-GM and 2.7 μ g/ml for CML CFU-GM (Table 3). Our findings are in agreement with the results obtained by HELLMANN and GOLDMAN¹⁰, who demonstrated that at low concentrations of CsA no significant inhibition of normal CFU-GM colony growth was observed, but 10 μ g/ml of CsA inhibited the proliferation of these cells in nearly 50%. HIRAO et al.¹¹ also observed that CsA had no toxicity against normal progenitor cells, neither CFU-GM nor burst forming unit-erythrocyte (BFU-E). Nevertheless, in hypoplastic refractory anemia other authors have demonstrated an

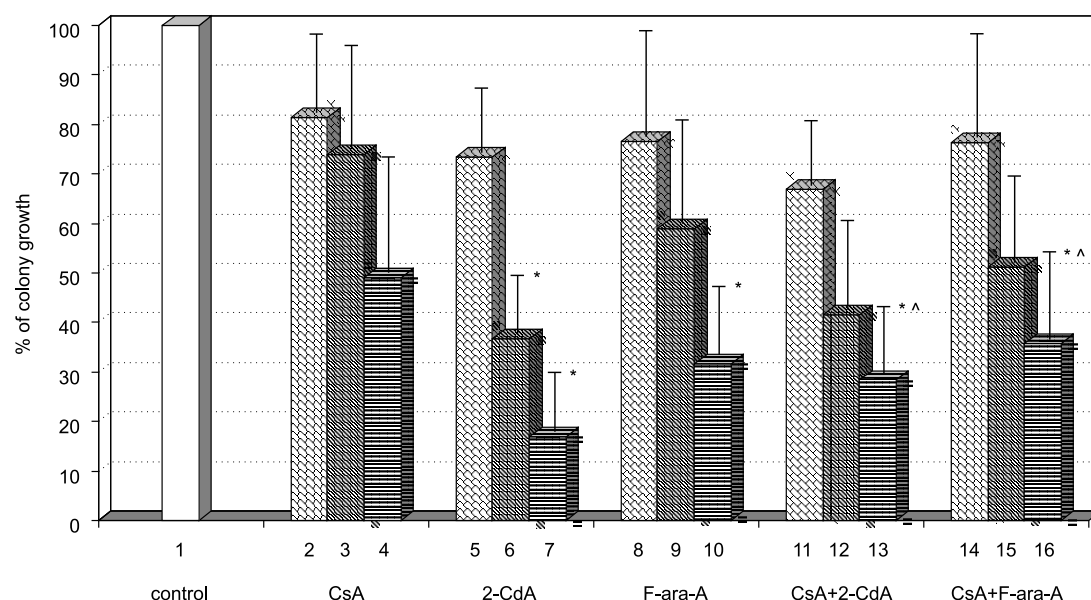


Fig. 1. The influence of CsA and purine analogues on normal CFU-GM colony growth. 1 – control, 2 – CsA 1 μ g/ml, 3 – CsA 2 μ g/ml, 4 – CsA 4 μ g/ml, 5 – 2-CdA 5 nM, 6 – 2-CdA 10 nM, 7 – 2-CdA 20 nM, 8 – F-ara-A 0.4 μ M, 9 – F-ara-A 0.8 μ M, 10 – F-ara-A 1.6 μ M, 11 – 4+5, 12 – 4+6, 13 – 4+7, 14 – 4+8, 15 – 4+9, 16 – 4+10. * Value statistically significant vs CsA used alone, ^ value statistically significant vs CML CFU-GM. The results are expressed as a percentage of colony number as compared with the control, $\bar{x} \pm$ SD

