This study was designed to investigate the effects of cyclosporine A (CsA) on a multidrug-resistance cultured cell line, and its effect on complete remission in patients with acute myeloid leukemia (AML). A multidrug resistant K562/ADM cell line and drug-sensitive K562 cell line was used. The intracellular concentration of daunorubicin and the accumulation of Rhodamine 123 (Rh123) in the K562/ADM and K562 cells were evaluated. Clinical effects of CsA were also studied in 65 patients with AML. In the K562/ADM cells, the 50% of inhibition concentration (IC50) of daunorubicin only group was 23.0±5.2μmol/L, which was greater than in other groups co-administered with CsA (1.2±4.8μmol/L), verapamil (1.5±5.4μmol/L) or CsA+verapamil (1.4±4.3μmol/L) (all P<0.01). The relative fluorescence intensity of Rh123 in the K562/ADM cells treated with CsA and daunorubicin was increased from 48.9% to 69.8% (P<0.05). CsA also improved the complete remission rate in the AML patients (72.7% vs 21.9%, P<0.01). We conclude that CsA can significantly diminish the multidrug resistance in K562/ADM cells. It also enhances the complete remission rates in patients with AML. CsA may be used as an integral part of the chemotherapy for AML.

Author Address: binzhang@csu.edu.au lwang@csu.edu.au

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Cyclosporine diminishes multidrug resistance in K562/ADM cells and improves complete remission in patients with acute myeloid leukemia

Guang-Yao Li¹, Ji-Zhu Liu¹, Bin Zhang¹, Le-Xin Wang², Chun-Bo Wang³, Shou-Guo Chen⁴

1. Liaocheng People's Hospital and Liaocheng Clinical School of Taishan Medical University, Liaocheng Shandong, 252000, P. R. China;
2. School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, Australia;
3. Department of Pharmacology, Qingdao University Medical College, Qingdao, P. R. China;
4. Haisheng Oncology Hospital of Qingdao, Qingdao, P.R. China

Corresponding authors:
Prof Lexin Wang, School of Biomedical Sciences, Charles Sturt University Wagga Wagga, NSW 2678, Australia. Phone: +61 2 69332905, Fax: +61 2 69 332587, E-mail: lwang@csu.edu.au

OR

Bin Zhang, Division of Hematology. Liaocheng People's Hospital, Liaocheng, Shandong Province, 252000, P. R. China. Phone: +86-635-8276317; Fax: +86-635-8277306; E-mail: lcrmyy@163.com.
Abstract:

This study was designed to investigate the effects of cyclosporine A (CsA) on a multidrug-resistance cultured cell line, and its effect on complete remission in patients with acute myeloid leukemia (AML). A multidrug resistant K562/ADM cell line and drug-sensitive K562 cell line was used. The intracellular concentration of daunorubicin and the accumulation of Rhodamine 123 (Rh123) in the K562/ADM and K562 cells were evaluated. Clinical effects of CsA were also studied in 65 patients with AML. In the K562/ADM cells, the 50% of inhibition concentration (IC50) of daunorubicin only group was 23.0±5.2µmol/L, which was greater than in other groups co-administered with CsA (1.2±4.8 µmol/L), verapamil (1.5±5.4µmol/L) or CsA+verapamil (1.4±4.3µmol/L) (all P<0.01). The relative fluorescence intensity of Rh123 in the K562/ADM cells treated with CsA and daunorubicin was increased from 48.9% to 69.8% (P<0.05). CsA also improved the complete remission rate in the AML patients (72.7% vs 21.9%, P<0.01). We conclude that CsA can significantly diminish the multidrug resistance in K562/ADM cells. It also enhances the complete remission rates in patients with AML. CsA may be used as an integral part of the chemotherapy for AML.

Key words: Leukemia; acute myeloid leukemia; cyclosporin A; multidrug resistance; cancer.
1. Introduction

Multidrug resistance to cytotoxic drugs is the main cause for the failure of chemotherapy of acute myeloid leukemia (AML). Cellular expression of the multidrug transporter, \textit{P-glycoprotein} is recognized as a biological mechanism contributing to treatment failure in AML [1, 2]. \textit{P-glycoprotein} is expressed in approximately 40% of relapsed AML, which was higher than other types of leukemia or normal lymphocytes [3]. In cells expressing \textit{P-glycoprotein}, there is an increased extrusion of cytotoxic drugs, leading to poor response to standard chemotherapy and adverse clinical outcomes in AML [1, 2].

