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ORIGINAL ARTICLE: RESEARCH

Flavokawain B inhibits the growth of acute lymphoblastic leukemia cells via p53 and caspase-dependent mechanisms

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Abstract

The development of novel chemotherapeutic drugs is needed for the treatment of patients with acute lymphoblastic leukemia (ALL). In this study, the anti-leukemic effect and the potential molecular mechanisms of action of flavokawain B on ALL were investigated. Flavokawain B was found to significantly inhibit the cellular proliferation of B-ALL and T-ALL cell lines in a dose-dependent manner. It also induced cellular apoptosis by increasing the expression of p53, Bax and Puma, and activating the cleavage of caspase-3 and poly ADP-ribose polymerase (PARP). Furthermore, the enhancement of p53-dependent apoptosis by flavokawain B could be rescued by pifithrin-α, a pharmacological inhibitor of p53 transcriptional activity. Moreover, the proliferation of leukemia blast cells from 16 patients with ALL was inhibited by flavokawain B, and tumor growth in xenograft mice was also suppressed by this drug. In conclusion, our results demonstrate the therapeutic potential of flavokawain B for the treatment of ALL.

Keywords: Flavokawain B, acute lymphoblastic leukemia (ALL), p53, proliferation, apoptosis

Introduction

Acute lymphoblastic leukemia (ALL), the most common childhood malignancy, accounts for approximately one-third of all pediatric malignancies [1]. Modern treatment strategies have led to remarkable improvements in the prognosis of ALL. Nearly 80% of children with ALL can achieve complete remission [2,3]. However, the number of cases that fail to achieve long-term disease-free survival is still high [4,5]. Thus, a significant need exists to find novel and less toxic drugs for the treatment of patients with ALL. Although several signaling pathways that regulate the occurrence and development of leukemia have been identified [6,7], the exact mechanisms remain unclear. The capacity for rapid proliferation and survival of leukemic cells is a crucial characteristic associated with disease progression [8]. Therefore, targeting anti-proliferative signals [9] or pro-apoptotic signals [10] may provide novel therapies for ALL. The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway is a pro-survival pathway that plays a central role in cell growth, apoptosis and survival [11–13]. The p53 pathway is a typical pro-apoptotic signaling pathway that plays a critical role as a tumor inhibitor [14–17].

Many standard chemotherapeutic agents such as vincristine and harringtonine have originated from natural sources [18]. The natural products either alone or in combination with other drugs that target the p53 pathway may improve therapeutic efficacy [19]. The consumption of traditional kava in the Pacific Islands has been associated with low incidences of cancer [20]. Flavokawains, extracted from kava, have shown anticancer activity by inhibiting cellular proliferation and inducing apoptosis in vitro and in vivo. Flavokawain B, one of the flavokawains, exhibited anticancer activity against osteosarcoma, prostate cancer, oral carcinoma and lung cancer cells [21–24]. However, the potential role of flavokawain B as an anticancer agent and its mechanism of action in ALL are unknown.

In this study, we investigated the effect of flavokawain B on the growth of leukemic cells in vitro and in vivo. We demonstrated the anti-leukemic effect of flavokawain B through activation of the p53-dependent apoptotic pathway in B-ALL and T-ALL cell lines and primary leukemic blasts from pediatric patients with ALL. We also showed that flavokawain B was effective in inhibiting the growth of leukemic cells in a xenograft mouse model of ALL. Our results indicated that flavokawain B might be a possible therapeutic drug for patients with ALL.
Materials and methods

Cell lines and cell culture
HK-2 (normal renal proximal tubular cell), CCRF-CEM (T-ALL), CEM-C1 (T-ALL), Jurkat (T-ALL) and RS4-11 (B-ALL) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Information regarding cell lines is detailed in Table I. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT). All cells were maintained in a humidified atmosphere with 5% CO\textsubscript{2} at 37°C.

Reagents and antibodies
Flavokawain B (Abcam, Cambridge, MA) and pifithrin-α (Selleck, Houston, TX) were dissolved in dimethyl sulfoxide (Sigma, St Louis, MO). The final concentration of dimethyl sulfoxide in the culture medium was 0.01%. Bax, Puma, cleaved caspase-3 (Asp175) and cleaved poly ADP-ribose polymerase (PARP) (Asp214) were from Cell Signaling Technology (Boston, MA); p53 was from Millipore (Billerica, MA); GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was from Sigma.

Cell viability assay
Cell viability was determined using the MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega, Madison, WI]. A total of 1 × 10\textsuperscript{4} cells per well were plated in 96-well sterile plastic culture plates and treated with the indicated concentrations of flavokawain B. After 24, 48 and 72 h incubation, cell viability was assessed using the MTS assay according to the manufacturer’s instructions.

