Recovery of PEX1-Gly843Asp associated peroxisome dysfunction by flavonoid compounds in fibroblasts from Zellweger spectrum patients

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ABSTRACT

Zellweger spectrum disorder (ZSD) is due to defects in any one of 13 PEX proteins, encoded by *PEX* genes, which are required for peroxisome biogenesis. ZSD features neurologic, hepatic and renal abnormalities; however, one common mutation, PEX1-p.Gly843Asp (G843D), confers a milder phenotype and represents 30% of all ZSD alleles. Thus, identifying treatments for this allele will benefit many patients. We recently showed that PEX1-G843D behaves as a misfolded protein amendable to improved function by chemical and pharmacologic chaperones. In a small molecule screen, we reported recovery of peroxisomal matrix enzyme import by acacetin diacetate, a flavonoid that may bind to the ATP binding site of PEX1-G843D to improve conformation. In order to develop a lead compound from this library ‘hit’, we evaluated an additional 34 flavonoids for peroxisomal matrix enzyme import recovery using a patient fibroblast cell line hemizygous for PEX1-G843D and expressing a GFP-tagged peroxisome targeting signal (PTS1-GFP reporter). Eight flavonoids were identified as potential pharmacologic chaperones. Based on these compounds, additional flavonoid derivatives were produced and tested to determine the minimal pharmacophore. Relevant functional groups that enhanced or decreased efficacy were identified from these compounds, with diosmetin as the most effective one. These findings will aid in determining structure activity relationships, as knowledge of chemicals that are effective and ineffective will be used to determine the interactions that are important in developing a pharmacophore model.

To determine how the flavonoids may be affecting PEX1 and associated proteins, PEX6 and PEX5, subcellular fractions were analyzed by SDS-PAGE and Blue Native PAGE to
evaluate protein localization and complex formation. This was first done in wild type and various null cell lines to determine what to expect in normal and diseased states. It was found that PEX1 and PEX6 are dependent on each other and on PEX26 for stable levels and peroxisomal localization, and that PEX5 is dependent on these three proteins for stable levels and cytosolic localization. It was also determined that PEX1 is present in the cytosolic fraction as a monomer and a trimer, and in the peroxisomal fraction as a hexamer and dodecamer. Flavonoid treatment did not appear to affect the localization of PEX1 or PEX6 or complex formation. Thus, we propose that the flavonoids interact with a partially folded population of PEX1 that is already able to interact with PEX6 and localize to the peroxisomal membrane. In addition, we propose that this interaction of protein and drug enhances the residual ATPase activity of the PEX1 complex, thus improving matrix protein import, as observed in the cell-based experiments.
RESUME

Le trouble du spectre de Zellweger (Zellweger Spectrum Disorder – ZSD) est dû à des defaults dans l’une des treize protéines PEX, codées par les gènes PEX, nécessaires pour la biogenèse des péroxysomes. ZSD a des caractéristiques neurologiques, hépatiques et des anomalies rénales. Cependant, une mutation fréquente, PEX1-p.Gly843Asp (G843D), confère un phénotype moins sévère et représente 30% de l’ensemble des allèles ZSD. Ainsi, l’identification de traitements pour cet allèle sera bénéfique à de nombreux patients. Nous avons récemment montré que PEX1-G843D se comporte comme une protéine mal conformée, dont la fonction peut-être améliorée par des protéines chaperons chimiques ou pharmacologiques. En examinant une série de petites molécules, nous avons observé une amélioration dans l’importation d’enzyme de la matrice du péroxysome par le diacétate d’acacétine, un flavonoïde qui peut interagir avec le site de liaison de l’ATP de PEX1-G843D, afin d’améliorer sa conformation. Afin de développer un composé de base à partir de cette bibliothèque de molécules, nous avons évalué trente-quatre autres flavonoïdes pour l’amélioration de l’importation d’enzyme de la matrice péroxysomale en utilisant une lignée de fibroblastes d’un patient hémizygote pour PEX1-G843D et qui exprime un signal de ciblage péroxysomal GFP (rapporteur PTS1-GFP). Huit flavonoïdes ont été identifiés en tant que protéines chaperons pharmacologiques potentielles. En utilisant ces composés comme base, des dérivés supplémentaires de flavonoïdes ont été produits et testés afin de déterminer le pharmacophore minimal. Les groupes fonctionnels pertinents, qui accroissent ou diminuent l’efficacité, ont été identifiés à partir de ces composés, la diosmétine étant la plus efficace. Ces résultats
permettront d’élucider les relations structure-activité, de même qu’une notion des produits importants dans le développement d’un modèle de pharmacophore.

Afin de déterminer comment les flavonoïdes pourraient affecter PEX1 et les protéines associées, PEX5 et PEX6, des fractions subcellulaires ont été analysées par SDS-PAGE et PAGE Bleu Natif, pour évaluer la localisation des protéines et la formation de complexe. Ceci fut tout d’abord effectué à l’aide de lignées nulles et de type sauvage, pour avoir une idée des états normaux et pathologiques. Il a été constaté que PEX1 et PEX6 sont dépendantes l’une de l’autre ainsi que de PEX26 pour leur stabilité et une localisation péroxysomale, et que PEX5 est dépendante de ces trois protéines pour sa stabilité et une localisation cytosolique. Il a été déterminé que PEX1 est présente dans la fraction cytosolique à l’état de monomère et de trimère, et dans la fraction péroxysomale à l’état d’hexamère et de dodécamère. Le traitement aux flavonoïdes n’a pas l’air d’avoir un effet sur la localisation de PEX1 et PEX6 ou sur la formation de complexe. Ainsi, nous proposons que les flavonoïdes interagissent avec une population de PEX1 qui a une mauvaise conformation, et qui est capable d’interagir avec PEX6 et de se localiser à la membrane péroxysomale. De plus, nous proposons que cette interaction protéine-composé améliore l’activité ATPase résiduelle du complexe PEX1, améliorant ainsi l’importation de protéines de la matrice, comme observé dans les expériences in vitro.
ABBREVIATIONS

AAA – ATPase associated with various cellular activities
BSA – Bovine serum albumin
GFP – Green fluorescent protein
NSF – N-ethylmaleimide-sensitive fusion protein
PBD – Peroxisome biogenesis disorder
PEX – Peroxin
PTS – Peroxisome targeting signal
RCDP – Rhizomelic chondrodysplasia punctata
VCP – Valosin-containing protein
ZSD – Zellweger Spectrum Disorder
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CHAPTER 1

Introduction
Peroxisome biogenesis disorders (PBD) are a heterogeneous group of autosomal recessive disorders that arise from genetic defects in any one of 13 PEX proteins, encoded by PEX genes, that are required for peroxisome biogenesis \(^1\,^2\). These proteins are involved in the import of peroxisomal matrix proteins, which are synthesized on free polyribosomes and post-translationally imported to the peroxisomal lumen \(^3\). This process includes recognition and binding of peroxisomal matrix proteins in the cytosol via one of two peroxisome targeting signals, PTS1 and PTS2, by their corresponding receptors, PEX5 and PEX7, respectively. This is followed by receptor-cargo docking at the peroxisomal membrane, translocation of the cargo across the membrane, and recycling of the receptor back to the cytosol for further rounds of import (Figure 1).

Mutations in genes in this pathway lead to the disruption of peroxisome assembly and consequently, various enzymatic pathways that occur in the peroxisomes. Such multiple enzyme deficiencies affect fetal development and cause Zellweger spectrum disorder (ZSD) and Rhizomelic chondrodysplasia punctata spectrum (RCDP). Mutations in PEX1 are the most common cause of ZSD, resulting in severe neurologic, hepatic and renal abnormalities, and can be lethal; however, one mutation, PEX1-p.Gly843Asp or G843D (c.2528G>A), if present on at least one allele confers a milder phenotype \(^4\,^5\). The PEX1-G843D allele has a high frequency due to a founder effect, and represents 12-30% of all ZSD alleles \(^6\,^8\). Thus, identifying treatments for this allele would benefit a significant number of patients.
Peroxisome matrix enzymes carrying peroxisome targeting signals, PTS1 and PTS2, are recognized and bound by their respective cytosolic receptors, PEX5 and PEX7. PEX5 has two isoforms as a result of alternative splicing, and the longer version also binds PEX7. The receptor-ligand complex docks at the peroxisome membrane through interactions with PEX13 and PEX14. Matrix enzymes are translocated across the membrane by formation of a large dynamic pore. PEX2, 10 and 12 are involved in preparing PEX5 for recycling, with the monoubiquitination of PEX5 by PEX2. The PEX1-PEX6 complex, anchored to the membrane by PEX26, pulls PEX5 and presumably PEX7 out of the membrane for additional rounds of import.