\textit{In vitro} studies have shown that cyclosporine A (CsA) is able to restore the concentration of chemotherapeutic drugs in target cancer cells [4]. CsA was found to enhance retention of the substrate drug mitoxantrone in cells overexpressing \textit{P-glycoprotein} and increase the cytotoxicity of mitoxantrone [5]. However, whether the restoration of intracellular drug concentration is related to CsA’s suppressive effect on \textit{P-glycoprotein} expression or function has been controversial. Some studies suggested that factors other than \textit{P-glycoprotein}, such as delays in systemic clearance of cytotoxic drugs, are responsible for the daunorubicin or idarubicin accumulation in AML blasts [6, 7].

In this study, we evaluated the drug-resistance reversing effect of CsA in a cultured cell line. The influence of CsA on intracellular concentration of daunorubicin as well as the function of \textit{P-glycoprotein} in these cells was also investigated. Finally, the clinical effects of CsA on the complete remission rates in a group of patients with AML were studied.
2. Materials and methods

This study was approved by the institution review board of Liaocheng People’s Hospital.

Written informed consent was obtained from all participant patients.

2.1. Cell lines and cell culture

K562/ADM cells were obtained from the Institute of Hematology at Peking Union Medical College. This multidrug-resistant leukemia cell line was derived from the parental K562 cell line by continuous exposure to increasing concentrations of adriamycin. The multi-drug resistant phenotype is not expressed by K562 but is over expressed in K562/ADM cells. The drug resistance of K562/ADM cell line was maintained by adding 1.0 µg/ml of adriamycin, which was ceased 3 days before the cell line was used for this study.

2.2. Effect of CsA on multidrug resistance in K562/ADM cells

This protocol involved four groups. The control group was treated with daunorubicin (2 µmol/L). Three study groups were treated with daunorubicin (2 µmol/L) and CsA 3 µmol/L (CsA group), or daunorubicin 2 µmol/L and verapamil 10 µmol/L (verapamil group), or daunorubicin 2 µmol/L, CsA 3 µmol/L and verapamil 10 µmol/L (combined drug group).

Trypan blue exclusion assay was performed to measure IC50, which was defined as the concentration of daunorubicin that causes 50% cytotoxicity. Trypan blue exclusion is a cell viability assay based on the ability of the cells to exclude the vital dye trypan blue, nonviable neurons failed to exclude the vital dye and showed trypan blue staining in their cytoplasm [8]. The values of IC50 were used as an indicator for multidrug resistance of the K562/ADM.
2.3. Influence of CsA on concentration of daunorubicin in K562/ADM cells

Multidrug-resistant K562/ADM cells (concentration 0.5×10⁶/ml) were divided into the following groups: 1) CsA group: treated with daunorubicin (2μmol/L) and CsA (3μmol/L); 2) positive control group: treated with daunorubicin (2μmol/L) and verapamil (10μmol/L); 3) Negative control group: K562/ADM cells treated with daunorubicin (2μmol/L); and 4) Drug-sensitive K562 cells were treated with daunorubicin (2μmol/L). Flow cytometry (EPICS, US Coulter Corp) was used to detect fluorescence intensity of daunorubicin.

2.4. Flow cytometry to detect the accumulation Rhodamine 123 (Rh123) in K562/ADM cells

Flow cytometry was used to detect the fluorescence intensity of Rh123 to reflect the function of P-glycoprotein. K562/ADM cells and parental K562 cells were washed with cold saline for 2 sec, adjusting the concentration to 0.5×10⁶/ml. After diluting the cells with serum-free RMPI1640, 10μmol/L of Rh123 were added. The positive control was treated with 10μmol/L of verapamil. The negative control group received no treatment. After treatment, the cells were incubated for 60min, and washed with cold saline for 2 sec. Flow cytometry (laser wavelength of 488 nm, emission wavelength of 530 nm) was used to detect fluorescence intensity of Rh123 in the K562/ADM and K562 cells.

2.5. Clinical trial

Forty-one patients with acute myeloid leukemia (AML) were recruited from Liaocheng
City People's Hospital, and 24 were from Qingdao University Hospital. Of the 65 patients (39 men, mean age 34±5.2 years), 38 were newly diagnosed cases, and 27 had relapse after conventional chemotherapy.

The classification of the leukemia was as follows: there was one patient for M0, M1 and M7, respectively. There were 19 for M2, 15 for M3, 14 for M4, 9 for M5, and 2 for M6. Three patients were of hybrid type of leukemia.