Apoptosis assay
The apoptotic rate was determined using an Annexin V-FITC [fluorescein isothiocyanate] Apoptosis Detection Kit (Nanjing Keygen, Nanjing, China). Cells were seeded at 1 × 10\textsuperscript{5} cells/mL in six-well plates and treated with various concentrations of flavokawain B. After 24 h incubation, cells were harvested and washed twice with cold phosphate buffered saline (PBS), then resuspended in binding buffer. The suspension was stained with 5 μL Annexin V–FITC before propidium iodide (PI). Finally, the suspension was incubated in the dark for 10 min at room temperature. The rate of apoptosis was determined using flow cytometry (Cytomics FC500 flow cytometer; Beckman Coulter).

Western blot assay
After treating cells with different concentrations of flavokawain B for 24 h, they were harvested, washed and lysed. The proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes. The protein bands were visualized using chemiluminescence.

Patients
This study enrolled 11 patients with B-ALL, five patients with T-ALL and four normal volunteers from the First Affiliated Hospital of Sun Yat-sen University. The patients were treated according to the modified ALLIC Berlin–Frankfurt–Münster (BFM) 2002 protocol. Clinical information about the samples is detailed in Table II. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. All samples were collected at the time of diagnosis before

| Table I. Characteristics of the cell lines used in this study. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell line       | Cell type       | Primary site    | Karyotype       | MLL status     | p53 status     | IC\textsubscript{50} (μM) |
| RS4-11          | B-ALL           | BM              | 46,XX,t(4;11)(q21;q23) | MLL/AF4        | Wild type      | 33.81            |
| CEM-C1          | T-ALL           | PB              | 47,XX           | Normal         | Het mut        | 36.64            |
| CCRF-CEM        | T-ALL           | PB              | 47,XX           | Normal         | Het mut        | 36.73            |
| Jurkat          | T-ALL           | PB              | 46,XY           | Normal         | Het mut        | 36.48            |
| HK-2            | PTC             | Kidney          | 47,XY           | Normal         | Wild type      | 185.80           |

| Table II. Clinical and biological features of patients in the study. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient         | Sex | Age (years) | WBC (10\textsuperscript{9}/L) | FAB category | Fusion gene | Blast cells (%) | p53 status |
| 1               | F   | 5           | 15.35            | L2, BII       | None         | 81.1            | Wild type  |
| 2               | F   | 7           | 47.03            | L2, BIII      | None         | 90.5            | Wild type  |
| 3               | F   | 3           | 4.96             | L1, IV        | None         | 87              | Not done   |
| 4               | M   | 6           | 121.74           | L2, BIII      | BCR/ABL      | 81              | Mutation   |
| 5               | M   | 1           | 5.2              | L1, BII       | None         | 92.5            | Not done   |
| 6               | M   | 2           | 4.5              | L2, BIII      | None         | 83              | Wild type  |
| 7               | M   | 3           | 7.95             | L2, BIII      | None         | 83              | Wild type  |
| 8               | F   | 7           | 15               | L2, BIII      | TEL/AML1     | 91              | Wild type  |
| 9               | F   | 2           | 43.05            | L2, BII       | MLL/ENL      | 88.5            | Wild type  |
| 10              | F   | 6           | 85.98            | L2, BIII      | None         | 87              | Wild type  |
| 11              | M   | 3           | 188.56           | L3, BIII      | TEL/AML1     | 89              | Not done   |
| 12              | M   | 11          | 121.61           | L2, T         | SIL/TAL1     | 81              | Wild type  |
| 13              | M   | 7           | 166.56           | L2, T         | SIL/TAL1     | 85              | Wild type  |
| 14              | M   | 5           | 65.45            | L3, T         | MLL/AF4      | 84              | Wild type  |
| 15              | F   | 8           | 78.59            | L2, T         | MLL/AF9      | 84.5            | Wild type  |
| 16              | M   | 7           | 84.75            | L3, T         | None         | 85.5            | Not done   |

F, female; M, male; WBC, white blood cells; FAB, French-American-British.
treatment. Mononuclear cells from bone marrow specimens of patients were isolated using Ficoll–Paque PREMIUM (GE Healthcare, Uppsala, Sweden). Samples that included more than 80% leukemic blasts were frozen and stored in liquid nitrogen before use.

**Xenograft mouse model**

Female Balb/c mice, 17–23 g, 4–5 weeks old, were housed in a specific pathogen-free barrier facility. All mice were maintained and cared for according to the guidelines established by the Animal Research Committee of Sun Yat-sen University, and all procedures were conducted in accordance with the established guidelines.