Braverman, D’Agostino, MacLean (2012)

Figure 1. Peroxisome matrix enzyme import
Peroxisome matrix enzymes carrying peroxisome targeting signals, PTS1 and PTS2, are recognized and bound by their respective cytosolic receptors, PEX5 and PEX7 (1). PEX5 has two isoforms as a result of alternative splicing, and the longer version also binds PEX7. The receptor-ligand complex docks at the peroxisome membrane through interactions with PEX13 and PEX14 (2). Matrix enzymes are translocated across the membrane by formation of a large dynamic pore (3). PEX2, 10 and 12 are involved in preparing PEX5 for recycling, with the monoubiquitination of PEX5 by PEX2. The PEX1-PEX6 complex, anchored to the membrane by PEX26, pulls PEX5 and presumably PEX7 out of the membrane for additional rounds of import (4).
Structure and function of PEX1 protein

The PEX1 protein is a member of the family of AAA ATPases (ATPases associated with various cellular activities). AAA ATPases typically consist of an N-terminal ligand or adaptor binding domain; one or two AAA cassettes (D1 and D2), each containing Walker A and Walker B sub-domains for ATP binding and hydrolysis, respectively; and other structural features, such as the second region of homology (SRH) domain. AAA ATPases typically form hexameric rings and interact with other molecules via the N-terminal domain and/or other adaptor proteins. ATP binding and hydrolysis confers conformational changes that enable mechanical movement of associated proteins.

PEX1 and PEX6 have been reported to display both cytosolic and peroxisomal membrane localization. PEX1 hetero-oligomerizes with PEX6, another AAA ATPase, and this complex is targeted to the peroxisome membrane via interactions between the N-terminal domain of PEX6 and PEX26, a peroxisomal membrane protein. Anchored to the membrane, this complex uses the energy of ATP hydrolysis to pull the peroxisome matrix enzyme receptors PEX5, and presumably PEX7, out of the peroxisome so that they can be recycled for additional rounds of import. Mono-ubiquitination of PEX5 occurs before export, potentially providing the handle for the PEX1-PEX6 complex to remove PEX5 and PEX7 from the membrane. Without the ability to recycle PEX5, PEX5 is polyubiquitinated at the peroxisome membrane and targeted for degradation. Thus, when there are defects in the PEX1-PEX6 complex, protein import is impaired and therefore peroxisome biogenesis is impaired.

The stoichiometry of the PEX1-PEX6 complex is not known, and may be novel, since most AAA ATPases form homo-hexamers, with the exception of the mitochondrial
Ytap/Ytap and the six Rpt proteins of the proteasome\textsuperscript{16,17}. In mammalian CHO cells, PEX1 has been reported to be present as a homo-trimer, and suggested to be present as a hexamer\textsuperscript{11}. Mixing overexpressed and purified yeast PEX1 and PEX6 proteins \textit{in vitro} revealed the presence of PEX1-PEX6 hexamers with 1:1 stoichiometry, PEX1 trimers and PEX6 monomers\textsuperscript{18}.

**Recovery of PEX1-G843D by chaperone therapy**

Studies on PEX1-G843D human fibroblasts suggest that PEX1-G843D is a misfolded protein that is amendable to proper folding when cells are grown at 30°C\textsuperscript{19} or when treated with chemical chaperones\textsuperscript{20}. Chemical chaperones are small molecules such as glycerol, DMSO, amino acids, and trimethylamines that non-selectively stabilize mutant proteins, while pharmacologic chaperones are small molecules designed to selectively bind target proteins. The latter include enzyme substrates or inhibitors that bind to and facilitate folding of non-native protein intermediates to their native state\textsuperscript{21}.

To test if the PEX1-G843D mutant protein function could be recovered by pharmacologic chaperones, a high throughput cell-based phenotype assay was designed using a patient fibroblast cell line with a hemizygous PEX1-G843D genotype, expressing a GFP-tagged PTS1 reporter that was cytosolic at baseline. After treatment, functional recovery of peroxisomal matrix enzyme import was demonstrated by the appearance of green punctate structures, indicating redistribution of the PTS1-GFP reporter from the cytosol to the peroxisomes. This phenotype was validated by three independent confirmatory assays that showed recovery of peroxisome biochemical functions. Using this method, potential pharmacological chaperones were identified by our laboratory, including two related flavonoids (acacetin and acacetin diacetate) and two related protein kinase C
inhibitors (GF109203X and Ro 31-8220). Flavonoids and protein kinase C inhibitors have been demonstrated to inhibit ATPase and enzyme activity by binding to the ATP binding pocket $^{22-24}$. This suggests a mechanism for their action as pharmacological chaperones by binding to PEX1-G843D, through the ATP binding domain, to improve its conformation.

As flavonoids demonstrated lower toxicity and are natural compounds present in our diet, we selected this group for further investigation. Flavonoids can be divided into several subclasses, including flavones, flavonols, flavanones, isoflavones, and chalcones (Figure 2). Flavones are comprised of three conjugated rings, with the A and C ring juxtaposed and B ring branched at position 2; flavonols contain an additional 3-OH substituent on the C ring, flavanones lack the C2-C3 double bond on the C ring, and therefore lack electron conjugation and ring planarity; isoflavones have the B ring branched at the 3 position instead of the 2 position on the C ring; and chalcones have an open C-ring and the numbering differs $^{23}$. 
Figure 2. Flavonoid subclasses
Objectives of the project

The aim of my Master’s thesis was to further investigate flavonoid efficacy on peroxisomal matrix enzyme import recovery in order to elucidate the minimal pharmacophore required for lead drug optimization and to test the proposed mechanism of action of these potential pharmacological chaperones.

To accomplish this, additional flavonoid compounds were purchased or synthesized and tested on the PEX1-G843D patient fibroblast cell line expressing PTS1-GFP for matrix enzyme import recovery. Dose response experiments were performed to confirm positive compounds and to differentiate between their efficacies. These results allow for analysis of the compounds to elucidate a structure-activity relationship, as certain classes of flavonoids may be more effective than others, and knowledge of chemicals that are effective and ineffective is useful in determining what interactions are important and developing a pharmacophore model.

To determine whether the flavonoids treatments are recovering protein import by recovering PEX1 folding, protein interactions, or localization, the subcellular localization of PEX1 and PEX6 were examined. If the chemicals recover protein folding, it may allow PEX1 to better interact with PEX6 and localize to the peroxisomal membrane. Additionally, PEX5 levels and localization are of interest, as recovery should lead to an improved ability of the PEX1-PEX6 complex to recycle PEX5 to the cytosol.

To determine localization, cells were cultured, harvested and fractioned using differential centrifugation to obtain the nuclear pellet, cytosolic fraction, and a peroxisome enriched fraction. These fractions were used to determine the subcellular localization of PEX1,
PEX5 and PEX6 by comparison of equivalent cell fractions by immunoblotting. Wild type, PEX1 null, PEX6 null, and PEX26 null fibroblasts, as well as wild type hepatocyte cell lines were prepared and fractioned to determine the localization of these proteins in normal and diseased states. To determine the effect of flavonoid treatment on localization, primary or immortalized PEX-G843D homozygous or hemizygous (PEX1-G843D/null genotype) cell lines were cultured and treated with various flavonoids, betaine as a positive control, and no chemical as a negative control, harvested, and fractioned for evaluation. If the flavonoids improve the folding of PEX1 and allow it to better interact with PEX6 and PEX26, recovery of PEX1 and possibly PEX6 in the peroxisome enriched fraction may be observed.

To examine the oligomerization of PEX1, PEX6, and PEX26, cell fractions were prepared as described above, and the fractions examined by Blue Native PAGE. In this process, solubilized proteins were prepared from cell fractions using mild detergents, and then Coomassie Brilliant Blue added. This is an anionic dye that binds to protein complexes during electrophoresis, allowing protein complexes to separate without denaturation. Immunoblotting allows the sizes of the complexes to be determined in order to elucidate interactions and stoichiometry. Effective treatment by flavonoids could show an increase of these proteins in the peroxisome enriched fraction and show recovery of oligomerization of the protein complex.
CHAPTER 2

Materials and Methods

Cell lines and culture conditions

Primary or immortalized fibroblast cells lines (Table 1) were cultured at 37°C, 5% CO₂ in DMEM with 10% (vol/vol) FBS. All patient samples were acquired at the Kennedy Krieger Institute Peroxisome Disease Laboratory and received Institutional Review Board approved consent for research use. Under this consent, cell lines could be donated to other peroxisome researchers. The cell lines were assigned Peroxisome Biogenesis Disorder identification numbers, listed in parentheses. Primary cell lines were used for around 5 passages, and then a new pellet was revived. Mycoplasma testing was performed for GM9503E and Johnson E and was negative.

