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Naturally occurring FANCF-Hes1 complex inhibitors from *Wrightia religiosa*

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The isolation and evaluation of inhibitors of Fanconi F protein (FANCF) - hairy and enhancer of split 1 (Hes1) complex are described. A high-throughput screening assay for small-molecule inhibitors of the FANCF-Hes1 complex was constructed. Successful complex formation between fluorophore (Cy3)-labeled human FANCF and immobilized rat or human HES1 on a microplate was established. Screening of our plant extract library using this system resulted in the isolation of eight natural products, including two new flavonoid glycosides (3 and 4), from *Wrightia religiosa*. Of these compounds, 3, 5, and 7 showed potent inhibition of the FANCF-Hes1 complex. Compound 7 disrupted the FANCF-Hes1 complex more efficiently than the Hes1 dimer.

**Introduction**

Fanconi anemia (FA) is an inherited anemia associated with bone marrow failure, progressive decline in hematopoietic stem cells, developmental defects, and a predisposition to cancer.1 A common cellular phenotype is the hypersensitivity to DNA cross-linking agents, such as mitomycin C2 and diepoxybutane,3 which suggests the presence of defects in DNA repair mechanisms. The FA core complex, which consists of eight proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM), is a key player in the DNA cross-link repair pathway and is referred to as the FA pathway. Mutations in any of the FA proteins cause the manifestation of clinical features. Although several proteins that interact with the FA core complex have been identified, such as Fanconi anemia-association protein 24 (FAAP24),4 FAAP100,5 FANCM-associated histone fold protein 1 (MHF1),6 MHF2,6 hairy enhancer of split 1 (Hes1),7 and C-terminal binding protein 1 (CtBP1),8 the details of this pathway remain unknown. Therefore small molecules that inhibit protein-protein interactions related to the FA pathway will not only further our understanding of this pathway, but may also provide novel therapeutic candidates for this complicated disease.

Hes1 is a repressor type basic helix-loop-helix (bHLH) factor that controls the fate of stem cells.9 It was revealed that Hes1 interacts with FANCA, FANCF, FANCG, and FANCL, which mediates the transcriptional regulation of Hes1-responsive genes.7 Inhibitors of the FA protein-Hes1 complex would enable the understanding and identification of unknown FA/Hes1 cross-talk. In the present study, we describe a rapid *in vitro* high-throughput system (HTS) for identifying inhibitors of FANCF complex formation with Hes1. The first FANCF-Hes1 complex inhibitors isolated from natural sources are presented here. The naturally occurring inhibitors were isolated from *Wrightia religiosa* using the screening method described herein.

**Results and discussion**

Glutathione-S-transferase fused human FANCF (GST-hFANCF; full length) was expressed in *Escherichia coli*, then
purified with glutathione sepharose 4B. A rat Hes1 protein (3-278 aa), with an amino acid sequence differing from human HES1 by only one residue outside the binding region with FANCF, was prepared as previously described. To prevent GST-GST interactions, which would result in false positives, GST-free Hes1 protein was prepared by GST cleavage with PreScission protease. The HTS plate assay was designed as shown in Figure 1. The assay was designed to use fluorophore-conjugated FANCF to detect the FANCF-Hes1 complex using fluorescent intensity. Hes1 protein was immobilized on the bottom of 96 well plates (Nunc ImmobilizerTM Amino Plate, Thermo). Hes1 protein was added to the plates and incubated overnight at 4 °C. After washes with buffer, the activated spacers remaining on the bottom of the wells were treated with ethanolamine for 2 h at room temperature. Hes1 immobilization was confirmed by measuring the Cy3 fluorescence intensity after detection of Hes1 antibody and Cy3-conjugated secondary antibody. It was found that 100 µl (20 µg/ml) of Hes1 resulted in a sufficient amount immobilization on the plate (data not shown). To form the FANCF-Hes1 complex, Cy3-conjugated GST-FANCF was added to Hes1-immobilized wells. The wells were scanned for their fluorescent intensity after 1 h incubation at room temperature, followed by removal of unbound Cy3-FANCF by washing with buffer and drying under reduced pressure. The FANCF-Hes1 complex was detected successfully, as shown in Figure 2 (lanes 3 and 4). The screening assay exhibited only low levels of non-specific binding by Cy3-GST (Figure 2, lanes 7 and 8). As the compounds were prepared in solutions containing DMSO, the effect of the solvent on binding was investigated. Fluorescent intensity of the FANCF-Hes1 complex was slightly reduced by addition of DMSO (2 %) (Figure 2, lane 4).

