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Flavonoid dimer as modulator of drug resistance in cancer

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Summary
Multidrug resistance (MDR) is a major problem in cancer chemotherapy. The best characterized resistance mechanism is the one mediated by the overexpression of permeability-glycoprotein (P-gp) and MDR related protein 1 (MRP1), both of which can pump a variety of anticancer drugs out of the cells, resulting in lowered intracellular drug accumulation. Development of MDR modulators against P-gp and MRP1 has attracted interests from both academia and industry. Unfortunately, the first and second generation MDR modulators have failed due to various reasons including toxicity and unpredictable drug-drug interaction. We are interested in designing novel synthetic flavonoid dimers to target P-gp and MRP1 with the aim of developing specific, non-toxic and efficient MDR modulators. Some naturally-occurring flavonoids have been demonstrated to be moderate MDR modulators. To increase the specificity and affinity of flavonoids towards P-gp and MRP1, we have successfully used bivalency approach to synthesize novel flavonoid dimers and demonstrated that they are potent P-gp and MRP1 modulators in cancers. The first generation flavonoid dimer is made up of two apigenin moieties linked together by a biocompatible linker with various length of polyethylene glycol. The second generation flavonoid dimers have higher efficacy with EC_{50} (effective concentration that reduces IC_{50} by half) at nM range for both P-gp and MRP1, which is comparable to that of the most potent MDR modulators available. Biochemical studies suggested that these flavonoid dimers are binding to the substrate binding site of MRP1. In summary, we have synthesized a series of novel flavonoid dimers and demonstrated that they are highly effective P-gp and MRP1 inhibitors.

Riassunto
La resistenza multifarmaco (MDR) è uno dei principali problemi nella chemioterapia antiblastica. Il miglior meccanismo caratterizzato di resistenza è quello mediato dalla iperespressione di una glicoproteina di membrana che regola la permeabilità (P-gp) e della proteina 1 correlata alla MDR (MRP1), entrambe in grado di pompare fuori delle cellule una varietà di farmaci antitumorali, con conseguente abbassamento dei loro livelli di accumulo intracellulare. Lo sviluppo di modulatori MDR

contro P-gp e MRP1 ha attirato gli interessi sia delle università che dell'industria. Purtroppo, i modulatori MDR di prima e seconda generazione hanno falito il loro compito a causa di vari motivi, tra cui la tossicità e un'interazione imprevedibile tra farmaci. Siamo interessati a progettare nuovi dimeri flavonoidi sintetici per designare come bersaglio P-gp e MRP1 con l'obiettivo di sviluppare modulatori MDR specifici, non tossici ed efficienti. Alcuni flavonoidi presenti in natura hanno dimostrato essere moderati modulatori MDR. Per aumentare la specificità e l'affinità dei flavonoidi verso P-gp e MRP1, è stato utilizzato con successo l'approccio bivalente per sintetizzare nuovi dimeri flavonoidi ed è stato dimostrato come essi siano potenti modulatori di P-gp e MRP1 nel cancro. La prima generazione di dimeri flavonoidi è costituita da due frazioni di apigenina collegate tra loro da un linker biocompatibile di varie lunghezze di polietilene glicole. La seconda generazione di dimeri flavonoidi ha una maggiore efficacia con un EC_{50} (la concentrazione efficace che riduce IC_{50} della metà) che varia in un range nanomolare sia per P-gp che per MRP1, che è paragonabile a quello dei modulatori MDR più potenti disponibili. Studi biochimici hanno suggerito che questi dimeri flavonoidi siano legati ad un sito legante il substrato di MRP1. In sintesi, sono stati sintetizzati una serie di nuovi dimeri flavonoidi ed è stato dimostrato come essi siano degli inibitori altamente efficaci di P-gp e MRP.

Multidrug resistance in cancer

ATP-binding cassette (ABC) transporter superfamily consists of a total of 49 human ABC members that can be further subdivided into 7 subfamilies (ABCA to ABCG). Most ABC members are ATP hydrolysis-dependent transporters that can translocate different substrates across biological membranes. ABCB1 (P-gp) (1), ABCC1 (MRP1) (2) and ABCG2 (BCRP) (3) are the three most important ABC members that are involved in mediating multidrug resistance (MDR) in human.

