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Bioflavonoid Hinokiflavone is a novel MDM2 inhibitor

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Abstract: MDM2 is an oncogenic RING domain E3 ubiquitin ligase found to be overexpressed in a number of human cancers, leading to poor prognosis. MDM2 negatively regulates the tumour suppressor p53 through ubiquitination of p53 and tagging it for proteasomal degradation. MDM2 also exhibits p53-independent oncogenic. Thus, inhibition of MDM2 in cancer cells could be a desired strategy in anti-cancer therapeutic development. Bioflavonoid Hinokiflavone was identified through an *in silico* screening targeting the MDM2 catalytic RING domain. Hinokiflavone was shown to physically bind the recombinant MDM2: MDMX RING domain heterodimer, and inhibit MDM2-mediated ubiquitination. This study provided biochemical and cellular evidence suggesting Hinokiflavone is a promising small molecule with anti-cancer effects through targeting MDM2.

Keywords: MDM2; E3 ligase inhibitor; p53; MDMX; ubiquitination

1. Introduction

The tumor suppressor protein p53 is inactivated by mutations of the *TP53* gene in over 50% of human primary cancers. However, in a few cancer types, such as leukemia, sarcoma and melanoma, genetic mutations of *TP53* are less common, and the loss of p53 function is often caused by alterations in p53-regulatory proteins, in particular by overexpression of MDM2 (HDM2 in humans) and MDMX (HDM4 or HDMX in humans). The oncogenic activity of MDM2 and MDMX has been exemplified by gene amplification and overexpression in more than 7% of human cancers, most frequently detected in sarcoma[1], glioblastoma[2] and breast cancers[3].

MDM2 and MDMX are key negative regulators of p53. MDM2 functions as a E3 ubiquitin (Ub) ligase targeting p53 for ubiquitination mediated proteasomal degradation[4]. MDMX is a homolog of MDM2, but lacks E3 ligase activity on its own. It heterodimerizes with MDM2 to enhance MDM2 mediated p53 ubiquitination. Both MDM2 and MDMX can interact with p53 to dampen its transcriptional activity. Thus, aberrant regulation of MDM2 and MDMX in cancer cells is a key mechanism of p53 inactivation and represents an important therapeutic target in many forms of malignancy.

In addition to suppressing the function of p53, increasing evidence suggests that MDM2 possesses p53-independent oncogenic potential [5]. MDM2 has shown to inhibit the tumor suppressor Rb and Foxo3a, altering cell cycle progression, and interfering with DNA replication and DNA repair[6-8]. The tumorigenic potential of MDM2 was further demonstrated by its ability to transform rodent fibroblasts, and to promote tumor formation and progression in nude mice or Eu-myc transgenic mice [5, 9, 10]. This inspired us to search for novel MDM2 inhibitors that could abrogate MDM2 E3 ligase activity and promote MDM2:MDMX destabilization.

MDM2 E3 ligase activity relies on homo-dimerization through its own RING domain or hetero-dimerization with MDMX. MDM2:MDMX heterodimer complex plays a critical role in the cell mediating p53 polyubiquitination and degradation [11-14], suggesting that the modulation of E3 ligase activity via dimerization is a plausible strategy for the development of inhibitors of MDM2 and MDMX. Using the crystal structure of MDM2:MDMX RING domain heterodimer as a template, we performed an *in silico* screening using a natural product library and identified the bioflavonoid Hinokiflavone as a candidate MDM2 inhibitor. Remarkably, Hinokiflavone causes destabilization of MDM2 and MDMX in the tested cancer cells. Hinokiflavone

demonstrated p53-dependent and -independent tumor suppressive activity, strongly suggesting its therapeutic potential as an anti-cancer compound through inhibition of MDM2 and MDMX.

2. Results

2.1. Hinokiflavone interacts with the MDM2:MDMX RING domain in vitro

MDM2 is a RING domain E3 ligase, which uses its RING domain to recruit a Ub conjugating enzyme E2 and stimulate transfer of Ub from the E2 to a lysine residue on MDM2 (autoubiquitination) or the target substrate proteins (substrate ubiquitination). Analysis of the crystal structural surface of the MDM2: MDMX RING heterodimer (PDB:2VJE) revealed a ligand binding site situated at a location distant from the active site for E2 binding, but close in proximity to MDM2:MDMX dimerization interface (Figure 1a). We performed a structure-based virtual screening using a natural product library and identified Hinokiflavone as one of the top-fitting chemicals to the targeted ligand binding site on the surface of the MDM2 RING domain (Figure 1b and 1c). Hinokiflavone, also known as 4′, 6′′- O-Biapigenin, belongs to a bioflavonoid family (Figure 1b), which could be isolated from several plants, including Selaginella tamariscina, Juiperus phoenica, and Rhus succedanea.