Null cell lines included a primary PEX1 c. 2097_2098insT/2097_2098insT (PEX1-602); a primary PEX6 c.2362G>A/? (PEX6-010); a transformed PEX6 c.1962-1G>A/2398-2417delinsT (PEX6-106); a transformed PEX6 c.511insT/517delA (PEX6-101); an immortalized PEX6 c.499_500delTG/2095-10-21del (PEX6-107); and a primary PEX26 c.296G>A/296G>A (PDL20674). Cell lines were transformed with a plasmid expressing SV40 large T antigen and immortalized with a plasmid expressing telomerase pBabePuro/hTERT 25.

PEX1-p.G843D cell lines included a hemizygous line PEX1 p.G843D/1700fs (PEX1-900), in which the second allele is null, and a homozygous line (PEX1-643). The hemizygous line was immortalized, and subsequently transfected to express GFP-PTS1.
and a clonal line selected, as described by Zhang et al\textsuperscript{20}. The homozygous line was immortalized with PA317-hTERT and PA317-E7 as per Antonicka et al\textsuperscript{26}.

We also used the transformed and immortalized wild type cell line, GM09503 (healthy fibroblasts from a 10 year old male, Corriel repository). This cell line was subsequently transfected using jetPEI and a PEX26-nmyc construct in pCDNA3.1 (inserted between BamHI/NotI restriction sites in the poly linker), to produce a wild type line expressing PEX26-nmyc. Additionally, the human liver carcinoma cell line, HepG2, was used.
Table 1. Cell lines used. All cell lines used were fibroblasts, with the exception of the hepatocyte line.

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<td>HepG2</td>
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</tbody>
</table>

$^a$ predicted null because of absent mRNA
Chemicals

A selection of commercial flavonoids was obtained by our colleague, Dr. Steve Steinberg (Appendix 1). Additionally, baicalein trimethyl ether was purchased from Extrasynthese Company. Flavonoids were resuspended in DMSO as 10 mM stock solutions and stored at -20°C. DMSO (Sigma-Aldrich) and anhydrous betaine (Orphan Europe) were obtained to be used as controls. Betaine (5 M stock solution) was dissolved in water, filter sterilized, and stored at -20°C. Four derivatives of diosmetin were synthesized by Zamboni Chemical Solutions Inc. (Dr. Robert Zamboni, affiliated with McGill University Department of Chemistry), ZCS-10-101, ZCS-10-102, ZCS-10-96, and ZCS-10-75. These were also resuspended in DMSO as 10 mM stock solutions and stored at -20°C.

GFP-reporter assays

Flavonoid screen

PEX1-G843D-GFP-PTS1 cells were seeded at 2200 cells/well in 96-well culture plates in a volume of 100 µL. Cells were counted using a haemocytometer. Flavonoid stocks were diluted to 20 µM in culture medium. Five hours after seeding, to allow for cell attachment, 100 µL of each flavonoid preparation was added in quadruplicate to each experimental well for a final concentration of 10 µM. Column one and 10 were reserved for negative (0.002%(vol/vol) DMSO or culture medium only) and positive (100 mM betaine) controls, respectively. The plates were cultured for 48 hours, in order to reach 60-80% confluence. Cells were washed with PBS, fixed with 3 % formaldehyde, washed with PBS, and stored in PBS. Plates were visually examined by fluorescence using a Leica DM IRB inverted microscope at 40X magnification. Images were taken using a Retiga 2000R camera and Image-Pro Plus 6.1 software. Initially wells were scanned, and
those wells containing greater than 50% importing cells were considered positive, ~20-50% were considered unknown and less than 20% were considered negative. Cases of high toxicity (cell death) were noted. An importing cell was defined as one containing ≥ 20 GFP positive punctate, peroxisomal structures.

We repeated this experiment using a final concentration of 20 µM for all flavonoids, except acacetin, which was used at 5 µM due to observed toxicity at higher doses. Cells were seeded at a density of 1000 cells/well and treatment was allowed to run for four days.

Flavonoid dose response

Eight flavonoids (diosmetin, acacetin, acacetin diacetate, apigenin, galangin, chrysin, and tamarixetin) showed peroxisome import recovery and were evaluated for a dose response at 0.5 µM, 5 µM, 10 µM, 20 µM and 30 µM concentrations after two or five days of treatment. Betaine and DMSO controls were added as described above. Plates were prepared as previously described, except that 500 cells/well were seeded and flavonoids were added 24 hours after seeding for the 2 day experiment, and 4 hours after seeding for the 5 day experiment. Upon visual examination, the numbers of importing and non-importing cells were counted for each well, scanning random fields of view from the top left to bottom right, until at least 100 cells were counted.

In order to blind the counting process, the dose response was repeated with the drugs and doses randomly distributed throughout the plate so there would not be a bias when working from low to high concentrations. Betaine, DMEM and DMSO controls were also randomly distributed. The less effective chemicals were also tested at concentration of 50
µM and 100 µM. 500 cells/well were seeded, chemicals added 24 hours later, and fixed after 5 days of treatment. Within each experiment there were 3 replicate wells for each flavonoid dose.

**Diosmetin synthetic derivative dose response**

The four synthesized diosmetin derivatives were evaluated for a dose response as previously described for the flavonoids. Cells were seeded at a density of 500 cells/well, and treated 24 hours later with ZCS-10-101, ZCS-10-102, ZCS-10-96, and ZCS-10-75 at concentrations of 0.5 µM, 5 µM, 10 µM, 20 µM and 30 µM for three days. Diosmetin and acacetin were also used at these concentrations as controls, as well as betaine, DMSO and DMEM controls. Treatments were randomly distributed and there were three replicate wells for each dose.

**Localization of PEX1 and associated proteins**

**Peroxisome enrichment**

Fibroblasts were grown in three T175 culture flasks to 80% confluence and harvested. For treatment experiments, cells were treated on the day following seeding with 10 µM flavonoid, 100 mM betaine as a positive control, or DMSO as a negative control and grown to 80% confluency. Media was changed every other day. Maintained at 4°C or on ice, cells were pelleted, washed with PBS, washed with 10 mL hypotonic lysis buffer (2mM EDTA, 2mM DTT, EDTA free protein inhibitor tablets), then resuspended in 1.5 or 3 mL hypotonic lysis buffer and iced x 10 min. Cells were lysed by expelling the fluid through a 25 gauge needle 5 times. 375 or 750 µL 4X isotonic buffer (200 mM Hepes/NaOH pH 7.4, 1 M sucrose, 4 mM ATP, 4 mM EDTA, 8 mM DTT, EDTA free
protein inhibitor tablets) was added to the lysate to recover isotonic conditions. The whole cell lysate was then centrifuged to remove debris and nuclei at 1000g x 5 min and 4°C. The nuclear pellet (P1) was resuspended in 1X isotonic buffer and the postnuclear supernatant (SN1) was centrifuged at 40 000 g x 45 min and 4°C. This produced a supernatant representing the cytosolic fraction (SN2) and a pelleted peroxisome enriched fraction (P2). All pellets (P1 and P2) were resuspended in the corresponding volume which they were pelleted from, so each sample represented the equivalent cell fraction.

**SDS-PAGE**

Laemmli buffer was added to each fraction sample and boiled x 15 min. Equivalent cell fractions were loaded on 7.5% SDS-PAGE mini-gels and run at 150V. Separated proteins were transferred to a nitrocellulose membrane at 100V x 1 hr. Membranes were blocked with 5% milk in TBS-T (TBS with 0.1% tween), and hybridized with PEX1, PEX5, PEX6, or PEX14 antibodies overnight at 4°C. Monoclonal mouse PEX1 antibodies were purchased from Becton-Dickinson (611719), monoclonal rabbit PEX5 antibodies were purchased from Proteintech Group (12545-1-AP), polyclonal rabbit PEX6 antiserum was a gift from G. Dodt, University of Tübingen, and polyclonal rabbit PEX14 antiserum was a gift from S. Gould, Johns Hopkins University. Washing was performed with TBS-T for 10 min x 3, and appropriate secondary antibodies were hybridized x 1 hr at RT, washing was repeated and the membrane was visualized by ECL.

