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Irisolidone, an isoflavone metabolite, represses JC virus gene expression via inhibition of Sp1 binding in human glial cells

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Abstract

Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease that results from an oligodendrocyte infection caused by the JC virus. Therefore, inhibiting the expression of JC virus is important for preventing and/or treating PML. This study found that irisolidone, an isoflavone metabolite, significantly inhibited the JC virus expression in primary cultured human astrocytes and glial cell lines. Studies examining the underlying mechanism revealed that a mutation of the Sp1 binding site downstream of the TATA box (Sp1-II) dramatically diminished the inhibitory activity of irisolidone. In addition, an irisolidone treatment repressed Sp1 binding to Sp1-II site, which is important for the basal JC virus promoter activity. The results suggest that the inhibitory effect of irisolidone against the JC virus may be attributed at least in part to the suppression of Sp1 binding to the JC virus promoter region. Therefore, the inhibition of the JC virus expression by irisolidone might provide therapeutic potential for PML caused by the JC virus.

Keywords: Progressive multifocal leukoencephalopathy; JC virus; Irisolidone; Gene expression; Promoter; Sp1

Under immunosuppressive conditions such as lymphoproliferative diseases and AIDS, an opportunistic pathogen, the JC virus, lytically infects the oligodendrocytes causing progressive multifocal leukoencephalopathy (PML), which is a demyelinating disorder of the central nervous system (CNS) [1,2]. With this disease, the JC virus accumulates in oligodendrocytes at high concentrations causing their destruction, and demyelination plaques in the cerebral white matter can be disseminated [1,3]. Before the advent of highly active antiretroviral therapy (HAART), less than 10% of PML patients who were HIV-positive survived for more than 1 year [4]. Cidofovir, a nucleotide analog, showed some efficacy against the JC virus and was administered in combination with HAART [5]. However, cidofovir has not only been proven to be ineffective when used as a sole treatment, but the value of cidofovir as a supplemental treatment to HAART is also unclear [2,6]. Recently, an antipsychotic agent, chloropromazine was reported to inhibit JC virus infection into the glial cell line, SVG-A [7]. However, further studies will be needed before it can be considered for use in PML patients. After the introduction of HAART, the disease prognosis has improved in association with the induced clearance of the JC virus DNA from the CSF [8]. Nevertheless, the 1-year mortality rate of PML is still 50%, and a significant number of patients do not respond to anti-retroviral therapy [9]. Therefore, a better understanding of the biology of the JC virus and a search for effective therapeutic agents would be very important for both the prevention and treatment of PML.

Isoflavones are biologically active compounds that are found in a variety of plants, and in relatively high levels in soybean. Recently, a series of isoflavonoids including kakkalide, glycitin, genistein, and daidzein were isolated

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from the flowers of *Pueraria thunbergiana* (Leguminosae), which is used in traditional Chinese medicine [10]. Because most traditional medicines are administered orally, their components inevitably come into contact with the intestinal microflora in the alimentary tract [11]. The intestinal bacteria transform most of the components before they can be absorbed through the gastrointestinal tract. Kakkalide was transformed into irisolidone by human intestinal bacteria [12,13]. The transformed irisolidone exhibited more potent hepatoprotective and anti-inflammatory activity than kakkalide [11,13]. This suggests that kakkalide is a prodrug that can be transformed into the active compound, irisolidone by human intestinal bacteria.

Preliminary data have shown that among the six types of isoflavones tested, irisolidone had most prominent inhibitory activity against the transcription of the JC virus in human primary cultured astrocytes and glial cell lines. Based on these results, this study further analyzed the inhibitory effect of irisolidone on the promoter activity of the JC virus and the detailed molecular mechanisms were investigated.

Materials and methods

Preparation of irisolidone. Kakkalide and irisolidone were isolated according to the previous methods [11]. Briefly, to obtain the metabolites of kakkalide by human intestinal microflora, kakkalide was anaerobically incubated with fresh human feces at 37 °C for 20 h, and then the reaction mixture was extracted three times with ethyl acetate. The EtOAc-soluble portion of the reaction mixture was dried on a rotary evaporator under reduced pressure and subjected to silica-gel column with $CHCl_3$/MeOH. From these fractions incubated with kakkalide, kakkalidone, and irisolidone were obtained and identified by comparison with the physicochemical data in the literature [11]. The chemical structures of kakkalide and irisolidone are shown in Fig. 1.