In 1976, Juliano and Ling first characterized the ATP-dependent efflux activity of the P-gp in colchicine-resistant Chinese hamster ovary cells (1). Clinically, P-gp detection correlates well with poor response to chemotherapy. P-gp can reduce intracellular drug concentrations which decreases the cytotoxicity of a broad spectrum of anticancer drugs including anthracyclines (e.g. doxorubicin), vinca alkaloids (e.g. vincristine and vinblastine), podophyllotoxins (e.g. etoposide) and taxanes (e.g. paclitaxel). P-gp has 1280 amino acids with a molecular mass of 170 kDa and is organized into two repeating units with each unit having six transmembrane helices and one highly conserved nucleotide binding domain (NBD).

In 1992, Cole et al. discovered MRP1 by virtue of its overexpression in a drug-selected human lung cancer cell line H69AR that did not overexpress P-gp (2). Association of MRP1 expression

with clinical cancer MDR has been documented particularly in lung cancer. The MRP1 substrates include anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins, nucleoside and nucleotide analogs, arsenicals and methotrexate. MRP1, compared to P-gp, has an extra five transmembrane helices at the N-terminal.

P-gp and MRP1 modulators

Development of reversers or modulators against P-gp and MRP1 has attracted interests from both academia and industry. Tsuruo and co-workers first demonstrated the ability of calcium channel blocker, verapamil and its derivatives to modulate P-gp (4). The disadvantage of these reagents is that they modulated P-gp at a very high concentration, ranging from 5 to 50 μM, and caused cytotoxicity to normal cells. Second generation P-gp modulators were designed to minimize the side effects of the first generation modulators. These include dexverapamil, non-imunosuppressive cyclosporine D derivatives (PSC833, valspodar; Novartis AG), dexniguldipine and VX-710. Although many of them have lower toxicity, partly due to their higher efficacy in modulating MDR, they were found to have drug-drug interaction with the co-administered cancer drug, resulting in changes in drug metabolism and clearance and toxicities (5). Third generation P-gp modulators include the cyclopropyldibenzosuberane modulator zosuquidar (LY335979; Eli Lilly), tariquidar (XR9576; NCI/Xenova/QLT Company), laniquidar (R101933; NCI/EORTC Inc.), the acridonecarboxamide GF120918 and the substituted diarylimidazole ONT-090. They were designed to specifically inhibit P-gp and did not inhibit other ABC transporters. Phase III clinical trials for some of these third generation MDR modulators are ongoing.

Flavonoids

Flavonoids represent a large family of polyphenolic compounds found naturally in fruits (especially in *Citrus*), vegetables, nuts, stems, flowers, wine and tea. There are more than 6500 different flavonoids identified. The general structure of flavonoid contains a flavin nucleus with two aromatic rings (A and B rings) interconnected by third heterocyclic ring (C ring). The most common flavonoids are flavone (with a double bond between C-2 and C-3 and a keto functional group at C-4 of C ring) and isoflavone (with the B ring at C-3). Flavonoids have been reported to have a wide range of biological activities, particularly as antioxidative and anticancer agents. As we consume large amounts of flavonoids every day, it is generally accepted that flavonoids are not toxic.[6] An example is apigenin (**2**) which is present in celery and has been found as a component in several Chinese medicinal herbs: *Lobelia chinensis*, *Dendranthema indicum var. aromaticum* and *Broussonetia papyrifera*.

Novel synthetic flavonoid dimer as P-gp modulator

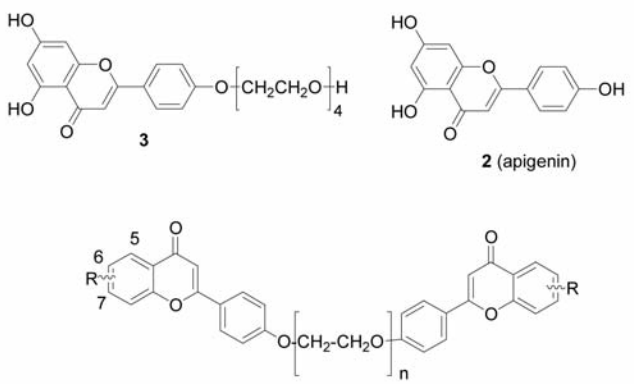
We are interested in developing specific and efficient P-gp and MRP1 modulators by taking advantage of the pseudodimeric nature and the multiple binding sites of P-gp and MRP1 using bivalent interactions. Polyvalent interactions in biological systems are characterized by the simultaneous binding of multiple ligands on one biological entity (7). Independent of and concurrent to our effort, this approach has been applied in designing MDR modulators with bivalent ligands like stipiamide and emetine (8, 9).