Our natural source extract library was then screened using the HTS assay. Of the extracts, the MeOH extract of Wrightia religiosa was found to contain naturally occurring inhibitors of the FANCF-Hes1 complex. The methanol extract of W. religiosa (28.5 g) was partitioned successively with EtOAc, nBuOH, and water. The active nBuOH layer (8.0 g) was subjected to ODS column chromatography and reversed-phase HPLC. Activity-guided separation yielded eight compounds (1-8), including two new compounds (3, 4) (Figure 3). The isolated compounds were identified as kaempferol 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (1),11 kaempferol 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (2),12 quercetin 4′-O-α-L-rhamnopyranosyl-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (5),13 rutin (6),14 quercetin 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (7),15 wrightiadione (8),16 based on comparisons of their spectral data with spectra in the literature. The new natural compound 3 was isolated as a yellow solid with the molecular formula C_{43}H_{52}O_{19}, as determined by HRAPCIMS (m/z 763.2040, calcd for C_{43}H_{52}O_{19}Na, [M+Na]$, $\Delta -2.2$ mmu). $^1H$ and $^{13}C$ NMR analyses indicated the presence of a kaempferol structure and three sugars (Table 1). HMBC correlations suggested the presence of glycosidic linkages between C-3 and glucose, C-4′ and rhamnose, and a 1,6-glycosidic bond between rhamnose and glucose (Figure 4). Compound 3 was designated kaempferol 4′-O-α-rhamnopyranosyl-3-O-α-rhamnopyranosyl-(1→6)-β-glucopyranoside. The new natural compound 4 was isolated as a yellow solid with the molecular formula C_{39}H_{48}O_{20}Na, $\Delta -0.7$ mmu as determined by HRAPCIMS (m/z 779.2004, calcd for C_{39}H_{48}O_{20}Na, [M+Na]$, $\Delta -0.7$ mmu). Analysis of $^1H$ and $^{13}C$ NMR spectra revealed the presence of a kaempferol as an aglycon and three sugars in compound 4 (Table 1). HMBC correlations suggested the presence of glycosidic linkages between C-3 and galactose, C-4′ and rhamnose, and a 1,6-glycosidic bond between rhamnose and galactose (Figure 4). Compound 4 was designated kaempferol 4′-O-α-rhamnopyranosyl-3-O-α-rhamnopyranosyl-(1→6)-β-galactopyranoside.

The inhibitory activities of the isolated compounds on the human FANCF-rat Hes1 interaction were examined (see the supporting information, Figure S1). Of the compounds tested, 3, 5, and 7 produced moderate inhibition at 50 µM. To precisely elucidate the inhibitory activity of the isolated compounds, we subsequently constructed a human HES1 interaction plate assay. Although the difference between human HES1 and rat Hes1 is only a single residue (human HES1 has 172S, which is absent in rat Hes1), examination of human protein interactions is more clinically relevant. Therefore, a human HES1 pGEX-6P-1 construct (1-280 aa, full length) was prepared and purified.
using the above method. After GST removal, human HES1 protein was immobilized on the plate. Immobilized HES1 was detected with anti-HES1 antibody and it was found that a concentration of 5 µg/ml human HES1 was sufficient for the assay. Addition of Cy3-human FANCF to wells containing immobilized HES1 resulted in the successful detection of the FANCF-HES1 interaction, as shown in Figure 5 (lane 2). A human HES1-human HES1 dimer complex was also clearly detected (Figure 5, lane 4). Non-specific interactions were assessed by addition of GST protein (Figure 5, lane 6).

**Table 1** $^1$H and $^{13}$C NMR data of compounds 3 (A) and 4 (B) ($\delta$ in ppm, J in Hz).

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<th>Position</th>
<th>$^1$H-NMR in DMSO-$d_6$</th>
<th>$^{13}$C-NMR in DMSO-$d_6$</th>
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<td>2</td>
<td>156.4 β-D-Glucose</td>
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<tr>
<td>3</td>
<td>133.9 1'' 5.27 (d, 7.2)</td>
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<td>4</td>
<td>177.4 2'' 74.2</td>
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<tr>
<td>5</td>
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<td>9</td>
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<td>2''' 70.3</td>
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**Fig. 3** Isolated natural products.