Cell culture and transient transfection assays. U87MG and U373MG human glioma cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), streptomycin, and penicillin and maintained at 37 °C with 5% CO_2. Primary cultured human fetal astrocyte cells (a kind gift from Dr. David J. Volsky, Columbia University, NY, USA) were maintained in DMEM-F-12 medium supplemented with 10% FBS and cells in passage 2 or 3 were used. Transfection was performed by a standard calcium phosphate method. Cells (2×10^5 in 60 mm-diameter dishes) were transfected with 4 μg of the reporter construct, 1 μg of pRSV β-gal, and PUC19 plasmid to a total of 10 μg of DNA. Plasmids used for transient transfection assays were prepared using Qiagen (Santa Clarita, Calif.) columns. After 48 h, cells were harvested and luciferase assays were performed as previously described [14]. To correct for differences in transfection efficiencies among different DNA precipitates, luciferase activity was normalized to β-galactosidase activity determined by ONPG assay. All transfection assays were performed at least three times in duplicate.

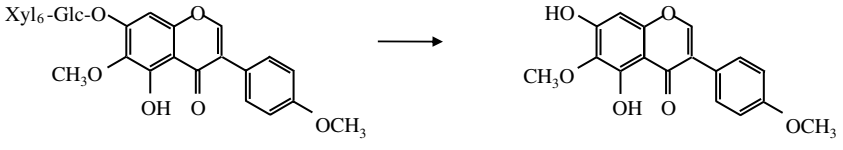
Site-directed mutagenesis. The pMH1long-luc reporter construct contains the 408 bp sequence upstream of the JC virus large T antigen gene fused to the firefly luciferase gene [15]. Base substitutions in the promoter region of the JC virus were generated in the context of the 408 bp upstream sequence, using a QuickChange PCR-based site-directed mutagenesis kit (Stratagene) according to the manufacturer's procedure. The oligonucleotide sequence for generating mutants of pentanucleotide, TATA, Sp1-I, Sp1-III, NF-1, and AP1 sites are described previously [14,16]. For site-directed mutagenesis of Sp1-II sequence, the following oligonucleotides were used: 5′-CGAGGCCGCCTCATATTCCAAGC TTA-3′ and 5′-GTAAGCTTGGAATATGAGGCGGCCTC-3′. The former primer represents coding-strand sequences of the promoter containing the desired mutations (underlined bases), and the latter represents the corresponding noncoding-strand sequences. Constructs with desired mutations were screened by restriction enzyme digestion and sequencing analysis.

Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from U87MG human glioma cells as described by Dignam et al. [17]. After treatment of irisolidone as described above, cells were washed twice with phosphate-buffered saline (PBS) and harvested by scraping. The cells were disrupted with cold lysis buffer (10 mM Tris–HCl [pH 7.9], 10 mM NaCl, 3 mM $MgCl_2$, and 0.1% NP-40) and the nuclei were pelleted by centrifugation at 13,000 rpm and resuspended in extraction buffer (20 mM Hepes [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol [DTT]) and dialyzed against the same buffer. The extracts were quick-frozen in liquid N_2 and aliquots were stored at −70 °C. Protein concentrations were determined by the Bradford method. The sequences of the Sp1-II oligomers are as follows; 5′-CCGAGGCCG CCTCCGCCTCCAAGCTTAC-3′ (sense) and 5′-GTAAGCTTGGAG GCGGAGGCGGCCTCGG-3′ (antisense). The oligonucleotides were annealed, labeled with [γ-^{32}P] ATP by using T4 polynucleotide kinase, and used as a probe. The probe (50,000 cpm) was incubated with 6 μg of nuclear extracts from U87MG cells in a final volume of 20 μl of 12.5% glycerol, 12.5 mM Hepes (pH 7.9), 4 mM Tris–HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT with 1 μg of poly(dI-dC) as described previously [14]. The reaction mixture was incubated at 4 °C for 30 min. The binding products were resolved on 5% polyacrylamide gel and visualized by autoradiography. For competition binding assays, binding reaction reagents, and nuclear extracts were mixed with nonradioactive oligonucleotides in molar excess and incubated before adding ^{32}P-labeled probe. For supershift assay, antibody was coincubated with the nuclear extract mix for 30 min on ice prior to addition of the radiolabeled probe. Antibodies against Sp1 and IRF were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

Results

Irisolidone suppresses JC virus promoter activity in human glial cells

In order to determine if irisolidone inhibits the JC virus gene expression, U87MG glioma cells were transfected with the JC virus reporter plasmid, which contained the MH1 type JC virus early promoter fused to the luciferase



Kakkalide Irisolidone

Fig. 1. Metabolic formation of irisolidone. Irisolidone (5,7-dihydroxy-4′,6-dimethoxyisoflavone) was formed from the transformation of kakkalide by human intestinal microflora.