**Blue Native PAGE**

Complexes were solubilized in each fraction by disruption and removal of membranes using lauryl malthoside at a final concentration of 1%, incubation on ice x 15 min, followed by centrifugation x 20 min at 20 000g and 4°C. The supernatant was then mixed
with NativePAGE™ Sample Buffer and NativePAGE™ 5% G-250 Sample Additive. Samples were run on NativePAGE 3-12 % Bis-Tris gels (Invitrogen) at 150 V with NativePAGE™ Anode Buffer and Dark Blue NativePAGE™ Cathode Buffer for migration through the first third of the gel, and then switched to Light Blue NativePAGE™ Cathode Buffer for the remainder of the run. Sample additives and buffers were prepared as per Invitrogen’s NativePAGE™ Novex® Bis-Tris Gel System User Manual, Version A, 20 January 2006. Gels were soaked in 0.1% SDS x 10 min and separated complexes were transferred to a PVDF membrane at 25 V x 1 hour. Membranes were incubated in 8% acetic acid and dried to fix proteins. Membranes were blocked with 5% milk or BSA in TBS-T (TBS with 0.1% tween), and hybridized with polyclonal rabbit PEX1 antiserum (gift from G. Dodt, University of Tübingen). Washes and hybridizations were the same as above. The membrane was visualized by ECL.

**Bioinformatic analysis**

**Sequence alignment and interaction diagram**

Human PEX1 and VCP protein sequences were aligned using Accelrys Discovery Studio 2.5 multiple sequence alignment protocol and the common AAA ATPase cassettes and motifs identified.

The crystal structure of the D2 subdomain of VCP with ADP bound was obtained from PDB ID: 3CF0 27. The protein and ligand were specified and a ligand interaction diagram was produced by Accelrys Discovery Studio 2.5. The sequence alignment was then used to compare the common interacting residues of VCP with the corresponding ones in PEX1.
CHAPTER 3

Results

Flavonoid efficacy on peroxisome protein import recovery

Previously our laboratory reported functional recovery of peroxisomal matrix enzyme import following flavonoid treatment as demonstrated by localization of a PTS1-GFP reporter in cultured fibroblasts from a patient with the PEX1-G843D genotype\textsuperscript{20}. In this small molecule screen, untreated cells showed that the GFP was distributed throughout the cytosol, while cells treated with betaine and flavonoids showed that the GFP localized to peroxisomes. Following the identification of acacetin diacetate as a hit compound, my project included testing an additional 34 commercially available flavonoids.

Flavonoid screen

In the initial flavonoid screen, wells treated with 10 $\mu$M of each flavonoid were scanned for an estimate of percent importing cells. Results showed that diosmetin and acacetin diacetate had clear import recovery, while acacetin appeared to have recovery, but was fairly toxic at this dose. After repeating this screen of all 34 flavonoids with an increased dose of 20 $\mu$M flavonoids or a decreased dose of 5 $\mu$M for acacetin and counting the proportion of importing to non-importing cells, eight potential hit compounds were identified. Observed recovery was 89% (range = 73-100%) for the betaine treated positive controls and for the negative controls recovery was 9% (1-20%) for DMSO treated and 9% (range = 3-15%) for untreated cells. Identified hit compounds showed greater than 50% importing cells (Figure 3), while all other treatments had below 20% importing cells. These compounds were diosmetin, acacetin, acacetin diacetate, apigenin,
galangin, chrysin, and tamarixetin (Figure 4). Functional groups common to the hit compounds are outlined and represent potential pharmacophores.
Figure 3. Effects of treatment on PTS1-GFP import in PEX1-G843D fibroblasts
Cells were treated with 10 μM flavonoid, 100 mM betaine (positive control), or untreated (negative control). Peroxisomal import recovery was observed by the redistribution of the PTS1-GFP from the cytosol to the peroxisomes (appearance of punctate structures).
Figure 4. Hit flavonoid compounds
Structures of the eight identified compounds. Common groups are indicated: green suggests groups that enhance binding, red suggests groups that reduce binding, and blue suggests groups that provide unique properties.
Flavonoid dose response

To confirm that these flavonoids were truly recovering import and to compare their efficacy, dose response experiments were performed after 2 or 5 days of treatment, and a blinded dose response experiment was performed to prevent any bias in counting or training of the eye when evaluating from low to high concentrations. Recovery of PTS1-GFP peroxisomal import was determined by visually scoring the number of cells with punctate vs. cytosolic GFP fluorescence, and reported as a percentage of importing cells (Figure 5, 6, and 7). Results showed that the two and five day dose response experiments showed similar trends: no further recovery of PTS1-GFP occurred at 5 days as compared to 2 days, and no apparent increase in toxicity was observed (Figures 5 and 6). Diosmetin achieved up to 100% recovery; acacetin, acacetin diacetate, and apigenin reached up to ~80% recovery; kaempferol reached 55-80% recovery; while, chrysin, galangin, and tamarixetin showed a lesser effect. In the blinded dose response experiment, at 20 µM the percentage of importing cells were approximately 90% for diosmetin, 80% for acacetin diacetate, 60% for apigenin, 50% for kaempferol, 45% for chrysin, 30% for galangin, and 20% for tamarixetin. Acacetin showed 50% recovery at 0.5 µM; however, at higher doses the toxicity was too high for reliable counts. EC$_{50}$ values for each experiment were determined by interpolation as the concentration at which 50% import recovery was achieved for each treatment and are summarized in Table 2.
Figure 5. Flavonoid dose response - 2 days
Dose response curve for PEX1-G843D fibroblasts under flavonoid treatment. Each treatment was performed in triplicate. The number of importing cells was scored visually, error bars indicate standard error. Average values for 100 mM betaine (+) and untreated (-) controls are shown. Positive controls showed 92% importing cells, and negative controls showed 7% (range = 2-13%) importing cells.
Figure 6. Flavonoid dose response - 5 days
Dose response curve for PEX1-G843D fibroblasts under flavonoid treatment. Each treatment was performed in triplicate. The number of importing cells was scored visually, error bars indicate standard error. Average values for 100 mM betaine (+) and untreated (-) controls are shown. Positive controls showed 89% importing cells, and negative controls showed 7% (range = 3-13%) importing cells.
Figure 7. Blinded flavonoid dose response - 4 days
Dose response curve for PEX1-G843D fibroblasts under flavonoid treatment. Each treatment was performed in triplicate and randomly distributed. Counts were blinded and the number of importing cells was scored visually, error bars indicate standard error. Average values for betaine (+) and untreated (-) controls are shown. Positive controls showed 96% (range = 87-100%) importing cells, and negative controls showed 10% (range = 5-20%) importing cells.
Synthesized derivatives of diosmetin efficacy on peroxisome protein import recovery

Overall the results showed that diosmetin was the most effective hit compound. The next step in drug screening projects is to identify the minimal pharmacophore so that lead compounds can be further developed. A minimal pharmacophore is defined as the minimal structure that is responsible for the compounds activity. In order to further evaluate structure-activity relationships, diosmetin derivatives were synthesized in collaboration with Zamboni Chemical Solutions. Compounds provided are shown in Figure 8. ZCS – 10 – 101 is equivalent to the previously tested acacetin and ZCS – 10 – 96 is equivalent to the previously tested chrysin. New compounds included ZCS – 10 – 75 (4’-methoxyflavone) and ZCS – 10 – 102 (flavone).
Figure 8. Diosmetin derivatives
Structures of compounds provided by Zamboni Chemical Solutions. Common groups found to be associated with binding are highlighted.
A dose response experiment was performed for these compounds as previously described. The positive control, betaine, averaged 94% importing cells, and the negative controls, DMEM/DMSO and DMEM averaged 6% and 4% importing cells, respectively. Diosmetin and acacetin were also included for comparison. Results showed that diosmetin was the most effective flavonoid, surpassing the positive control and reaching 97% importing cells, with an EC$_{50}$ of 3.4 µM. ZCS-10-101, which is equivalent to our commercially purchased acacetin, and our acacetin were comparable, reaching ~70% and ~60% recovery at 10 µM, and EC$_{50}$ values of 7 µM and 4.5 µM, respectively. As these drugs showed more toxicity, counts were more difficult, leading to more variability, especially at higher doses. ZCS-10-96, which is equivalent to our commercially purchased chrysin, demonstrated a small recovery, peaking at ~30%, which nears that of our findings for chrysin which reached up to ~50% recovery. ZCS-10-75 (4’-methoxyflavone) showed little if any recovery, only a bit above the negative control, and did not appear to respond to increasing doses, while ZCS-10-102 (flavone) showed no recovery whatsoever.
Figure 9. Diosmetin derivative dose response - 4 days

The number of importing cells was scored visually. Each treatment was performed in triplicate and was blinded for two of the three counts. The number of importing cells was scored visually, error bars indicate standard error. Positive controls showed 94% (range = 89-96%) importing cells, and negative controls showed 5% importing cells: DMSO had 6% (range = 1-12%) and DMEM only had 4% (range = 1-9%).
Table 2. Calculated EC<sub>50</sub> values for the efficacious flavonoids. EC<sub>50</sub> values were determined as the concentration at which 50% of the maximal response was achieved.