Female Balb/c mice were treated with busulfan (Sigma) and cyclophosphamide (Sigma) to suppress their immune system and facilitate the establishment of the xenograft model. All mice were inoculated intravenously (i.v.) with $1 \times 10^7$ CCRF-CEM cells via the tail vein. Seven days after inoculation, all mice were randomly separated into two groups ($n = 5$). The treatment group received daily i.v. injections of flavokawain B (0.75 mg/kg) and the control group received vehicle only. Body weight was measured every 3 days. After 14 days of treatment, the mice were killed and their spleens photographed. Peripheral blood smears were stained with Wright’s Giemsa stain using standard procedures. Spleens were fixed in 10% neutral-buffered formalin.

**Figure 1.** Flavokawain B suppresses the viability and proliferation of ALL cells. (A) The chemical structure of flavokawain B. (B) HK-2, CCRF-CEM, CEM-C1, Jurkat and RS4-11 cell lines were exposed to different concentrations of flavokawain B for 24 h, and cell viability was evaluated using the MTS assay. (C) ALL cells were treated with 25, 50 and 100 μM flavokawain B for 24, 48 and 72 h. *$p < 0.05$ and **$p < 0.001$ vs. control. Results are presented as mean ± SD of three independent experiments.
Flavokawain B inhibits leukemia cell growth

for 24 h, and transferred to 70% ethanol before being embedded in paraffin. Glass slides with 3 μm tissue sections were prepared and stained with hematoxylin and eosin. On days 7 and 22, blood was drawn from the tail veins of mice in each group and collected into ethylenediaminetetraacetic acid (EDTA)-lined tubes for complete blood counts (CBCs). The experiment was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (Chicago, IL).

Results were expressed as mean ± standard deviation (SD). For normally distributed data sets, the differences between two groups were analyzed using Student’s t-test. Differences in the case of data not from normally distributed populations were determined using the Wilcoxon rank-sum test. Comparisons of multiple groups in which data were from normal populations and showed homogeneity of variance were analyzed using one-way analysis of variance (ANOVA); alternatively, the Kruskal–Wallis test was performed. Multiple comparisons were analyzed using the Bonferroni test. All tests were two-sided. Differences were considered statistically significant at *p < 0.05, **p < 0.01 and ***p < 0.001.

Results

Flavokawain B inhibits proliferation of various types of ALL cell lines

To investigate the effects of flavokawain B on the growth of human normal cell and ALL cells, we first examined the viability of HK-2 cells and CCRF-CEM (T-ALL), CEM-C1 (T-ALL), Jurkat (T-ALL) and RS4-11 (B-ALL) cell lines exposed to various concentrations of flavokawain B for 24 h using the MTS assay. As shown in Figure I(B), flavokawain B markedly inhibited the proliferation of ALL cell lines in a dose-dependent manner. However, flavokawain B had no obvious effect on HK-2 cells. We also analyzed the inhibition
of proliferation of ALL cells exposed to 25, 50 and 100 μM flavokawain B for 24, 48 and 72 h. Flavokawain B significantly inhibited the viability and proliferation of all four ALL cell lines at different treatment times [Figure 1(C)].

**Flavokawain B induces apoptosis of ALL cell lines**

To examine whether the growth inhibition of ALL cells by flavokawain B was mediated by its apoptotic effects, we tested the rate of apoptosis in three ALL cells, exposed to 25, 50 and 100 μM flavokawain B for 24 h by flow cytometry. Compared with the control group, flavokawain B treatment resulted in a significant increase in both early and late apoptotic populations in CCRF-CEM, RS4-11 and Jurkat cells [Figures 2(A)–2(C)].

**Flavokawain B increases caspase-3 activity and PARP cleavage and promotes expression of p53, Bax and Puma in ALL cells**

To assess the underlying molecular mechanism of cell apoptosis caused by flavokawain B in ALL cells, we investigated the expression of cleaved caspase-3, cleaved PARP, p53 and downstream target proteins. Western blot analysis showed that flavokawain B treatment induced dose-dependent increases of caspase-3 and PARP cleavage in CCRF-CEM, RS4-11 and Jurkat cell lines [Figures 3(A)–3(C)]. The expression levels of p53, Bax and Puma were also markedly increased in a dose-dependent manner in CCRF-CEM and RS4-11 cells after treatment with flavokawain B [Figures 3(D) and 3(E)].