To examine the physical interaction between MDM2:MDMX and Hinokiflavone, recombinant GST-MDM2: His-MDMX RING heterodimer was prepared through sequential GST and His affinity chromatography (Figure 1d) and subject to a Biolayer Interferometry (BLI) assay. Hinokiflavone was shown to physically bind to the MDM2: MDMX RING heterodimer with a Kd value of 12 μM (Figure 1e).

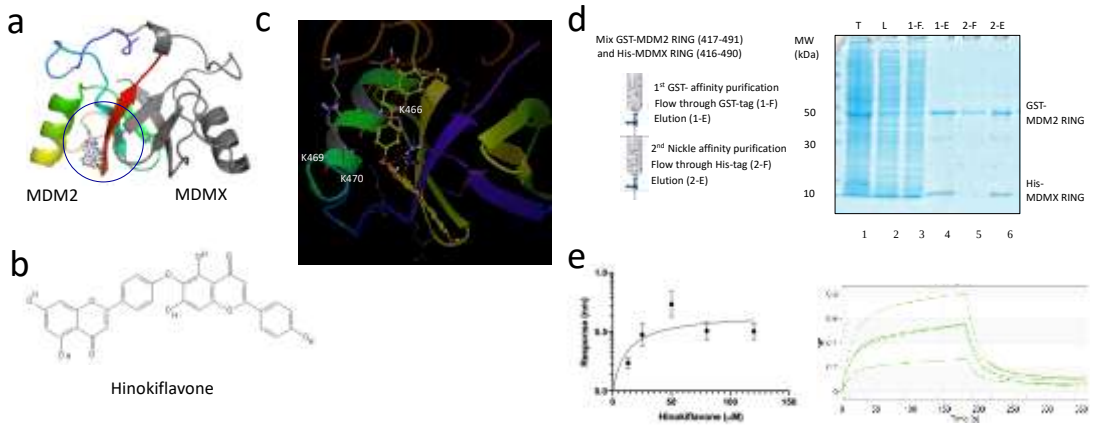

Figure 1. Hinokiflavone interacts with MDM2:MDMX RING domain. (a) The surface pocket of MDM2:MDMX RING heterodimer used for in silico screening. MDM2:MDMX RING domain heterodimer cartoon model with MDM2 and MDMX are shown in rainbow and grey, respectively. (b) Chemical structure of Hinokiflavone. (c) Docking of Hinokiflavone onto the MDM2:MDMX RING heterodimer. Hinokiflavone is modeled into MDM2 RING domain at the selective Site. Hinokiflavone is shown in ball and stick. MDM2 RING domain Lysine residues K466, 469 and K470 are shown in stick form. (d) Recombinant MDM2:MDMX RING heterodimer was purified using sequential GST- and Nickle- affinity chromatography. (e) The physical interaction between Hinokiflavone and MDM2:MDMX RING heterodimer was measured by Biolayer Interferometry (BLI) assay. The BLI response (nm) to the immobilized MDM2:MDMX RING heterodimer with the addition of Hinokiflavone (0-120 μM) was depicted in the left. The BLI association and dissociation curves of MDM2:MDMX RING heterodimer in the presence of Hinoflavone (0-120 μM) were shown in the right.

2.2. Hinokiflavone inhibits the MDM2 RING domain mediated ubiquitination

To examine whether Hinokiflavone inhibits MDM2 E3 ligase activity, an *in vitro* ubiquitination assay was performed, in which ATP, E1, E2, Ub and MDM2 were provided to facilitate MDM2 ubiquitination [15], and candidate compounds or DMSO (vehicle control) were added to examine their ability to block MDM2 E3 ligase activity. Hinokiflavone was found to inhibit MDM2 ubiquitination with an IC_{50} value of ~13 μM, demonstrating more potency than a known MDM2 RING domain antagonist HLI373 (Figure 1 a-c) [16].

To determine the effect of Hinokiflavone on MDM2 and p53 ubiquitination in the cell, the proteasome inhibitor MG132 was employed to enrich the cellular ubiquitinated MDM2 and p53 using a leukemia line AML-2. Decreased ubiquitinated MDM2 and p53 levels were observed when cells were treated with increasing doses of Hinokiflavone (6-25 μM) and MG132 (Figure 1d), indicating that Hinokiflavone could attenuate MDM2 and p53 ubiquitination in AML-2 cells. To examine the effect of Hinokiflavone on the cellular levels of MDM2 and MDMX, AML-2 and HL-60 cells were treated with Hinokiflavone (0-8 μM) and analyzed with immunoblotting. Hinokiflavone was found to induce a transient and mild increase of MDM2 and MDMX at low doses, while moderate to high doses of Hinokiflavone induced a gradually decline of both MDM2 and MDMX protein levels (Figure 2e).