<table>
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<th>Flavonoid</th>
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<th>5 day</th>
<th>blinded</th>
<th>diosmetin derivatives</th>
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Localization and complex formation of PEX1 and associated proteins

Subcellular fraction localization of PEX proteins

In order to determine if our flavonoids were working to recover peroxisome import by interacting with PEX1-G843D and changing its localization or the localization of associated PEX proteins, we first sought to determine what would normally be expected. Wild type and various null patient fibroblast cells lines were grown and fractioned to determine the localization of PEX1 and associated proteins in normal and diseased states (Figure 10). Determination of the distribution of endogenous PEX1, PEX6 and PEX5 proteins in wild type and mutant cells is not yet specifically known. PEX14 levels were also evaluated to ensure clean separation of peroxisomal and cytosolic fractions and equal loading of lysates and peroxisomal fractions.

Following SDS-PAGE, immunoblotting results showed that for wild type fibroblasts, PEX1 levels were greater in the peroxisomal fraction than cytosolic, PEX6 levels were approximately equal in the two fractions, and PEX5 was mostly cytosolic. In PEX1 null cells, PEX1 was absent, PEX6 levels were reduced and mostly cytosolic, and PEX5 levels were below detectable levels. In PEX6 null cells, PEX6 was absent, PEX1 levels were reduced and mostly cytosolic, with a small amount peroxisomally localized, and PEX5 was reduced and mostly peroxisomal. These findings show that PEX1 and PEX6 require each other for stable levels and to localize to the membrane. This data also supports previous data that has shown that if either of these components of this complex are missing, then PEX5 remains stuck at the membrane, it cannot be recycled to the cytosol, and is ultimately degraded 28; 29. In PEX26 null cells, PEX1 and PEX6 were reduced and mainly cytosolic, highlighting the importance of PEX26 for stable levels and
peroxisomal localization of PEX1 and PEX6; however, small amounts of both were peroxisomal.

Additionally, hepatocytes were examined, using the HepG2 human liver carcinoma cell line. As liver cells have greater number of peroxisomes compared to fibroblasts, we were interested to see if this increased the total peroxisomal protein levels to allow for better detection on the immunoblots. Results showed that there was no overall increase for these PEX proteins, and the localization of PEX1 and PEX5 were similar to that for fibroblasts; however, PEX6 had little if any cytosolic localization and was predominantly peroxisomal in liver cells. The lack of an increase in these peroxisomal protein levels may be because there are not actually more peroxisomal proteins. There may be more peroxisomes, but smaller. The explanation for this different localization for PEX6 is unclear, but may reflect different functional roles in cells in which peroxisomes are more actively dividing. In fact, in yeast, PEX6 has been suggested to play a role in membrane fusion of premature peroxisomal vesicles \(^{30}\), as well as other functions unrelated to the peroxisome involved in the suppression of different cell death mechanisms \(^{31-33}\).

Considering the nonspecific bands detected by the PEX6 antibody in PEX6 null cell lines, we evaluated several additional PEX6 null cell lines to confirm which band represented PEX6 protein (Figure 11). PEX6 null cell lines had more non-specific bands present than wild type, possibly due to lack of specific antigen in that microenvironment.
Figure 10. Subcellular localization of selected PEX proteins
Immunoblots showing PEX1, PEX5, PEX6 and PEX14 (peroxisome marker) levels in cell fractions from various null and wild type cell lines. Protein concentrations loaded for each lysate are indicated, and volumes corresponding to the equivalent cell fraction were loaded for the remaining fractions for each line. (L - whole cell lysate, S - postnuclear supernatant, C - cytoplasmic fraction, P - peroxisome enriched fraction)
Figure 11. Specificity of PEX6 antibody

Immunoblots showing PEX1 and PEX6 levels from various PEX6 null cell lines and wild type cell line expressing PEX26-nyc (A) and verification of specific and non-specific bands with further separation (B). Protein concentrations loaded for each lysate are indicated, and volumes corresponding to the equivalent cell fraction were loaded for the remaining fractions for each line. (L - whole cell lysate, C - cytoplasmic fraction, P - peroxisome enriched fraction)
**Complex formation**

The prepared fractions from the various cells lines were also subjected to Blue Native PAGE in order to determine complex formation and their localization in normal and diseased states. Despite having two antibodies for both PEX1 and PEX6, and a cell line expressing a myc-tagged PEX26, only our polyclonal PEX1 antibody was able to detect complexes with confidence by Blue Native PAGE.

This antibody indicated the presence of a band of ~146 kDa, suggestive of monomeric PEX1, present in the cytosolic fraction for wild type and PEX6 null cell lines that was absent in PEX1 null cell lines (Figures 12 and 13). Interestingly, there appeared to be more monomeric PEX1 in the PEX6 null cell line than wild type (Figure 13B). The PEX26 null cell line did not have the PEX1 monomer present, although it could be present, but undetected, because reduced amounts of protein were loaded for this cell line (Figure 13A). Two additional bands, slightly larger than the monomer were also present, but were determined to be non-specific, as they were present in the PEX1 null cell line (Figures 12 and 13).

In some, but not all cases, a band between 480 and 720 kDa, indicative of a trimer, was present in the cytosolic fraction of the PEX6 null cell lines (Figure 13A and B) and potentially the wild type cell line (Figure 13B); and a band of ~720 kDa, indicative of a hexamer, was present in the peroxisomal fraction of the wild type and PEX6 null cell lines (Figure 13B). The absence of these bands in some experiments may simply be due to detection limitations. The blot shown in Figure 13B was the only one probed in BSA, which may explain the enhanced ability to detect bands.
A high molecular weight smear, between 1048 and 1236 kDa or greater, was observed in the wild type peroxisomal fraction, indicating the potential for a dodecamer that may contain a hexamer of PEX1 with a hexamer of PEX6. Interestingly, this dodecamer was also observed in the PEX6 and PEX26 null cell line. Perhaps complexes in this size range can contain a combination of PEX1 and PEX6 in the wild type and/or PEX26 null, or PEX1 alone in the PEX6 null. This however does not explain how the complexes are peroxisomally localized. Investigators have previously shown that PEX1 depends on PEX6 to interact with the transmembrane protein PEX26, in order to anchor it to the membrane. The HepG2 hepatocyte cell line also had the high molecular weight complex, but did not appear to have hexameric or monomeric PEX1 (Figure 12B).

These are results from 3 individual fractionation preps: samples used in Figures 12A and 12B were fractioned and blotted together under identical conditions, Figure 13A samples were fractioned in another experiment, and for the Figure 13B blot PEX6 null (3) samples were the same as those used for Figure 13A, PEX6 null (4) samples were the same as those used in Figure 12A, and the wt+PEX26nmyc samples were fractioned in another experiment.
Figure 12. PEX1 containing dodecamers and monomers

Immunoblots showing complexes and monomers from cell fractions separated by blue native PAGE in PEX1 null, PEX6 null and wild type fibroblasts (A), and wild type hepatocyte and wild type fibroblasts (B). Protein concentrations loaded for each lysate are indicated, and volumes corresponding to the equivalent cell fraction were loaded for the remaining fractions for each line. (L - whole cell lysate, C - cytoplasmic fraction, P - peroxisome enriched fraction)
Figure 13. PEX1 containing complexes and monomers
Immunoblots showing complexes and monomers, separated by blue native PAGE in PEX6 null and PEX26 null fibroblast cell lines (A), and wild type expressing PEX26-nmyc and two PEX6 null fibroblast cell lines (B). Protein concentrations loaded for each lysate are indicated, and volumes corresponding to the equivalent cell fraction were loaded for the remaining fractions for each line. (L - whole cell lysate, S - postnuclear supernatant, C - cytoplasmic fraction, P - peroxisome enriched fraction)
Localization and complex formation of PEX1 and associated proteins following flavonoid treatment

Reasoning that recovery of PEX1-G843D folding by flavonoids in the patient fibroblasts cell lines would improve PEX1-PEX6 interaction and peroxisomal localization, we examined the subcellular localization of PEX1 and PEX6 with and without drug treatment. A PEX1-G843D hemizygous cell line (PEX1-G843D/null-E) was treated with diosmetin, acacetin diacetate, kaempferol, apigenin, acacetin, betaine as a positive control, and DMSO as a negative control, and peroxisome enriched fractions were isolated by differential centrifugation.