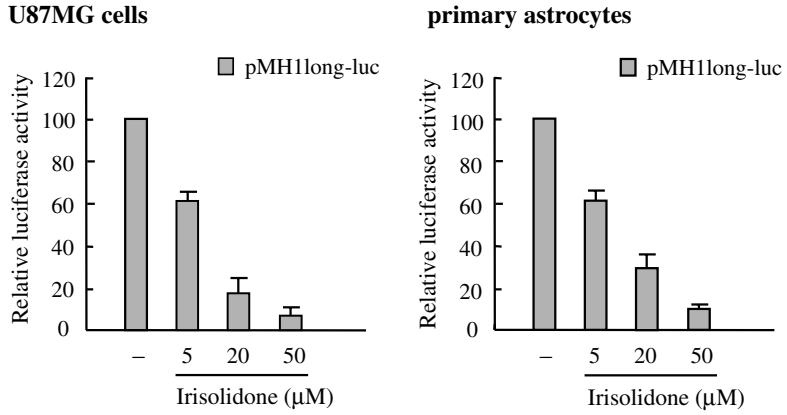


Fig. 2. Inhibition of JC virus promoter activity by irisolidone in human glial cells. Human astroglioma U87MG cells or primary cultured astrocytes were transfected with the reporter plasmid containing the early promoter of the MH1 type JC virus and were treated with various concentrations of irisolidone for 24 h before harvesting. Irisolidone decreased the JC virus promoter activity in both U87MG cells and primary astrocytes in a dose-dependent manner.

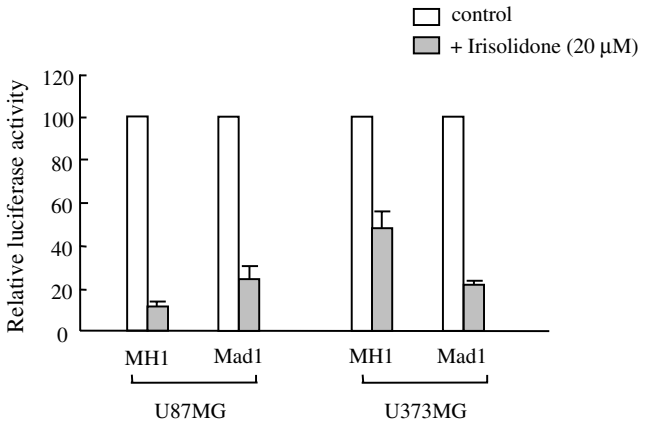


Fig. 3. Comparison of the inhibitory effects of irisolidone on the two types of JC virus promoters in human glial cell lines. U87MG or U373MG cells were transfected with the reporter plasmid containing the MH1 or Mad1 type JC virus promoter, and treated with irisolidone for 24 h prior to harvesting. Luciferase assays revealed that irisolidone inhibited the transcriptional activity of the early promoter of both types of JC viruses in the two glial cell lines.

gene (pMH1long-luc). Different concentrations of irisolidone were added to the transfected cells 24 h before harvesting and their promoter activities were compared. As shown in Fig. 2, irisolidone remarkably repressed the promoter activity of the JC virus in a dose-dependent manner. The IC_{50} was approximately 7.5 μM, and the promoter activity was inhibited by more than 90% in the presence of 50 μM irisolidone. Irisolidone also markedly suppressed the promoter activity of the JC virus in primary cultured human astrocytes (Fig. 2).

In order to address whether or not the inhibitory effect of irisolidone is common to the JC virus subtypes, the reporter plasmids containing the MH1 or Mad-1 JC virus promoter were transfected into U87MG or U373MG glial cells, and the promoter activity of these two subtypes was compared. Both the MH1 and Mad-1 type JC virus promoters were inhibited to some extent by irisolidone in two glial cell lines (Fig. 3). Therefore, irisolidone appears to be a potent inhibitor of JC virus transcription in glial cells.