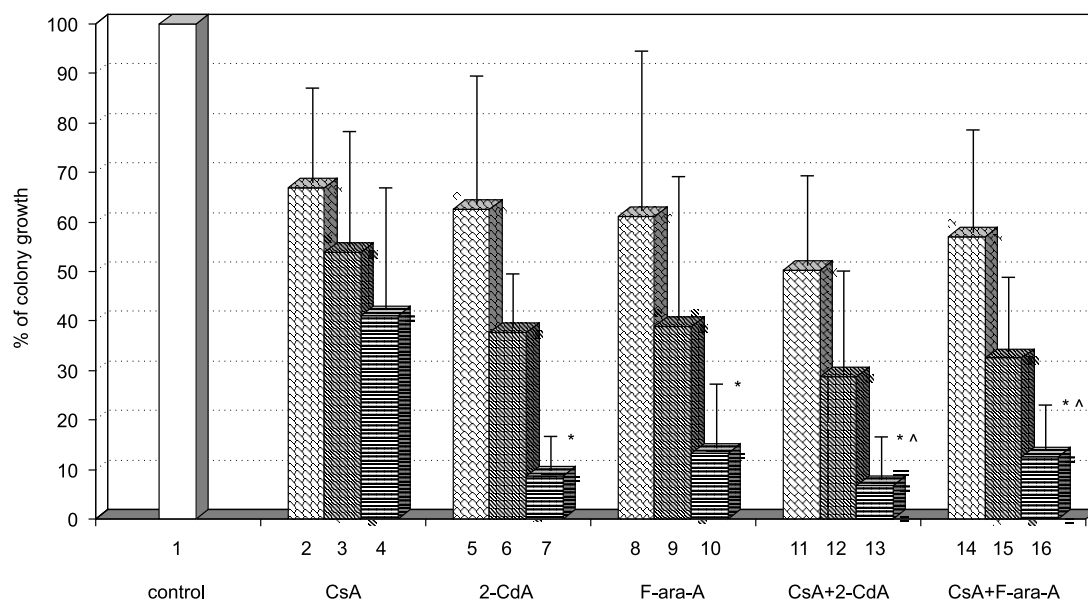


Fig. 2. The influence of CsA and purine analogues on CML CFU-GM colony growth. For legend see Fig. 1

Table 1. Comparison of the effect of CsA used alone or in combination with 2-CdA on normal and CML CFU-GM colony numbers in cultures *in vitro*

Type of colonies	Control	CsA			2-CdA			CsA+2-CdA			P
		1 µg/ml	2 µg/ml	4 µg/ml	5 nM	10 nM	20 nM	4+5	4+6	4+7	
	1	2	3	4	5	6	7	8	9	13	
Normal CFU-GM	35.0±7.4 (28–42)	29.4±6.4 (22–40)	28.8±4.8 (23–38)	17.5±10.3 (15–34)	25.0±3.0 (21–29)	12.8±2.9 (10–16)	6.1±3.6 (0–10)	22.9±3.3 (17–26)	13.9±3.1 (8–16)	9.5±3.2 (6–12)	6v1=0.002;7v1=0.002; 8v1=0.002;9v1=0.003; 10v1=0.006;6v3=0.005; 7v4=0.0007;10v4=0.004
CML CFU-GM	52.1±13 (42–81)	34.6±16.7 (30–65)	28.2±13.9 (15–50)	21.7±12.9 (12–36)	32.6±12.1 (19–49)	19.5±8.4 (9–36)	3.2±6.8 (2–9)	27.7±9.9 (17–58)	15.0±8.3 (10–38)	3.9±3.6 (0–11)	2v1=0.02;3v1=0.005; 4v1=0.0001;5v1=0.003; 6v1=0.0006;7v1=0.0002; 8v1=0.0005;9v1=0.0006; 10v1=0.0004;7v4=0.007; 9v4=0.01;10v4=0.001

Values are expressed as a mean number ±SD, ranges in parentheses.

Table 2. Comparison of the effect of CsA used alone or in combination with F-ara-A on normal and CML CFU-GM colony numbers in cultures *in vitro*

Type of colonies	Control	CsA			F-ara-A			CsA+F-ara-A			P
		1 µg/ml	2 µg/ml	4 µg/ml	0.4 µM	0.8 µM	1.6 µM	4+5	4+6	4+7	
	1	2	3	4	5	6	7	8	9	10	
Normal CFU-GM	35.0±7.4 (28–42)	29.4±6.4 (22–40)	28.0±4.8 (23–38)	17.5±10.3 (15–34)	26.3±3.9 (18–30)	20.0±3.0 (15–23)	10.5±4.1 (8–16)	25.8±3.7 (22–30)	17.0±2.5 (15–21)	12.9±3.9 (4–16)	6v1=0.0005;7v1=0.0006; 8v1=0.001;9v1=0.0001; 10v1=0.0001;6v3=0.002; 7v4=0.009;10v4=0.03
CML CFU-GM	52.1±13 (42–81)	34.6±16.7 (30–65)	28.2±13.9 (15–50)	21.7±12.9 (12–36)	34.4±14.4 (15–52)	21.2±16.0 (16–49)	7.4±4.9 (8–15)	35.4±20.2 (17–41)	18.8±8.7 (12–34)	7.2±5.8 (3–8)	2v1=0.02;3v1=0.005; 4v1=0.0001;6v1=0.003; 7v1=0.0006;9v1=0.0009; 10v1=0.0005;7v4=0.009; 10v4=0.01

Values are expressed as a mean number ±SD, ranges in parentheses.

Table 3. Comparison of IC₅₀ and type of interaction for the normal and CML CFU-GM

Type of colonies	IC ₅₀						O/E	
	CsA (µg/ml)	2-CdA (nM)	F-ara-A (µM)	CsA + 2-CdA		CsA + F-ara-A		(type of interaction)
				CsA (µg/ml)	2-CdA (nM)	CsA (µg/ml)	F-ara-A (µM)	CsA + 2-CdA CsA + F-ara-A
Normal CFU-GM	3.9	8.1	1.01	3.42	6.45	3.11	0.81	1.70 (Sa) 1.60 (Sa)
CML CFU-GM	2.7	7.5	0.63	2.8	5.25	2.96	0.44	1.73 (Sa) 1.78 (Sa)

Sa – subaddition.

increase of both CFU-GM and BFU-E colonies when CsA was added to the culture². On the other hand, ITO et al.¹³ have shown that CsA was toxic against primary leukemic cells obtained from ALL patients, maintained in stroma-based culture. Similarly, leukemia cells from B cell CLL at advanced clinical stage were the most sensitive to CsA¹⁷.

