A Systems Biology Approach to Dissection of the Effects of Small Bicyclic Peptidomimetics on a Panel of Saccharomyces cerevisiae Mutants*  

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Received for publication, March 19, 2010, and in revised form, May 24, 2010 Published, JBC Papers in Press, May 25, 2010, DOI 10.1074/jbc.M110.125153  

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In recent years, an approach called “chemical genetics” has been adopted in drug research to discover and validate new targets and to identify and optimize leads by high throughput screening. In this work, we tested the ability of a library of small peptidomimetics to induce phenotypic effects with functional implications on a panel of strains of the budding yeast Saccharomyces cerevisiae, both wild type and mutants, for respiratory function and multidrug resistance. Further elucidation of the function of these peptidomimetics was assessed by testing the effects of the compound with the most prominent inhibitory activity, 089, on gene expression using DNA microarrays. Pathway analysis showed the involvement of such a molecule in inducing oxidative damage through alterations in mitochondrial functions. Transcriptional experiments were confirmed by increased levels of ROS and activation of mitochondrial membrane potential. Our results demonstrate the influence of a functional HAP1 gene in the performance of S. cerevisiae as a model system.

Pharmaceutical and biotechnological fields are constantly looking for new drugs characterized by innovation and economy (1). New approaches emerging from chemical and biological knowledge, such as “chemical genetics” (2), consist of the study of biological systems by systematic investigation of the effects of small molecules on a specific cellular model rather than perturbing with gene mutations (3–5). This new approach has been divided into forward and reverse chemical genetics; the former is based on the research of a desired phenotypic effect following the administration of small molecules to biological systems of interest, whereas the latter is based on the investigation of the effects of small molecules against a specific target (6–8). To make these new approaches time- and money-saving, the set-up of easy, rapid, and economical chemical synthesis and biological screening processes is essential (9, 10). The budding yeast Saccharomyces cerevisiae, which has been defined as an “honorary mammal” (11), is a suitable model organism for this aim, mainly because of the high degree of conservation with human cells concerning main biological processes (11). Thanks to its ease of manipulation, it can be successfully used to identify molecules of pharmacological interest (4, 12). Moreover, once active compounds have been selected, functional information about their mode of action can be inferred from screening ~5000 viable yeast haploid deletion mutant strains for hypersensitivity or hyperresistance to each molecule, thus identifying pathways that influence the cellular response to tested compounds (13–16). However, so far, chemical genetic approaches have used only one genetic background, that of laboratory strain S288c, which is limited by an accumulation of genetic defects, such as the inability to sustain proper respiratory metabolism of this strain (4, 17–19). In this work, we selected a panel of strains in which it is possible to investigate the influence of a library of molecules on growth rate, respiratory metabolism (HAPI), and multidrug-membrane function (ERG6, SNQ2, and PDR3).

Peptidomimetics (20) play a prominent role as candidate compounds to induce phenotypic effects on biological systems. In fact, side-chain recognition dominates biological interactions of almost all cellular processes; thus, peptidomimetics, initially developed for their property of preventing degradation and improving oral bioavailability of peptide-based drugs, have been envisaged as a tool for perturbing such interactions and identifying protein function. Small peptide-based agents have attracted wide interest as cancer-targeting and anti-infective agents, and there is a need to develop new high affinity and high specificity peptidomimetic or small molecule ligands in such widespread pathologies. Success in this area depends on the ability to create novel complex molecular structures of a peptidomimetic nature as tools for probing protein-protein interactions. Diversity-oriented synthesis (21) is the concept of choice for the rapid exploration of the chemical space. Our efforts are oriented to the development of efficient synthetic strategies for the production of rigid and polyfunctional heterocyclic templates using elements from the chiral pool, such as carbohydrates and amino acids, which guarantee a high stereochemical and functional diversity (22–26). The library of small peptidomimetics herein taken into account was synthesized by focusing on a
bicyclic scaffold based on the 6,8-dioxa-3-azabicyclo[3.2.1]octane core and diverse substituents, in order to hypothesize a connection between specific groups and biological effects.