Sp1 binding site downstream of TATA is important for irisolidone-mediated inhibition of JC virus promoter activity

The location of the transcription factor binding site(s) regulated by irisolidone was determined by site-directed mutagenesis. The level of inhibition by irisolidone was compared with that of the wild type promoter in U87MG glial cells and primary astrocytes (Fig. 4). A mutation of the pentanucleotide or TATA sequence, which are important for T antigen-mediated activation [16], did not produce any significant change in the decrease caused by irisolidone. Moreover, a mutation of the AP1 and NF-1 binding sites on the enhancer region or NF-κB binding sites did not cause any significant changes. An alteration of the two Sp1 binding sites (mSp1-I and mSp1-III) also did not cause any change. However, a mutation of the Sp1 site downstream of the TATA box (mSp1-II) resulted in much less inhibition (only 30% inhibition) by irisolidone than the wild type. These results suggest that the Sp1-II site is important for regulating the JC virus expression by irisolidone.

Irisolidone appears to repress JC virus promoter activity by inhibiting Sp1 binding to Sp1-II sequences

An electrophoretic mobility shift assay (EMSA) was performed to determine if irisolidone represses the promoter activity of the JC virus by inhibiting Sp1 binding to the JC virus promoter. The ^{32}P-labeled probe harboring the Sp1-II sequences (Fig. 5A) was incubated with the nuclear extracts prepared from U87MG cells treated with irisolidone. As shown in Fig. 5B, pretreating the cells with irisolidone significantly reduced Sp1 binding to the Sp1-II site in a dose-dependent manner. The specificity of the Sp1 protein–DNA complexes was demonstrated by competition and antibody supershift assays (Fig. 5B). The basal