Apigenin (**2**) is a flavone that has been demonstrated to have a low MDR modulating activity (10-12). We are interested in applying the bivalent approach to increase the potency of apigenin as MDR modulators. To this end, we have synthesized a panel of apigenin di-

mers of general structure **1**, linked by ethylene glycol chain of various chain lengths (Fig. 1) (13). These synthetic apigenin dimers can modulate paclitaxel resistance in human breast cancer (LCC6/MDR, a resistant cell lines which overexpressed P-gp) and mouse leukemia cells (P388/ADR) with the optimal number of ethylene glycol units equaled to 4 (**1d**) (Fig. 1). Treatment with 5 μM **1d** can lower the IC_{50} of paclitaxel of LCC6/MDR cells by about 26-folds to a level close to that of the sensitive cells. The EC_{50} of **1d** in lowering IC_{50} for paclitaxel is 900 nM. We also demonstrated that **1d** can increase drug accumulation in MDR cells and enhanced cytotoxicity of paclitaxel, doxorubicin, daunomycin, vincristine and vinblastine in drug resistant breast cancer (LCC6/MDR) and leukemia cells (P833/ADR) *in vitro*, resulting in reduction of IC_{50} by 5-50 times (13).

Apigenin dimers with fewer (**1a** to **1c**) or more (**1e** to **1i**) ethylene glycol units have lower P-gp modulating activities (Fig. 1) (13). This "U" shaped relationship between the linker length and MDR modulating activity suggested that there was a relatively 'rigid' distance between the two apigenin-binding sites. Monomers with 4 ethylene glycol linkers (compounds **3**), even when double concentration was added, did not have any MDR modulating activity, suggesting that bivalency approach was successful in making the otherwise ineffective monomers to become effective MDR modulator.

Figure 1 - A partial list of flavonoid dimers used in reversing P-gp and MRP1-mediated cancer drug resistance



Cpd	ethylene glycol units (n)	5	6	7	[modulator] μM	LCC6MDR1 cells IC_{50} (paclitaxel)	LCC6MDR1 cells IC_{50} (paclitaxel)	2008/MRP1 cells IC_{50} (doxorubicin)
						115 to 131 #	115 - 131 #	577 *
1a	1	OH	H	OH	5	76		
1b	2	OH	H	OH	5	18		
1c	3	OH	H	OH	5	21		
1d	4	OH	H	OH	5	4		
1e	5	OH	H	OH	5	84		
1f	6	OH	H	OH	5	86		
1g	8	OH	H	OH	5	77		
1i	9	OH	H	OH	5	139		
4a	4	H	H	H	1		8.9	
4b	4	H	Me	H	1		4.9	
4c	4	H	Et	H	1		5.4	
4d	4	H	H	Me	1		6.6	
4e	4	H	H	F	1		12.4	
5a	5	H	Me	H	0.5			42
5b	6	H	Me	H	0.5			54
5c	5	H	H	Me	0.5			47
5d	6	H	H	Me	0.5			46

Notes
IC_{50} (paclitaxel) for LCC6 MDR1 cells is 115 to 131 nM
* IC_{50} (doxorubicin) for 2008/MRP1 cells is 577 nM

Lead optimization of flavonoid dimer as P-gp modulator

Since **1d** has the highest P-gp modulating activity, we have

synthesized derivatives of **1d** by maintaining the polyethylene glycol linker number to 4. When all OH groups are removed from the apigenin ring (**4a**), the P-gp modulating activity is significantly higher (Fig. 1) (14). Activity can be further improved when methyl group is added at position 6 (**4b**), ethyl group at position 6 (**4c**), methyl group at position 7 (**4d**) and fluorine at position 7 (**4e**). Limited structure-activity-relationship studies suggested that flavonoid dimers with non-polar and hydrophobic substituents (e.g. methyl, ethyl) generally showed more potent reversing activity than that of dimers with polar and hydrophilic substituent (e.g. hydroxyl) for positions 3, 6 and 7, but not at position 5 (14). All the above compounds can lower the IC_{50} of LCC6/MDR cells to that of the parental level when 1 μM of modulator is added. The EC_{50} of **4b** was determined to be 360 nM. This is about 3-fold more potent than **1d**.