Immunoblotting of these fractions showed that wild type cell fractions had almost equivalent levels of peroxisomal and cytosolic levels for both PEX1 and PEX6. In PEX1-G843D/null cell fractions total levels of PEX1 were reduced, but were still almost equivalent in the peroxisomal and cytosolic fractions, and PEX6 levels were only reduced slightly, if at all, but were predominantly cytosolic (Figures 14 and 15). Following treatment, the same distribution was observed and total amounts of PEX1 and PEX6 do not appear to improve. Diosmetin, acacetin, and apigenin treatment may have slightly improved PEX1 and/or PEX6 localization to the peroxisomal fraction, although these findings were not dramatic.
Figure 14. Effects of flavonoid treatments on localization of select PEX proteins in PEX1-G843D/null fibroblasts

Immunoblots showing PEX1, PEX6 and PEX14 (peroxisome marker) levels in cell fractions from PEX1-G843D/null fibroblasts with or without treatment (0.1% DMSO solvent) and a wild type cell line. (L-whole cell lysate, C-cytoplasmic fraction, P-peroxisome enriched fraction)
Figure 15. Peroxisome associated PEX1 and PEX6 levels by densitometry analysis of immunoblots shown in Figure 14.
To see if a cell line that may have more PEX1-G843D protein would show a more pronounced response, and to confirm that findings were equivalent in primary and immortalized cell lines, primary and immortalized PEX1-G843D homozygous cell lines (PEX1-G843D and PEX1-G843D-E, respectively) were treated with the most promising compound, diosmetin, betaine as a positive control, and DMSO as a negative control. Immunoblotting of the prepared fractions showed that at baseline in PEX1-G843D cell fractions both PEX1 and PEX6 are predominantly peroxisomal and below detection in the cytosolic fraction (Figures 16 and 17). The same distribution was observed following treatment. However, this experiment needs to be repeated with the inclusion of a wild type control. There may be some enhanced PEX1 and PEX6 in the peroxisomal fraction following flavonoids treatment; however, as in the findings for the hemizygous cell line, the difference between treated and untreated were not dramatic.

These fractions were also evaluated by Blue Native PAGE (Figure 18), which revealed high molecular weight complexes, suggestive of PEX1-containing dodecamers in the peroxisomal fraction; however, no difference between treated and untreated cells was observed.
Figure 16. Effects of flavonoid treatments on localization of select PEX proteins in PEX1-G843D fibroblasts

Immunoblots showing PEX1, PEX6 and PEX14 (peroxisome marker) levels in cell fractions from primary (A) or immortalized (B) PEX1-G843D fibroblasts with or without treatment (0.1% DMSO solvent). Protein concentrations loaded for each lysate are indicated, and volumes corresponding to the equivalent cell fraction were loaded for the remaining fractions for each line. (L-whole cell lysate, C-cytoplasmic fraction, P-peroxisome enriched fraction)
Figure 17. Repeat of immunoblots for better visualization of PEX proteins

Immunoblots showing PEX1, PEX6 and PEX14 (peroxisome marker) levels in cell fractions from primary (A) or immortalized (B) PEX1-G843D fibroblasts with or without treatment (0.1% DMSO solvent). Protein concentrations loaded for each lysate are indicated, and volumes corresponding to the equivalent cell fraction were loaded for the remaining fractions for each line. (L-whole cell lysate, C-cytoplasmic fraction, P-peroxisome enriched fraction)
Figure 18. Effects of flavonoid treatments on PEX1 containing complex formation
Immunoblots showing PEX1 containing complexes and monomers separated by blue native PAGE in cell fractions from primary (A) or immortalized (B) PEX1-G843D fibroblasts with or without treatment (0.1% DMSO solvent). Protein concentrations loaded for each lysate are indicated, and volumes corresponding to the equivalent cell fraction were loaded for the remaining fractions for each line. (L - whole cell lysate, C - cytoplasmic fraction, P - peroxisome enriched fraction)
Evaluation of PEX1 D2 domain and its role in ligand binding

An alignment of PEX1 and valosin-containing protein (VCP or p97), a closely related AAA ATPase that has been crystallized, demonstrated the high conservation of the second AAA cassette (D2), including conservation of the Walker A and Walker B motifs and the glycine residue which is mutated in PEX1-G843D (Figure 18). A ligand interaction diagram for the crystallized D2 subdomain of VCP with ADP bound (PDB ID: 3CF0), produced using Accelrys Discovery Studio 2.5, shows that the glycine residue in VCP, which corresponds to the PEX1-G843 residue, directly interacts with the bound ADP molecule (Figure 19). This model shows the importance of the PEX1-G843D mutation in the ATP binding site, thus giving potential future insight to the mechanism for flavonoids chaperone action.
Figure 19. VCP and PEX1 sequence alignment for the D2 AAA cassette. Glycine 843 (*) and corresponding residues found to interact with ADP are indicated.

Figure 20. Interaction diagram depicting several residues of VCP interacting with bound ADP.
**Discussion**

In this project, our goal was to evaluate additional flavonoids for their ability to recover peroxisomal enzyme import function in PEX1-G843D patient fibroblast cell lines, and to compare their efficacies to elucidate a structure-activity relationship. Eight flavonoids were identified, with diosmetin as the most effective, and various structure-activity relationships were determined based on the comparison of their structures and the structures of negative compounds.

We additionally sought to determine if the flavonoids were recovering import by affecting the localization and complex formation of PEX1 and associated proteins. This was first analyzed in various null and wild type cell lines in order to determine what would be normally expected, and then was compared in treated and untreated PEX1-G843D cell lines. From this, we find that flavonoid treatment does not appear to alter PEX1 or PEX6 amounts, localization or complex formation. Rather, we suggest that the flavonoids improve PEX1-G843D function by interacting with a partially folded population of the protein that is stable, able to form complexes and localize to the membrane, but is only partially functional, and improve its ability to act as an ATPase.

**Flavonoids as pharmacologic chaperones**

In my initial experiments on flavonoid dose response, I demonstrated that certain flavonoids effectively recover peroxisomal enzyme import in a PEX1-G843D hemizygous cell line, with diosmetin as the most effective compound. Additional tested compounds allowed for the elucidation of important functional groups by comparison of structures of negative compounds and of positive compounds with varying levels of efficacy. Functional groups of all relevant compounds discussed in the following section...
are summarized in Table 6. Backbone structures of the classes are depicted in Figure 2, and other structures are depicted in Figures 4 and 8.

Within the group of eight identified positive flavonoids, there were three flavones and their three corresponding flavonols, and two other similar compounds. The lack of efficacy for flavone (ZCS-10-102) shows that the basic core flavonoid structure alone was not sufficient. Structure comparisons showed that flavones (diosmetin, apigenin and chrysin) were more effective than their corresponding flavonols (tamarixetin, kaempferol and galangin). Methoxylation or hydroxylation at the 4’ position increased efficacy, with the former having a greater influence (acetatin>apigenin>chrysin). All compounds also shared 5,7-dihydroxy groups, except for acetatin diacetate, which had acetate groups at these positions, highlighting their importance. The reduced to negligible efficacy in chrysin (ZCS-10-96) and 4’-methoxyflavone (ZCS-10-75) further demonstrates the significance of the hydroxyl groups at the 5 and 7 positions and the methoxy group at the 4’ position. The fact the chrysin (ZCS-10-96) retained a better ability to recover than 4’-methoxyflavone (ZCS-10-75) suggests that one of or both of these two hydroxyl groups may be more important than the methoxy group; however, the methoxy group does contribute to efficacy since acetatin (ZCS-10-101) shows much better recovery than chrysin (ZCS-10-96).