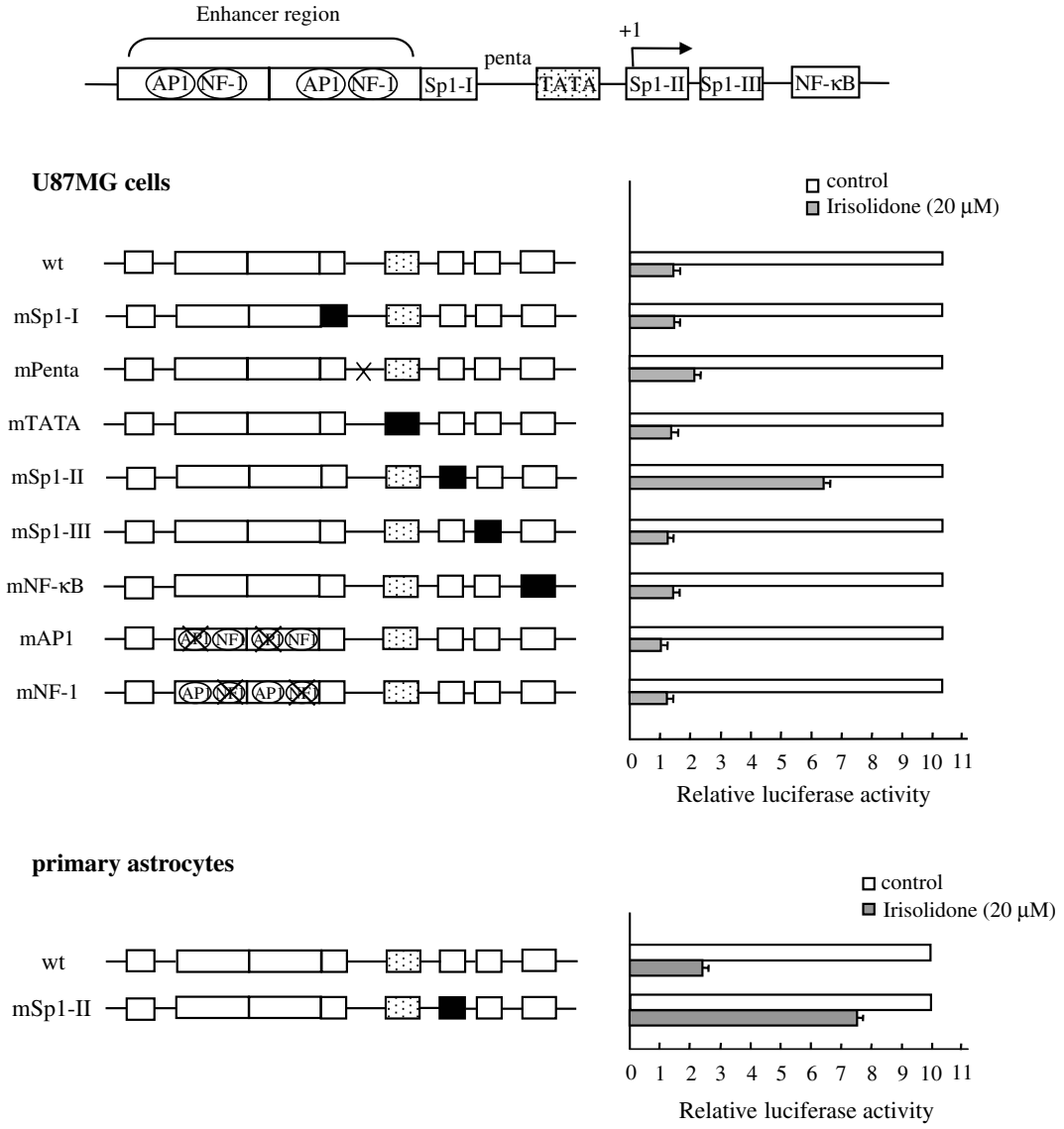


Fig. 4. Sp1 binding site downstream of the TATA box is essential for the irisolidone-mediated inhibition of the JC virus early promoter. U87MG cells and primary astrocyte cells were transfected with pMH1long-luc plasmid carrying mutations, as shown on the left and treated with 20 μM irisolidone for 24 h before harvest. An alteration of the Sp1 site downstream of the TATA box (Sp1-II) significantly diminished the irisolidone-mediated inhibition of the JC virus promoter activity in both the U87MG cells and primary cultured astrocytes. However, a mutation of the other sites did not alter the inhibition of promoter activity by irisolidone.

promoter activity of the Sp1-II mutant and wild type reporter plasmid was compared in order to confirm that Sp1 binding to the Sp1-II site is important for regulating the JC virus expression. A mutation of the Sp1-II site significantly suppressed the transcriptional activity of the JC virus both in U87MG cells and primary cultured astrocytes (Fig. 5C). Therefore, the inhibitory effect of irisolidone against the JC virus appears to be mediated at least in part through the specific inhibition of Sp1 binding to the Sp1-II site on the JC virus promoter.

Discussion

Among the biologically active compounds found in herbal medicines, flavonoids including isoflavones have a wide variety of biological activity including anti-inflammatory, and anti-tumor effects in addition to their ability to prevent atherosclerosis and stroke [13,18,19]. In particular, among the foods consumed by humans, soybeans contain the highest concentration of isoflavones. Because isoflavones exhibit estrogenic activity, they are also known as phytoestrogens. Recently, genistein, a major phytoestrogen found in soybean, has been suggested to be a potential alternative to estrogen in the treatment of Alzheimer's disease because it has comparable neuroprotective effects to estrogen, with minimal side effects [20]. It has also been shown to have neuroprotective effects in several neurodegenerative disease models [19,21].

This study reports for the first time the inhibitory effect of irisolidone against glial cell-specific JC virus expression