The cellular effect of CsA action depends on its binding to a cytosolic protein, cyclophilin, and the complex of CsA and cyclophilin targets the Ca²⁺ and calmodulin-dependent protein phosphatase calcineurin. The resulting interference with Ca²⁺-dependent signaling is accompanied by a block in transcriptional activity and suppression of other regulatory molecules, such as IL-2 and tyrosine kinase^{13, 27}. ITO et al.¹³ suggested that the mechanism of CsA cytotoxicity in childhood ALL cells is associated with activation of apoptosis, which is suppressed by phorbol myristate acetate but not by phosphatidyl inositol-3 kinase or tyrosine kinase activities. In CLL cells, CsA has also been shown to inhibit cytokine-induced proliferation⁴.

We have also demonstrated that 2-CdA and F-ara-A used alone inhibited the colony growth of normal and CML CFU-GM in a dose-dependent manner compared with the control (Table 1,2). In addition, in the case of CML CFU-GM a significant colony growth inhibition was observed after the use of the highest concentrations of 2-CdA and F-ara-A compared with CsA used alone ($p=0.002$, $p=0.005$, respectively; Fig. 2). In the case of normal CFU-GM, the differences between the two higher concentrations of 2-CdA and CsA were also significant ($p=0.006$; $p=0.001$; Fig. 1). However, no significant differences in colony growth inhibition between both PNAs used together with CsA were observed. In the case of 2-CdA, IC₅₀ for normal and CML CFU-GM were 8.1 and 7.5 nM, and in the case of F-ara-A it was 1.01 and 0.63 µM, respectively (Table 3). The present studies confirmed our previous *in vitro* observations that 2-CdA used alone inhibited the col-

ony growth of CML CFU-GM to a higher degree than their normal counterparts^{15, 19, 25}.

It is known that PNAs play an effective role in the activation of apoptosis. MARTINELLI et al. demonstrated the induction of apoptosis *in vitro* on fresh CML cells subjected to the activity of 2-CdA and F-ara-A. The authors suggested that the BCR-ABL fusion protein observed in CML cells is resistant to the induction of apoptosis by a number of agents and that the elevated expression of BCR-ABL tyrosine kinase activity may act to suppress apoptosis. The BCR-ABL anti-apoptosis activity may be opposed by the apoptosis-inducing effect of PNAs²¹. In addition, CONSOLI et al.⁴ suggested that the mechanism of F-ara-A action in proliferating cells is mainly cell cycle-specific, and incorporation of F-ara-A into the DNA during the S-phase is required for the induction of apoptosis in a T lymphoblastic cell line.

In the second part of our studies we have shown that CsA at a concentration of 4 µg/ml added to the culture medium 30 min prior to the three different concentrations of 2-CdA or Fara-A inhibited the colony growth of both normal and CML CFU-GM in a dose-dependent manner. After the use of CsA in combination with the highest concentrations of either 2-CdA or F-ara-A, statistically significant differences, compared with CsA used alone, were observed ($p=0.008$, $p=0.03$ for CsA with 2-CdA, and $p=0.0007$, $p=0.005$ for CsA with F-ara-A, respectively, for normal and CML CFU-GM). We have also shown that the highest concentrations of 2-CdA and F-ara-A used jointly with CsA inhibited CML CFU-GM colony growth to a greater degree than normal CFU-GM ($p=0.002$ and 0.005 , respectively). However, we suspect that this effect is probably connected with PNAs action and not with CsA. We did not observe any statistical significances in colony growth inhibition between CsA used in combination with PNAs and PNAs used alone. In contrast, MAIA et al.²⁰ have shown that etoposide used alone was partially

toxic to the cells obtained from the blastic phase of CML patients, but its toxicity increased nearly 6-fold when it was combined with CsA.

In conclusion, in our present study we observed that the differences in the inhibition of both types of CFU-GM colony growth among the combinations of CsA with either 2-CdA or F-ara-A and the PNAs used alone were similar. In addition, CsA at the concentrations used, added to the culture medium 30 min prior to 2-CdA or F-ara-A, did not enhance the antileukemic effect of PNA in chronic-phase CML patients. However, the highest concentrations of 2-CdA and F-ara-A used jointly with CsA inhibited CML CFU-GM colony growth to a greater degree than normal CFU-GM. Finally, we suggest that the combination of CsA with PNAs may be more useful in patients before allogeneic bone marrow transplantation to intensify the antiproliferative effect connected with the prophylaxis of graft-versus-host disease than in the treatment of CML patients. Nevertheless, further studies connected with the molecular analysis of MDR1 expression on normal and CML progenitor cells are necessary.

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