**Flavokawain B activates p53-mediated apoptotic pathway in ALL cell lines**

To further elucidate the mechanisms of the dose-dependent increase of p53 expression in apoptotic ALL cells caused by flavokawain B, we studied the viability of ALL cells exposed to 25, 50 or 100 μM flavokawain B with or without 10 μM pifithrin-α, a pharmacological inhibitor of p53 transcriptional activity [25]. Pifithrin-α blocked the inhibitory effect of flavokawain B and rescued a large proportion of ALL cells [Figures 4(A)–4(D)]. Additionally, p53 expression was suppressed by pifithrin-α in flavokawain B-treated cells [Figure 4(E)].

Figure 3. Flavokawain B up-regulates the expression of cleaved caspase-3, cleaved PARP, p53, Bax and Puma. (A–C) ALL cell lines CCRF-CEM (A), RS4-11 (B) and Jurkat (C) were incubated with 25, 50 and 100 μM flavokawain B for 24 h. Cells were harvested, lysed and the expression of cleaved caspase-3 and cleaved PARP was detected by Western blot analysis, using the expression of GAPDH as a loading control. (D, E) CCRF-CEM and RS4-11 cells were treated with 25, 50 and 100 μM flavokawain B for 24 h. The expression of p53, Bax and Puma was determined by Western blot analysis, using GAPDH as a loading control.
Flavokawain B inhibits leukemia cell growth

Mice showed an excess of circulating leukocytes, and after treatment, flavokawain B-treated mice had fewer leukocytes than control mice (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.976819). Similarly, the white blood cell (WBC) count showed a statistically significant decrease after treatment, compared with the control group, but the blood platelet counts of the two groups were not significantly different [Figure 6(A)]. In addition, we clearly noted splenomegaly in control mice but not in flavokawain B-treated mice. Flavokawain B treatment significantly reduced splenomegaly compared with the control group and the weights of spleens in the treatment group were significantly less than in the control group [Figure 6(B)]. We observed that spleen samples from flavokawain B-treated mice had less leukemic infiltration than control mice (Supplementary Figure 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.976819). We also determined whether the weight loss of mice was correlated with disseminated leukemia, and found that the weight loss of control mice was significantly greater than that of flavokawain B-treated mice [Figure 6(C)].

Flavokawain B inhibits growth of patient-derived ALL blasts ex vivo

To determine the effect of flavokawain B on normal mononuclear cells, primary B-ALL blasts and T-ALL blasts, we isolated cells from the bone marrows of normal volunteers and patients with ALL, then exposed these cells for 24 h to 25, 50 or 100 μM flavokawain B. The cell viability was analyzed using the MTS assay. As shown in Figure 5(A), the proliferation of B-ALL blasts was significantly inhibited in a dose-dependent manner. A similar effect was obtained in T-ALL blasts [Figure 5(B)]. However, flavokawain B had no obvious effect on normal mononuclear cells [Figure 5(C)]. Western blot analysis of leukemic blasts from patient 1 showed similar results to those obtained in ALL cell lines. Flavokawain B led to a marked increase of p53, Bax and Puma expression [Figure 5(D)]. The results for other patients are detailed in Table III.

Flavokawain B suppresses xenografted human ALL in mice

To examine the effect of flavokawain B on ALL in vivo, we injected ALL cells systemically into Balb/c female mice and treated the mice with flavokawain B. Before treatment, the
Figure 5. Flavokawain B inhibits the proliferation and induces p53-mediated apoptosis in patient-derived ALL blasts \textit{ex vivo}. (A–C) Cells isolated from the bone marrows of 11 patients with B-ALL (A), five patients with T-ALL (B) and four normal volunteers (C) were exposed \textit{ex vivo} to 25, 50 and 100 \( \mu \text{M} \) flavokawain B for 24 h. Cell viability was measured using the MTS assay. \( **p < 0.001 \) vs. control. Results represent mean \( \pm \) SD of triplicates. (D) ALL cells from patient 1 were treated with flavokawain B for 24 h and the expression of p53, Bax and Puma were tested using Western blot with the indicated antibodies. The expression of GAPDH served as a loading control.

Discussion

Based on previous reports suggesting that flavokawain B has anti-carcinogenic properties in some types of cancer, we hypothesized that flavokawain B may have an anti-leukemic effect on ALL cells [21–24, 26–29]. In the present study, we investigated the anti-leukemic activities of flavokawain B.