Patients were randomly (based on random draw of a number from a container) divided into CsA group (n=33) who were treated with CsA (16 mg/kg/day) and a course of standard chemotherapy, and the control group (n=32), who were treated with a course of standard chemotherapy but no CsA. The standard chemotherapy used in this study consisted of idarubicin (12mg/m²/day for 3 days) and cytarabine (100mg/m²/day for 7 days).

2.6. Statistical analysis

Data were expressed as means ± SD. SPSS12.0 software was used for data analysis. Numerical data were analyzed with one-way ANOVA. Categorical data were analyzed with Chi-square test. P<0.05 was considered statistically significant.

3. Results

3.1. Effect of CsA on multidrug resistance in K562/ADM cells

As shown in Table 1, the IC50 of daunorubicin in the K562/ADM cells treated with CsA, verapamil, or CsA+verapamil was significantly less than the IC50 in the control (daunorubicin only) group (P<0.01). There was no significant difference in the IC50 between CsA, verapamil and CsA+verpamil groups (P>0.05).
3.2. Effect of CsA on intracellular concentration of daunorubicin in K562/ADM cells

As shown in Fig 1, the intracellular concentration of daunorubicin in the multidrug resistant K562/ADM cells was significantly lower than in the drug-sensitive K562 cells. CsA increased the concentration of daunorubicin in the K562/ADM cells; after 6 hours the daunorubicin concentration in the K562/ADM cells was almost similar to that in the drug-sensitive K562 cells.

3.3. Accumulation of Rh123 in K562/ADM cells

Table 2 shows the ratio of the fluorescence intensity of Rh123 between K562/ADM cells and in the drug-sensitive K562 cells. The fluorescence intensity of Rh123 in K562/ADM cells was about half of that in the K562 cells. After CsA treatment, the fluorescence intensity of Rh123 intensity in the K562/ADM cells increased to 69.8% (P<0.05), whereas verapamil treatment increased the Rh123 intensity to 52% (P<0.05).

3.4. Clinical effects of CsA in AML patients

There were no mortalities during the induction. As shown in Table 3, complete remission rate and total effective rate in the CsA group was significantly higher than in the control group (P<0.01).

Complete remission in the 24 patients lasted from 28 weeks to 208 weeks (mean 91±12 weeks, median 66 weeks). The numbers of patients remaining in complete remission at 6, 12, 18, 24 and 36 months were 24 (100%), 16 (67%), 10(50%) and 8(33%).
4. Discussion

The main findings of this study are: 1) CsA had a significant suppressing or reversing effect on multidrug resistance in K562/ADM cells in vitro; 2) CsA increased the intracellular concentration of daunorubicin in the multidrug resistant K562/ADM cells; 3) CsA treatment increased the fluorescence intensity of Rh123 in the K562/ADM cells; 4) Addition of CsA to the conventional remission induction regimen increased the rate of complete remission and total effective rate in patients with AML.

Overexpression of the multidrug resistance protein G-glycoprotein has been associated with treatment failure in AML and other malignancies. Therefore, co-administration of conventional chemotherapy and multidrug resistant modulators that block P-glycoprotein-mediated drug efflux is potentially an effective strategy for relapsed or refractory AML [5]. Several pharmacological modulators of P-glycoprotein have been tested, such as calcium channel blockers (verapamil, nifedipine and diltiazem), steroid hormones (tamoxifen and progesterone), antibiotics (ceftriaxoe), protein kinase inhibitors (staurosporine, imatinib mesulate), PSC-833 and monoclonal antibodies (MRK16, UIC-2) [9]. These modulators share a various degree of success in suppressing drug resistance in vitro, but their clinical efficacy and safety profiles are either undesirable or yet to be determined [10].

CsA is a noncytotoxic multidrug resistant modulator that diminishes the extrusion of cytotoxic drugs in the targeted cancer cells [4-7]. The present study used K562/ADM and K562 cells to detect the cytotoxicity of daunorubicin with or without concurrent CsA. K562/ADM cells were induced by gradually increasing the concentrations of doxorubicin in
the cell culture. These cells share a number of multidrug resistant characteristics with other tumor cells such as human MCF-7 breast cancer cells [10]. The IC50 of daunorubicin in the K562/ADM cells was more than 20 times higher than in the K562/ADM cells treated with CsA, or verapamil, or both. The intracellular concentration of daunorubicin in the K562/ADM cells was significantly lower than in the drug-sensitive K562 cells. However, treatment with CsA increased the daunorubicin concentration in the K562/ADM cells to the levels seen in the drug-sensitive K562 cells (Fig 1).