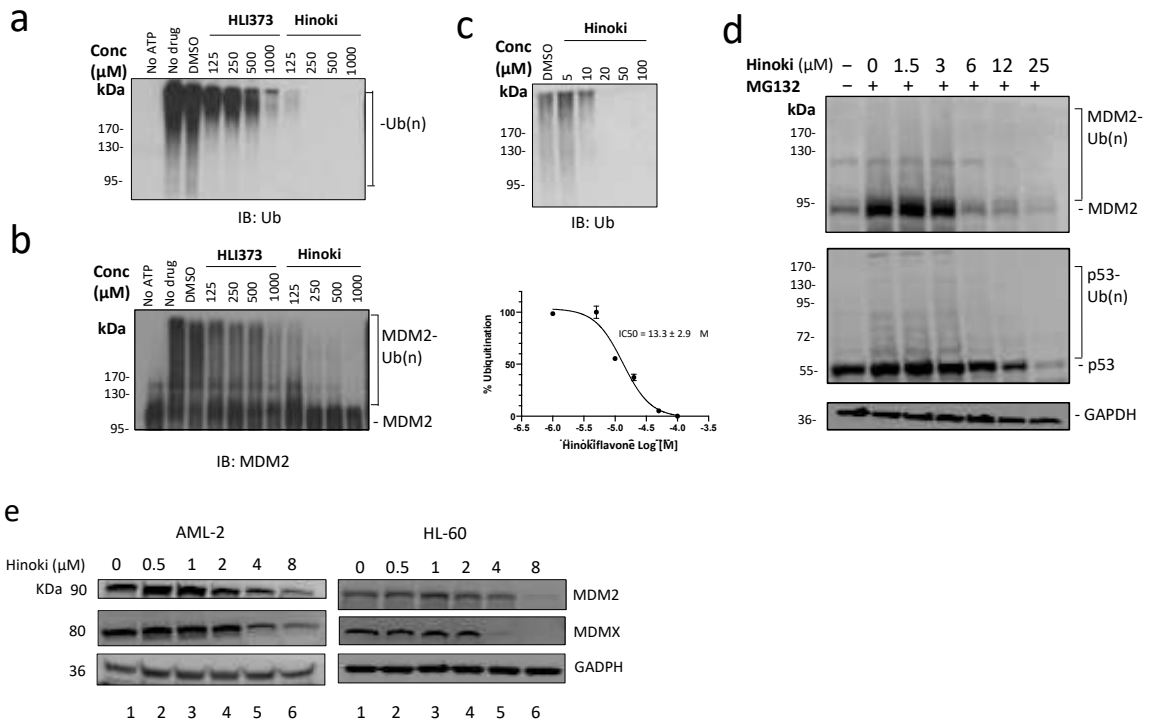

Figure 2. Hinokiflavone inhibits MDM2 mediated ubiquitination and destabilize MDM2:MDMX. (a) Ubiquitination activity of MDM2 was studied in the presence of Hinokiflavone or MDM2 inhibitor HLI373 (0 – 1 mM) using *in vitro* ubiquitination assay. A monoclonal Ub antibody used for detection of poly-Ub chains in this assay. (b) MDM2 poly-ubuquitination was detected using MDM2 antibody in the presence of Hinokiflavone or HLI373 (0 – 1 mM). (c) Evaluation of Hinokiflavone IC_{50} on the ubiquitination activity of MDM2. The MDM2 *in vitro* ubiquitination assay was performed in the presence of Hinokiflavone (5 - 100 μM). The ubiquitination levels were quantified by densitometry using Fiji and the inhibitory effect of Hinokiflavone was calculated and plotted using Prism 9.0. (d) Hinokiflavone attenuates MDM2 and p53 ubiquitination in AML-2 cells. AML-2 cells were treated with Hinokiflavone (0 - 25 μM) for 24 h followed by 4 h treatment with protease inhibitor MG132 (10 μM). MDM2 and p53 were detected by immunoblot with MDM2 and p53 specific antibodies. (e) AML-2 and HL-60 were treated with various concentrations of Hinokiflavone for 24 h. The levels of MDM2, MDMX were detected by immunoblotting. GAPDH was used as a loading control.