Table III. Relative expression of p53, Puma and Bax in samples exposed to 25, 50 and 100 \( \mu \text{M} \) flavokawain B.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>p53</th>
<th>Puma</th>
<th>Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con 25 50 100</td>
<td>Con 25 50 100</td>
<td>Con 25 50 100</td>
</tr>
<tr>
<td>1</td>
<td>0.46 0.63 1.16 2.03</td>
<td>0.06 0.26 0.73 0.90</td>
<td>0.02 0.17 0.74 0.98</td>
</tr>
<tr>
<td>2</td>
<td>0.24 0.53 0.95 1.67</td>
<td>0.13 0.32 0.65 0.87</td>
<td>0.04 0.21 0.52 0.87</td>
</tr>
<tr>
<td>3</td>
<td>0.32 0.43 1.23 1.93</td>
<td>0.05 0.16 0.52 0.63</td>
<td>0.05 0.24 0.43 0.65</td>
</tr>
<tr>
<td>4</td>
<td>0.34 0.51 0.84 1.68</td>
<td>0.15 0.34 0.63 0.52</td>
<td>0.08 0.35 0.63 0.73</td>
</tr>
<tr>
<td>5</td>
<td>0.16 0.32 0.56 0.86</td>
<td>0.23 0.36 0.85 0.82</td>
<td>0.14 0.57 0.86 0.75</td>
</tr>
<tr>
<td>6</td>
<td>0.23 0.43 0.78 2.53</td>
<td>0.34 0.25 0.56 0.84</td>
<td>0.32 0.25 0.64 0.86</td>
</tr>
<tr>
<td>7</td>
<td>0.52 0.58 0.84 1.96</td>
<td>0.12 0.35 0.53 0.75</td>
<td>0.42 0.84 0.78 0.93</td>
</tr>
<tr>
<td>8</td>
<td>0.43 0.74 1.32 1.84</td>
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</tr>
<tr>
<td>9</td>
<td>0.48 0.82 1.43 2.12</td>
<td>0.04 0.21 0.64 1.52</td>
<td>0.32 0.43 0.94 1.83</td>
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<td>0.21 0.42 0.56 0.73</td>
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<tr>
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<td>0.43 0.52 0.48 0.85</td>
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<tr>
<td>13</td>
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<td>0.52 0.46 0.83 1.78</td>
<td>0.35 0.41 0.52 0.86</td>
</tr>
<tr>
<td>14</td>
<td>0.58 0.59 1.32 2.21</td>
<td>0.08 0.41 0.92 1.53</td>
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<td>0.61 0.62 0.83 1.85</td>
</tr>
<tr>
<td>16</td>
<td>0.69 0.83 0.93 2.69</td>
<td>0.17 0.45 1.34 1.23</td>
<td>0.41 0.51 0.94 2.12</td>
</tr>
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</table>

Con, control.
Evidence suggests that p53 induces apoptosis through many molecular mechanisms involving activation of its target genes [36]. p53-mediated apoptosis can occur through both transcription-dependent and -independent pathways [37]. In the transcription-dependent pathway, p53 triggers apoptosis through post-translational modifications, nuclear translocation and subsequent transactivation of pro-apoptotic genes, such as Noxa, Puma, Bak and Bax, as well as inhibition of anti-apoptotic genes, such as Bcl-xL [38–41]. The transcription-independent pathway involves rapid translocation of p53 directly to the mitochondria and physical interaction with Bcl-2 family members [42].

Our study showed that flavokawain B increased the expression of cleaved caspase-3 and cleaved PARP in ALL cell lines in a dose-dependent manner (Figure 3). Flavokawain B induced apoptotic cell death through activation of caspase-3 and degradation of PARP. The results suggested that flavokawain B induced apoptosis of ALL cells via the intrinsic apoptotic pathway.
Flavokawain B could also inhibit the proliferation of primary B-ALL and T-ALL blasts tested ex vivo; however, flavokawain B had no evident effect on normal mononuclear cells (Figure 5). The anti-proliferative effect of flavokawain B we observed on ALL blast cells needs to be further examined in a larger number of samples, ideally in clinical trials. In addition, the results of in vivo experiments showed that flavokawain B also inhibited tumor growth in an animal model (Figure 6). Flavokawain B-treated mice showed statistically lower numbers of circulating leukocytes than control mice (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.976819). Additionally, flavokawain B-treated mice showed less leukemic infiltration than control mice (Supplementary Figure 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.976819). This xenograft mouse model represented a clinically relevant preclinical paradigm. Altogether the results indicated that flavokawain B was efficacious in the treatment of ALL.

In conclusion, our results demonstrate that flavokawain B acts as a novel agent that inhibits the growth of human ALL cells through the induction of cell apoptosis mediated by modulation of p53 in vitro and in vivo. The data suggest that flavokawain B, used alone or in combination with other therapeutic methods, may be a promising agent for the treatment of patients with ALL.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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References


Flavokawain B inhibits leukemia cell growth


Supplementary material available online

Figures showing further results.