We used Rh123, a P-glycoprotein-transported fluorescent dye to measure the function of P-glycoprotein in the K562/ADM and K562 cells. The unidirectional efflux of R123 is believed proportional to the level of P-glycoprotein expression and can be reduced by P-glycoprotein inhibition [11]. In the present study, the fluorescence intensity of Rh123 in the K562/ADM cells was less than half of that in the drug-sensitive K562 cells. CsA treatment significantly increased the fluorescence intensity of Rh123 in the K562/ADM cells, indicating inhibition of P-glycoprotein function.

A potential limitation of this in vitro study is that, CsA is also a modulator of apoptosis via permeability transition pore [12]. Therefore, increased cytotoxicity of daunorubicin in CsA treated K562/ADM cells may not be solely due to inhibition of P-glycoprotein and accumulation of intracellular daunorubicin concentration. Further studies are required to clarify the role of apoptosis in the CsA-induced actions of daunorubicin on the multidrug resistant K562/ADM cells.

Several randomized clinical trials have been completed to assess the effect of CsA in adult patients [13, 14] or in children with AML [15]. In adult patients with relapsed or
refractory AML, and in those with secondary AML, treatment with CsA significantly reduced the frequency of resistant disease or the need for second induction [14]. Relapse-free survival was also significantly improved with the addition of CsA, but the rate of complete remission was only marginally improved [14]. In children with AML, addition of CsA to consolidation chemotherapy did not prolong the durations of remission or improve overall survival [15]. However, overexpression of \textit{P-glycoprotein} was only 14\% in this pediatric population [15], which was much lower than in the adult population of approximately 40\% [3]. Therefore, \textit{P-glycoprotein} inhibition in pediatric population may not be as effective as in the adult AML patients in revising multidrug resistance. In the present study, administration of CsA improved the complete remission rate from the conventional remission induction regimen (cytarabine and daunorubicin); suggesting multidrug resistance modulation with CsA offers at least some short-term benefits.

Several limitations may be present in this study. The clinical evaluation of CsA involved a small group of 65 patients. The expression of \textit{P-glycoprotein} in the AML cells was not assessed. However, this may not have significant impact on the results of the clinical trial since the study and the control groups were comparable in age (average 33-34 years) and sex. Finally, due to time constraint the effect of CsA on relapse free survival rate was not investigated.

In conclusion, CsA appears to be an effective and safe multidrug resistance modulator both \textit{in vitro} and \textit{in vivo}. It increases the intracellular accumulation of cytotoxic agents such as daunorubicin, by inhibiting the drug-extrusion \textit{P-glycoprotein} on the leukemia cells. When used concurrently with the standard remission induction regimen, CsA improves the
completion remission rate in patients with AML. Further studies are required to assess the influence of CsA on the relapse free survival and adverse effects.

Acknowledgement

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References


Table 1. Effect of cyclosporine A (CsA) on the IC50 of daunorubicin in the multidrug resistant K562/ADM cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>IC50 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.0±5.2</td>
</tr>
<tr>
<td>CsA</td>
<td>1.2±4.8*</td>
</tr>
<tr>
<td>Verapamil</td>
<td>1.5±5.4*</td>
</tr>
<tr>
<td>CsA+verapamil</td>
<td>1.4±4.3*</td>
</tr>
</tbody>
</table>

* Compared with the control, \( P<0.01 \).
Table 2. Fluorescence intensity of Rh123 in K562/ADM cells (relative to that in the drug-sensitive K562 cells)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fluorescence intensity of Rh123</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>52.3%±0.04*</td>
</tr>
<tr>
<td>CsA</td>
<td>69.8%±0.05*</td>
</tr>
<tr>
<td>Negative control</td>
<td>48.9%±0.03</td>
</tr>
</tbody>
</table>

* Compared with the negative control, $P<0.05$
Table 3. Impact of cyclosporine on the clinical outcomes of patients with acute myeloid leukemia.

<table>
<thead>
<tr>
<th>Group</th>
<th>CsA (n=33)</th>
<th>Control (n=32)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>33±3.8 (16-64)</td>
<td>34±6.3 (18-57)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>19 (57.6%)</td>
<td>20 (62.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Complete remission</td>
<td>24 (72.7%)</td>
<td>7 (21.9%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Partial remission</td>
<td>5 (15.2%)</td>
<td>6 (18.8%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Total efficiency</td>
<td>30 (87.9%)</td>
<td>12 (40.7%)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure legends

Fig 1. Effect of cyclosporine on intracellular concentration of daunorubicin (DNR) in K562/ADM cells. CsA: cyclosporine; VRP: verapamil. K562/S: DNR-sensitive parental K562 cells.
Figure 1.