**Fig. 4** Key HMBC and COSY correlations for compounds 3 and 4.

**Fig. 5** Human FANCF-human HES1 complex formation in the microplate assay. All wells were treated with ethanolamine after HES1/blank immobilization, and were then incubated with Cy3-proteins followed by washing with buffer. Excitation was 544 nm and emission was 590 nm. Error bars represent SD (n = 3). Background (each well) was subtracted. 1, Cy3-GST-hFANCF without immobilized hHES1 (control with DMSO); 2, Cy3-GST-FANCF with immobilized hHES1; hHES1/Cy3-GST-hFANCF complex was detected; 3, Cy3-GST-hHES1 without immobilized hHES1 (control with DMSO); 4, Cy3-GST-hHES1 with immobilized hHES1; hHES1/Cy3-GST-hHES1 complex was detected; 5, Cy3-GST without immobilized hHES1 (control with DMSO); 6, Cy3-GST with immobilized hHES1, non-specific interactions was minimal. Immobilization; hHES1 (5 µg/ml), Cy3-proteins; Cy3-GST-hFANCF (20 µg/ml, 0.3 µM), Cy3-GST (7.4 µg/ml, 0.3 µM).
Using the HTS assays described here, the inhibition of human FANCF-human HES1 interactions by the isolated compounds (3, 5 and 7) was examined (Figure 6). The activity of 8 was shown in Figure S2 (supporting information). Wnt signal is one of the important signals that control stem cell fate. The selectivity in these stemness control signals would be useful. Therefore to check non-specific inhibition, TCF-β-catenin complex, which is a key complex in Wnt signalling was examined. The TCF-β-catenin plate assay was performed using the compounds 3, 5, and 7, which showed moderate inhibition of human FANCF-rat Hes1 interaction. All compounds produced dose-dependent inhibition of FANCF-human HES1 interaction. Interestingly, inhibition by compounds 5 and 7 was improved to 40% at 10 µM, greater than what was observed with rat Hes1. The IC\textsubscript{50} values of 5 and 7 were 23.6 µM and 35.8 µM, respectively. Although the inhibition levels were moderate, to the best of our knowledge, these are the first reported inhibitors of the interaction between FANCF and HES1.

To determine the specificity of the inhibitors on HES1 complexes, human HES1-human HES1 (HES1 dimer) interactions were also assessed. Because the difference between the structures of 3 and 5 is only a C-3’ phenolic OH, this OH appears to enhance inhibition of both FANCF-HES1 interactions and HES1 dimerization. Our results show that compound 7 inhibited FANCF-HES1 complex more efficiently than HES1 dimer. The structural differences between 5 and 7 are a rhamnose at the 4’ position of the aglycon and a galactose at the 3 position. The stable structures and electrostatic potential energy of isolated compounds were calculated (see Supporting Information, Figure S3). Interestingly, the internal hydrogen bonds between rhamnose and rhamnose (compound 5) and between rhamnose and OH at the 4’ position (compound 7) were observed to make the folding structures. There is difference in size of cavities which were made by hydrogen bonds (Figure S4).

Next, we investigated the effect of compound 5 on colony formation in hematopoietic stem/progenitor cells. Briefly, bone marrow cells were collected from femurs of wild-type and Fanconi A-deficient mice and stem/progenitors were selected using the StemSep negative selection procedure, according to the manufacturer’s protocol (Stem Cell Technology). Two to 5 x 10\textsuperscript{3} stem/progenitor cells per ml were seeded in complete methylcellulose medium (Stem Cell Technology), with or without compound 5 (50 µM), and incubated at 37 °C in 5% CO\textsubscript{2}. The total number of colonies was counted and presented as colony forming cells (CFC). Results show that wild-type stem/progenitor cells incubated with compound 5 exhibited a 40% reduction in colony forming ability, consistent with
disruption of the FA pathway. FancA-deficient cells incubated with compound 5 showed reduced CFC compared to FancA cells incubated with DMSO, whereas no effect was observed with FancC-deficient cells (Figure 7). The degree of reduction in WT and FancA-deficient cells was equivalent in the absence and presence of 5. This result indicates that CFC inhibition by 5 is not dependent on FancA. Because inhibition by 5 was absent in FancC-deficient cells, compound 5 might also interact with FANCC. These results suggest that compound 5 may be useful in discriminating specific functions of FA proteins and/or the role of HES1 dimers in hematopoietic function. Indeed, the lack of effect by compound 5 in FancC-deficient cells supports previous studies suggesting roles of FANCC in distinct pathways. 18

Conclusions

To identify FANC-F-HES1 complex inhibitors derived from natural sources, we constructed a high-throughput plate assay using recombinant FANC-F and Hes1 proteins. This assay led to the isolation of eight compounds, including two new flavonoid glycosides (3 and 4). Compounds 3, 5, and 7 showed moderate inhibition of FANC-F-HES1 interactions. We also investigated the selectivity of the inhibition using HES1-HES1 dimer and TCF-β-catenin plate assays. Compound 5 exhibited selective inhibition of HES1 complexes (FANC-F-HES1 and HES1 dimer), while compound 7 produced selective inhibition of the FANC-F-HES1 complex, and to a lesser extent HES1-HES1, without affecting TCF-β-catenin complex formation. To the best of our knowledge, this is the first report of inhibitors of FANC-F-HES1 interactions. The search for more active inhibitors from natural sources continues.

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Notes and references


17 (a) M. Lepourcelet, Y. -N. P. Chen, D. S. France, H. Wang, P. Crews, F. Petersen, C. Bruseo, A. W. Wood and R. A. Shivdasani, *Cancer Cell*, 2004, **5**, 91-102; (b) The condition was slightly modified (see supporting information). The reliability of the assay was confirmed using a known TCF4/β-catenin complex inhibitor, calphostin C (PKF115-584)\(^{17a}\). In the dark condition, PKF115-584 didn’t inhibit the complex.