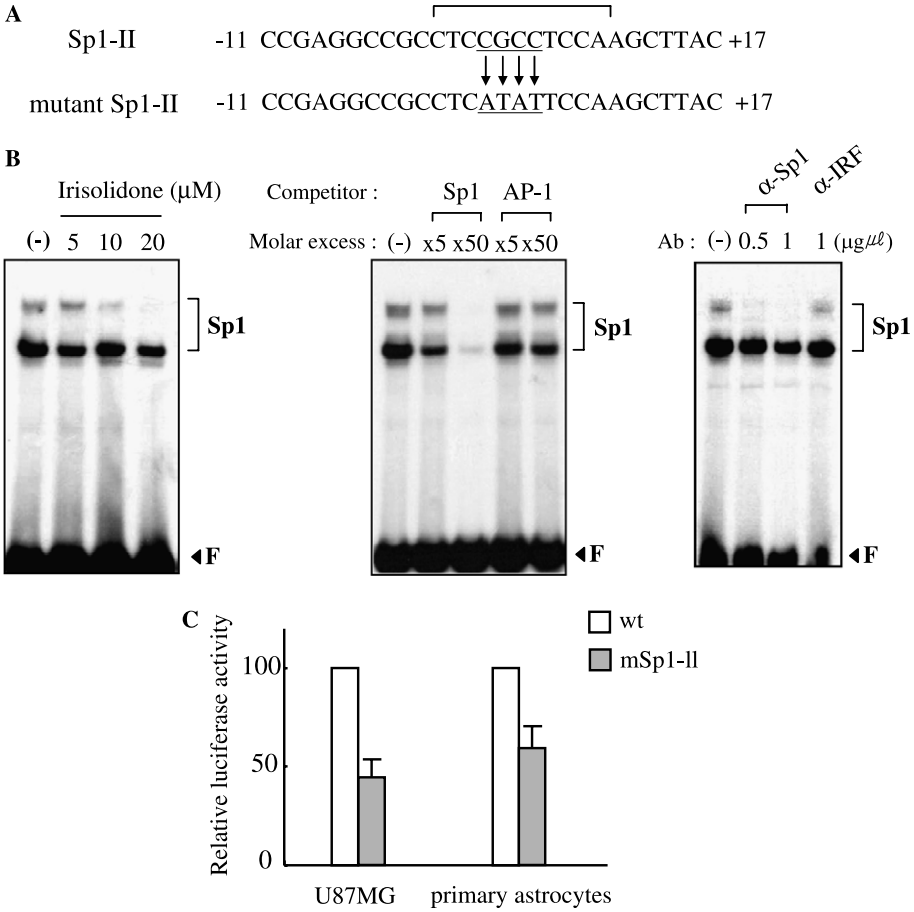


Fig. 5. Irisolidone decreased Sp1 binding to the Sp1-II sequences on the JC virus promoter, which are important for its basal promoter activity. (A) Nucleotide sequences and the location of Sp1-II on MH1 type JC virus promoter. The Sp1-binding motif residing in Sp1-II is indicated by brackets. Base substitutions within Sp1-II that were analyzed by transient transfection assays are also indicated. (B) Nuclear extracts were prepared from U87MG cells treated with irisolidone and incubated with a ^{32}P-labeled oligonucleotide probe harboring the Sp1-II sequence. Irisolidone decreased the Sp1 binding in a dose-dependent manner. Competition assay revealed that the DNA-protein complex contained Sp1 because it was diminished by an excess amount of cold oligonucleotide of its own but not by the nonspecific oligonucleotide, AP1. In accordance with this, coincubation with the Sp1-specific antibody but not with the IRF diminished formation of the DNA-protein complex. (C) U87MG and primary astrocyte cells were transfected with the wild type and mutant Sp1-II reporter plasmids. After 48 h, the cells were harvested and the basal expression levels of each promoter were compared.

in primary cultured human astrocytes and glial cell lines. Molecular mechanism studies revealed that the inhibitory effect of irisolidone was mediated via the suppression of Sp1 binding to the Sp1-II sequence downstream of the TATA box on the JC virus promoter region. Site-directed mutagenesis and transient transfection analysis revealed that the Sp1-II sequence is important for the basal promoter activity of the JC virus in primary astrocytes and glial cell lines. In contrast, the Sp1-I or Sp1-III sites did not appear to be involved in inhibiting the JC virus by this compound. The results suggest that besides Sp1, other regulatory factors interacting with Sp1 protein and/or Sp1-II sequences may also mediate the inhibitory effect of irisolidone. Sp1 is a transcriptional factor that has several functions in controlling the JC virus. Sp1 has been reported to activate the early promoter of the JC virus [14,15], presumably by maintaining methylation-free CpG islands and playing an important role in the T antigen-mediated transactivation of viral early genes [16,22]. Furthermore, Sp1 has been reported to be involved in regulating the transcription and replication of other viruses such as HIV-1 and human cytomegalovirus (HCMV) [23,24].

Besides irisolidone, several flavonoids or their related compounds have anti-viral activity by inhibiting gene expression or viral replication. Quercetin is known to have inhibitory effects against the herpes simplex virus [25], and 3-methylkaempferol, a flavonoid from *Psiadia dentata*, inhibited the genomic RNA synthesis of the poliovirus [26]. Baicalein was shown to have inhibitory effects on the replication of the human cytomegalovirus [27]. Considering that these isoflavones including irisolidone are natural compounds with a low toxicity and fewer side effects in the body [20,28], they might be potentially useful treatments for various viral diseases.

In conclusion, irisolidone inhibits the JC virus gene expression by controlling the Sp1 activity in both glial cell lines and primary astrocytes. Nevertheless, besides the inhibition of Sp1, there may be other factors underlying the

inhibitory effect of irisolidone because mutation of Sp1 site did not fully reverse the inhibition by irisolidone. Further research will be needed to investigate the precise mechanism for the inhibitory effect of irisolidone against the JC virus expression. Overall, the strong inhibition of the JC virus by irisolidone may provide a new therapeutic modality for PML and other CNS disorders caused by this virus.

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