Flavonoid dimer as MRP1 modulator

Besides P-gp, MRP1 is also responsible for most of the efflux-based cancer MDR. Others have reported that flavonoids have a low MRP1 reversing activity. For example, 10 μM of quercetin can reduce IC_{50} of vincristine of MRP1-HeLa cells by 4-fold (15). In contrast, LY465803 has a much higher efficacy *in vitro* (EC_{50} = 93 nM in reversing doxorubicin resistance in MRP1-transfected HeLa-T5 cells (16, 17). The low efficacy of flavonoids has prevented the further development of them as effective MRP1 modulators.

Since the secondary structure of P-gp is similar to the MRP1, we hypothesize that the apigenin dimers and their derivatives will also bind to and modulate MRP1. We found that the apigenin dimers (**1b – 1h**) can reverse doxorubicin resistance in 2008/MRP1 in a linker length dependent manner (18). Flavonoid dimers bearing 5 or 6 ethylene glycol (EG) units with 6-methyl (**5a**, **5b**) or 7-methyl (**5c**, **5d**) substitution on the ring A of flavonoid dimers have the highest modulating activity for doxorubicin against MRP1 (Fig. 1) with an EC_{50} ranging from 73 to 133 nM.[18] At 0.5 μM, the flavonoid dimer **4e** was sufficient to restore doxorubicin accumulation in 2008/MRP1 to parental level. The EC_{50} for **4e**, **5e** and **5f** have also been determined to be 73, 137 and 114 nM respectively. The efficacy is comparable to that of the highly potent MRP1 inhibitor LY465803 (EC_{50} = 93 nM in reversing doxorubicin resistance of MRP1-transfected HeLa-T5 cells) (16, 17)

How does flavonoid dimer inhibit P-gp and MRP1?

It has been demonstrated that monomeric flavonoids can bind to the nucleotide binding region of P-gp (19) and the substrate binding region of MRP1 (20). Our data suggested that flavonoid dimer is binding to the substrate binding region of P-gp and MRP1. First, **1d** stimulated P-gp's ATPase activity by about 3 fold, suggesting that **1d** was binding to the substrate-binding site of P-gp rather than the NBDs (13). This is similar to another P-gp inhibitor verapamil which is also binding to the substrate binding region of P-gp. Second, flavonoid dimer is a competitive inhibitor of doxorubicin transport in 2008/MRP1 cells with a K_i=0.2 mM (18). We hypothesize that flavonoid dimer can inhibit P-gp and MRP1 by binding to the substrate binding region present on the transmembrane helices, resulting in either the prevention of the binding of substrates and/or passage of drugs through the interior channel of P-gp and MRP1.

Are the two binding sites of each flavonoid moiety similar to each other? We have compared the P-gp modulating activities of flavonoid homodimers with those of heterodimers (14). We have synthesized five groups of compounds, with each group consi-

sting of heterodimers (**A-B**) and their homodimers (**A-A** and **A-B**) where **A** and **B** represent the monomeric flavonoid. We reason that if the two binding sites on P-gp are similar, the heterodimer (**A-B**) will have an intermediate modulating activity between the two homodimers (**A-A** and **B-B**). We found that the P-gp modulating activity for heterodimer A-B is approximately intermediate between **A-A** and **B-B** in 4 out of 5 groups. The results suggest that the two binding sites on P-gp are quite similar to each other.

Summary

We have successfully used bivalency approach to generate a new class of P-gp and MRP1 modulator with very high effiacy. Lead optimization has resulted in some flavonoid dimers with EC_{50} in nM range which is similar or even higher than some of the most potent MRP1 modulators. We provided evidence to suggest that these flavonoid dimers are binding to the substrate binding region of these transporters, thereby preventing the substrate from binding onto it or block the efflux process. We are in the process of developing this new class of P-gp and MRP1 modulators to be used in the future to reverse clinical cancer drug resistance.

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