Flavanones, isoflavones, and chalcones were not found to be effective. From evaluation of similar flavonoid structures in which there was no recovery in this screen, it was determined that lack of efficacy for flavanones could be attributed to the absence of the C2-C3 double bond of ring C, resulting in a loss of planarity (hesperetin and naringenin as compared to diosmetin and apigenin), and the presence of the B ring at the 3 position,
as opposed to the 2 position, of the C ring could result in the lack of efficacy for isoflavones (genistein as compared to apigenin). In addition, only aglycones displayed efficacy, while flavonoid glycosides did not (diosmetin as compared to diosmin). This suggests that the glycosides may interfere with binding; however, since the glycoside functional group is removed by the intestinal flora and the flavonoid is absorbed as its aglycone, these compounds may be effective in vivo, but not in our cell based assay.

Only one chalcone, neohesperidin dihydrochalcone, was tested and was ineffective, and its corresponding flavanone, neohesperidin, was also ineffective. Therefore, the lack of efficacy could not be attributed to the differences in the chalcone core structure without testing chalcones of corresponding positive flavones.

Overall, these structure activity relationships were consistent with findings from other investigators who evaluated the ability of flavonoids to bind and inhibit ATP binding sites in proteins. The similar findings were: (1) activity of flavones > flavonols, though some studies do suggest the reverse, suggesting some protein specificity to flavonoid efficacy; (2) flavanones are ineffective, suggesting the importance of the C2-C3 double bond; (3) 5, 7-OH groups enhance activity; (4) 4’ substitutions enhance activity, with OCH₃ > OH; (5) isoflavones are less effective; (6) and glycosides are less effective. Interestingly, the previously identified PKC inhibitors, GF109293X and Ro31-8220 and kaempferol have been shown to bind to the ribosomal S6 kinase 2 (RSK2) ATP-binding site by modeling and a structure-based pharmacophore model was proposed in which the 5,7–OH groups are involved.

Unique to our assay was that hydroxylation at the 3’ position made diosmetin a more effective compound than acacetin, whereas reports from other investigators indicated that
such substitutions reduce ATPase inhibition and therefore ATP site binding. Also, evaluation of compounds with acetate groups at the 5 and 7 positions, as in acacetin diacetate, have not been previously reported, while the importance of hydroxyl groups at these positions have been repeatedly shown. Taken together, this suggests that acacetin may be the most potent compound for ATP site binding, but the addition of the hydroxyl group to form diosmetin, or the addition of acetate groups to make acacetin diacetate, make these molecules better suited for our specific target. Thus, we suggest that molecules that bind very strongly are strong inhibitors of ATPases, while in our case, weaker binding may allow them to bind reversibly. In this fashion they can act as chaperones to properly fold the protein, and then be released so that the protein can function better as an ATPase.

The decreasing order of efficacy of the flavonols, kaempferol and galangin, follows the decreasing efficacy of their corresponding flavones, apigenin and chrysin. However, the flavonol, tamarixetin, was the least effective of the three flavonols, even though its corresponding flavone, diosmetin, was most effective. This further supports my idea that the 3’-OH on diosmetin is reducing binding, or enhancing the reversibility of diosmetin binding. Each of the flavonols is less effective than the corresponding flavone due to the additional 3-OH, which defines it as a flavonol. However, the reduction of efficacy for tamarixetin is even greater because of the 3’-OH. The lack of efficacy of luteolin also supports the idea that the 3’-OH reduces binding, as it is equivalent to apigenin with a 3’-OH. Taken together, this information suggests that the 3’-OH that makes diosmetin differ from acacetin improves the efficacy by reducing its binding ability, rather than increasing it.
A micronized purified flavonoid fraction (Daflon®), which contains 90% micronized diosmin and 10% hesperidin is currently available as an oral phlebotic drug, and it has been found that diosmetin is rapidly and widely distributed throughout the body. This could prove to be a promising treatment trial for ZSD patients with the PEX1-G843D genotype.
<table>
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<th>Functional groups of selected flavonoids.</th>
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<td>Luteolin</td>
</tr>
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<td>Genkwanin</td>
</tr>
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<td>Diosmin</td>
</tr>
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<td>ZCS - 10 - 75 (4'-'methoxyflavone)</td>
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<td>ZCS - 10 - 102 (flavone)</td>
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<td><strong>Flavonol</strong></td>
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</tr>
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<td>Hespereretin</td>
</tr>
<tr>
<td>Naringenin</td>
</tr>
<tr>
<td><strong>Isoflavone</strong></td>
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<tr>
<td>Genistein</td>
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</table>
**Peroxisomal protein localization and complex formation**

In order to determine the mechanism of action of the flavonoids, PEX protein localization and complex formation were examined. In these experiments it was also important to determine these parameters in wild type and null cell lines, which had not previously been done, to determine what would normally be expected.

In these experiments, we found that protein distribution across cell fractions in the various null cell lines show that PEX1 and PEX6 are dependent on each other for stable levels and peroxisomal localization, as they are reduced and mostly cytosolic in the absence of each other. Both proteins are also dependent on PEX26 for stable levels and peroxisome localization. This suggests that the complex of the three at the peroxisomal membrane may be the dominant form, and in its absence the individual components are less stable. PEX5 is also dependent on this complex for stable levels and cytosolic localization. In the PEX1 null cells, PEX5 was mostly degraded and below levels of detection, and in the PEX6 null cells it was mostly degraded and what remained was stuck at the peroxisome membrane. As PEX5 levels have been previously shown to vary within PEX1 and PEX6 nulls and they were only examined in one of each PEX1 and PEX6 null cell lines and not in a PEX26 null cell line in these experiments, it remains unclear whether one or another has a greater impact on PEX5 levels.

Similar to my findings, Tamura et al. 11, using CHO cells and HA-tagged PEX1 and PEX6, showed that these are mostly peroxisomal, some cytosolic and depend on each other for localization. However, Weller et al. 39 found that PEX1 was not reduced in PEX6 null and PEX26 deficient patient cell lines. Using overexpressed epitope-tagged
constructs in wild type fibroblasts, they found that PEX6 mostly cytosolic, PEX1 mainly cytosolic, some peroxisomal

Contrary to previous data which suggested that PEX1 and PEX6 are recruited to the membrane by PEX26, with PEX6 acting as a bridge between the other two, we found some peroxisomal PEX1 in the PEX6 and PEX26 null cell line. Some PEX6 also appears to be peroxisomally localized in the PEX26 null cell line, but not in the PEX1 null cell line. The N-terminal domain of PEX1, as well as other AAA-ATPases, such as VCP and NSF, was shown to bind phosphoinositides. All three proteins are organelle membrane associated, and it was suggested that this protein-lipid interaction is what allows for membrane binding. It may be that this is the mechanism by which PEX1 and possibly PEX6 are targeted to the membrane, and the interactions with PEX26 are what allow it to be firmly anchored to the membrane during receptor recycling. Work by Weller et al. also suggests that PEX1 is able to target the peroxisomal membrane, independently of PEX6 and PEX26 peroxisomal localization.

Further to support the idea that PEX1 is capable of targeting itself and/or PEX6 to the peroxisomal membrane is the peroxisomal localization of the potential PEX1-containing dodecamers in the PEX6 and PEX26 null cell lines and PEX1-containing hexamers in the PEX6 null cell lines. PEX1-containing dodecamers were observed in the peroxisomal fraction of all cell lines tested, except the PEX1 nulls: various PEX6 nulls, PEX26 null, wild type, wild type + PEX26nmyc, and wild type hepatocytes. PEX1-containing hexamers were only observed in the peroxisomal fraction of PEX6 and wild type cell lines; however, this complex was only observed on one blot, which was prepared using BSA and overall showed stronger signals. As this band was faint, it may have been
present but undetected on other blots for other cell lines as well. The fact that these complexes are peroxisomal in the PEX6 and PEX26 nulls supports that PEX1 is capable of peroxisomal targeting, independent of PEX6 and 26.

The composition and stoichiometry of these complexes remains unclear. Limited work has been done in this area, with the exception of Saffian et al. 18, who suggested that PEX1 and PEX6 form a hexameric complex with a 1:1 stoichiometry in yeast. Other studies of heteromeric AAA proteins, predominately Rvb1-Rvb2, have also come up with controversial results, sometimes indicating the formation of single heteromeric hexameric rings or dodecamers composed of two heteromeric or two homomeric rings, each with a 1:1 stoichiometry 41. It was suggested that the different conformations may be the result of the artificial expression systems, such as buffer conditions, presence or absence of nucleotides, protein construct and expression. As my experiments look at the composition of endogenous protein levels in their native state, and both hexamers and dodecamers were observed, it may be that both conformations are present in the cell; however, the dodecamer appears to be the predominant form. The different complexes may represent different functional roles or intermediates of the fully formed complex.