2.3. Hinokiflavone decreases cell viability of cancer cells

To examine the cytotoxic effect of Hinokiflavone on cancer cells, a panel of cancer cell lines including leukemia AML-2 and HL60, osteosarcoma U2OS, breast cancer MCF-7, colorectal cancer HCT116, and a normal human fibroblast cell line BJ (BJ-FB) were treated with Hinokiflavone (0-50 μM) and analyzed for cell viability (Table 1). Hinokiflavone induced concentration-dependent decreases in cell viability of the tested cancer cells (AML-2, HL60, U2OS, MCF-7, HCT116), with the leukemia AML-2 and HL-60 cells showing highest sensitivity to Hinokiflavone treatment. In contrast to the tested cancer cells, BJ fibroblast cells showed little response to Hinokiflavone treatment (Table 1), suggesting that BJ fibroblasts were more tolerant to Hinokiflavone treatment. The observation that Hinokiflavone showed an increased inhibitory potency in p53 wild type cells relative to p53

null cells suggests that the cytotoxic effect of Hinokiflavone towards cancer cells was due to the p53-dependent as well as p53-independent responses .

Table 1. Summary of the cytotoxic effect of Hinokiflavone on a selective panel of human cells

Cell line	p53 status	IC_{50} (μM)
AML-2	wildtype	4.93 ± 1.16
HL-60	null	10.95 ± 0.19
HCT116	wildtype	14.19 ± 2.04
HCT116 p53null	null	32.66 ± 0.31
U2OS	wildtype	15.90 ± 2.07
MCF-7	wildtype	17.33 ± 1.90
BJ-FB	wildtype	ND[1]

[1] Cell viability was evaluated using *CellTiter-Glo 2.0*. IC_{50} results were represented by Mean ± SEM of three trials. ND, inhibition not detected under the tested doses.

3. Discussion

Hinokiflavone was identified using an *in silico* approach as an allosteric binder to the MDM2: MDMX RING heterodimer. It was hypothesized that Hinokiflavone would inhibit the MDM2 RING domain and might have an effect on MDM2 mediated ubiquitination. Indeed, Hinokiflavone was shown to block MDM2 mediated ubiquitination, supporting its role as an inhibitor of MDM2 RING domain.

A cell viability assay was performed in osteosarcoma, leukemia, colorectal cancer, breast carcinoma and normal fibroblast cell lines. Hinokiflavone induced cytotoxicity in the tested carcinogenic cells but showed little effect in the normal BJ-FB cells. Among the carcinogenic cell lines used in this study, leukemia cells were found to be most sensitive to Hinokiflavone treatment. These results agreed with previously reported anti-cancer effects of Hinokiflavone. Hinokiflavone is a naturally derived bioflavanoid known to possess anti-inflammatory, antioxidant and antitumour activity. Several mechanisms have been proposed for the pharmacological activities of Hinokiflavone including inhibition of mRNA spliceosome, activation of mitochondrial ROS/JNK/caspase signaling pathway, as well as inhibition of Matrix Metalloproteinase 9 [17-20]. This study presented a novel mechanism for Hinokiflavone mediated anti-cancer potential through inhibition and destabilization of MDM2 to promote cancer cell apoptosis and growth arrest.

4. Conclusions

Hinokiflavone was identified as a plant-based bioflavonoid compound that could interact with MDM2:MDMX RING domain heterodimer. This study investigated the anti-cancer effects of Hinokiflavone through targeting MDM2. We provided biochemical and cellular evidence suggesting Hinokiflavone is a promising small molecule with anti-cancer potential through inhibition and destabilization of MDM2 in cancer cells.

5. Experimental Material and Methods

Cell Culture and Antibodies

The cell lines MCF-7 cells and BJ-FB normal fibroblast cells were grown in DMEM medium supplemented with 10% FBS. p53 wild type leukemia AML-2 suspension cells were grown in MEM Alpha (1X) Minimum Essential medium containing 10% FBS. p53 null leukemia HL-60 suspension cells were grown in RPMI Medium 1640 (1X) with 10% FBS. Colorectal HCT-116 adherent cancer cells were grown in McCoy's 5A Medium (1X) Modified supplemented with 10% FBS. All cells were incubated at 37°C and with 5% CO_2.

In vitro ubiquitination assay

Experiments were performed as previously described[21].

Cell Viability Assay

Cell viability assay was performed using the *Celltiter Glo 2.0* kit (Promega, Cat. G9242) as per the manufactures' instructions.

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