The composition of these complexes is questionable, as similar sized bands are observed in the PEX6 and PEX26 null cell lines as in the wild type. The dodecamer was observed in all three, and the hexamer was observed in both the PEX6 null and wild type (it may have been present in the PEX26 null as well; however, this complex was not detected in the conditions of this experiment). As PEX26 is small (34 kDa), it is possible that the complexes formed in the PEX26 null is the same as in the wild type, except lacking PEX26, and this change is undetectable in this size range. As PEX1 and PEX6 are similar
in size, it may be that the hexamers and dodecamers in the wild type and PEX6 null have a different composition, but form similar sized complexes. As we expect a 1:1 stoichiometry, in the wild type we may be observing a heteromeric hexamer comprised of 3 of each PEX1 and PEX6 and a dodecamer formed by two heteromeric or homomeric rings comprised of 6 of each PEX1 and PEX6. In the PEX6 null, with the absence of PEX6, PEX1 may contribute completely to these complexes, forming homomeric hexamers and dodecamers.

Saffian et al.\(^ {18}\) suggest that the PEX1 trimer may recruit 3 PEX6 monomers to form the hexamer. This may be the case, as in my experiments PEX1 trimers were observed in the cytosolic fractions of wild type and PEX6 null cells. Since there appears to be a stable PEX1 homomeric hexamer in the PEX6 null cell line, it is also possible that this is an intermediate to the formation of the dodecamer and it may be present in the wild type cells as well; however, the composition of the hexamer in the wild type could not be determined and it is unclear if the homomeric PEX1 hexamer is normally present, or only in the absence of PEX6. Blue Native-PAGE performed by Tamura et al.\(^ {11}\) also suggest that PEX1 is present as a homomeric trimer in the cytosol and a homomeric hexamer at the peroxisomal membrane; however, due to limitations of their ability to detect PEX6, it cannot be ruled out that it was a constituent of the observed hexamer.

PEX1 was also observed as a cytosolic monomer in wild type and PEX6 null cell lines, although it was below detection in PEX26 null cell lines and hepatocytes. The levels of PEX1 monomer were much greater in the PEX6 null cell line. The differences in levels of trimers and hexamers in these two cell lines were not apparent, as the levels were so low, but it did appear that there may be less dodecamer in the PEX6 null cell line than the wild
type. It is likely that PEX1 less efficiently forms homomeric complexes than the heteromeric complexes with PEX6, leaving more uncomplexed monomer in the PEX6 null cell line.

**Mechanism of PEX1-G843D dysfunction recovery by flavonoids**

Interestingly, the PEX1-G843D hemizygous cell line showed equal or a bit less peroxisomal than cytosolic PEX1, and less peroxisomal than cytosolic PEX6; while the homozygous cell line showed mostly peroxisomal PEX1 and PEX6. However, an increase in peroxisomally associated PEX1 or PEX6 was not clearly observed following flavonoid treatment for either cell line and no change in complex formation was observed following treatment of the homozygous cell line (complex formation was not evaluated in the hemizygous cell line). As such, it could not be determined if treatment is affecting complex formation and localization. There may be slight changes that were below detection by Western blot or changes dealing with alterations in complex composition that could not be determined, and these changes display a larger functional impact.

More likely, it is that the mutant protein is able to form complexes and localize to the peroxisomal membrane; however, it is less functional. This is consistent with previous findings that showed reduced temperature and chemical treatment could lead to recovery, but only the temperature reduction increased PEX1 levels. It was suggested that there were two populations of PEX1: one that is completely misfolded and targeted for degradation and another that is partially functional. Examination of PEX1 and PEX6 in a two-hybrid system and coimmunoprecipitation experiments by Geisbrecht et al. suggest that the PEX1-G843D mutation reduces its ability to interact with PEX6;
however, these experiments did not take into account the possibility of the presence of two populations.

While both the degraded and partially folded populations can be recovered by a decrease in temperature, flavonoid treatment is only effective on the latter. It is this population that is able to form complexes, and therefore not be degraded, that the flavonoids are acting on to improve their ability to function, thereby leading to recovery.

The fact that flavonoids are known to bind to ATP binding sites and the demonstration of the importance of the mutated residue in PEX1-G843D in this pocket, as depicted by the interaction diagram of ADP with the corresponding residue in VCP, further supports the idea that the mutant protein may be in a conformation that nearly resembles the normal state. We propose that these compounds are interacting with the mutant protein via the ATP binding site to improve function, so something close to the correct conformation may be required in order for this binding to occur.
Conclusions

The following summarizes and highlights some of the main findings of my work:

Flavonoid SAR

1. Flavones > flavonol (3-OH reduces efficacy), flavanones ineffective, isoflavones ineffective, only tested chalcone ineffective
2. 5,7–OH and 4’OCH₃ are important functional groups
3. 3’-OH – may reduce binding, but improves diosmetin efficacy

PEX1, 6, 26 and 5 localization in control and patient cell lines

1. In wild type fibroblasts, PEX1 and PEX6 are present at higher levels in the peroxisomal fraction, although there is a significant amount also in the cytosolic fraction. PEX5 is almost exclusively cytosolic.
2. PEX1 and PEX6 are dependent on one another for stable levels and peroxisomal localization. In the absence of one another, their levels are reduced and mostly cytosolic. Both are also dependent on PEX26 for stable levels and peroxisomal localization.
3. In the PEX6 and PEX26 null cell lines, some PEX1 is peroxisomal. In the PEX26 null cell line, some PEX6 is peroxisomal. In the PEX1 null cell line peroxisomal PEX6 is negligible.
4. PEX5 is degraded in the PEX1 null cell line, and its levels are reduced and mostly peroxisomal in the PEX6 null cell line.
5. In wild type hepatocytes, PEX6 is mainly peroxisomal and almost none is cytosolic.
Complex formation (detected by PEX1 antiserum)

1. In wild type fibroblasts there are dodecamers, hexamers (only observed on BSA blot), trimers and monomers.
2. In PEX1 null cells, there are no complexes.
3. In PEX6 null cells, all complexes are detected: dodecamers, hexamers (only observed on BSA blot), trimers (only observed on BSA and one other blot) and monomers. Also, in PEX6 null cells there is more monomer than in wild type cells.
4. In PEX26 null cells, only the dodecamer was detected; however, the protein levels were at low concentration and BSA was not used.
5. In hepatocytes, only the dodecamer was detected; however, BSA was not used.

Flavonoid and betaine treatment effects on localization and complexes

1. No strong effects on localization of PEX1 and PEX6 between treated and untreated cells.
2. For both treated and untreated cells, PEX1-G843D hemizygous cells appeared to have equal levels of peroxisomal and cytosolic PEX1, and some peroxisomal, but mostly cytosolic PEX6; whereas PEX1-G843D homozygous cells appeared to have more peroxisomal and negligible cytosolic levels of both PEX1 and PEX6.
3. For both treated and untreated PEX1-G843D homozygous cells, there was no apparent change in complex formation – dodecamers were observed in all. Since PEX1-G843D levels are reduced, the other complexes and monomers may be below detection.
Future Directions

1. Additional flavonoids are currently under evaluation to further refine the SAR.
2. Evaluation of combination drug therapy with pharmacological chaperones (e.g., diosmetin) and a proteasome inhibitor, Velcade.
3. Flavonoid effects on plasmalogen levels will be evaluated to further confirm recovery.
4. Evaluate PEX1, PEX6 and PEX5 levels in fractions of another PEX1 null and PEX26 null to confirm findings and differentiate between effects of the various protein deficiencies, still including the originally tested PEX1, PEX6 and PEX26 nulls.
5. Repeat PEX1-G843D hemizygous and homozygous cell line treatments and include a wild type control.
6. Repeat Blue Native PAGE for various null cell lines and treated and untreated PEX1-G843D cell lines and include second dimension to see composition of complexes.
BIBLIOGRAPHY


APPENDICES

**Appendix 1.** List of analyzed flavonoid compounds

<table>
<thead>
<tr>
<th>Common name</th>
<th>Class</th>
<th>IUPAC name</th>
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<td>3',4'-dihydroxyflavone</td>
<td>flavone</td>
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