**EXPERIMENTAL PROCEDURES**

**Molecules**—In this study, a preconstituted library, composed of bicyclic peptidomimetics, bicyclic from tartaric acid and amino acids (BTAa), was used for assays on selected yeast strains (supplemental Table 1).

**Yeast Strains and Media**—Three wild-type strains were used: W303 (MATa can1-100 ade2-1 his3-11,15 leu2-3,112 trpl-1, ura3-1), BY4742 (MATa his3 leu2 lys2 ura3), and 0117. The deletion strains used were BY4742Δsnq2 (MATa his3 leu2 lys2 ura3 snq2::kanMX), BY4742Δpdr3 (MATa his3 leu2 lys2 ura3 pdr3::kanMX4), and BY4742Δerg6 (MATa his3 leu2 lys2 ura3 erg6::kanMX4). Yeast strains used to simulate the HAP1 pattern of BY4742 were FY2607 (MATa ural3 his3 leu2 lys2-128TM hap1T), FY2609 (MATa ural3 his3 leu2 lys2-128TM) and FY2611 (MATa ural3 his3 leu2 lys2-128TM hap1kanMX4). For growth inhibition tests, yeast cells were inoculated at 1 × 10⁶ cells/ml in YPD medium (yeast peptone dextrose, yeast extract 1% (w/v), peptone 2% (w/v), buffered at pH 4.8 with citric acid, 2% glucose).

**First Level Assays**—First level assays allowed the selection of a restricted number of active molecules. W303 and 0117 strains were grown in YPD. The different cultures were dispensed at 10⁵ cells/ml in two replicates. After 3 days, the concentration of the cells was determined, and 100 cells were plated (100 cells/plate) on YPD agar in two replicates. After 5 days, colony-forming units were calculated, and the concentration-effect curve of the treatment was plotted.

**Determination of Survival Rate**—This test was carried out with the LIVE/DEAD® yeast viability assay (Molecular Probes), following the manufacturer’s instructions, and with the methylene blue assay determining the survival rate of the wild-type W303 and BY4742 strains and of the BY4742Δerg6, BY4742Δsnq2, and BY4742Δpdr3 strains. Cells were grown in YPD supplemented with 089 at concentrations of 1.0, 0.4, 0.2, and 0.1 mM; a positive control was also carried out without the compound. The assay cultures were incubated at 28°C, and after 2 and 4 h, aliquots were processed with the LIVE/DEAD and methylene blue assays, permitting calculation of the survival rate (number of live cells counted × 100/total number of cells in the positive control culture).

**Microarray Hybridization**—Yeast microarrays were constructed as described previously (27). Yeast cells (BY4742, BY4742Δerg6, and BY4742Δsnq2 strains) were treated in YPD (pH 4.8) supplemented with 089 at 0.2 mM concentration and incubated at 28°C with shaking for 4 h. RNA was extracted from about 1 × 10⁷ cells using the hot acid phenol/chloroform protocol. RNAs were quantified spectrophotometrically using Nanodrop and by measuring the absorbance at 230, 260, and 280 nm and then calculating the 260/230 and 260/280 ratios.

**Gene Expression Analysis**—Fluorescent cDNA bound to the microarray was detected with a GenePix 4000B microarray scanner (Axon Instruments), using the GenePixPro6.1 software package to quantify microarray fluorescence. The control microarray spot quality required the following features: 50% as the minimum percentage of pixels for which the foreground intensity was greater than the background intensity + 2 S.D. values; 80 pixels as the minimum number of pixels; absence of saturated pixels. Median values for the foreground and background of each channel were normalized using the MIDAW Web tool (28). A -fold change cut-off of 1.5 filtered by variance coefficient was used to select differentially expressed genes. Genes within each group were examined for functional enrichment using Gene Ontology categories.

**Pathway Analysis**—Normalized transcriptional data were analyzed with Eu.Gene Analyzer 5.1 (29), using Fisher’s exact
test (FET) and then visualized with T-MEV 4.4 (30). This approach describes each pathway as up- or down-regulated, considering the relative number of up- or down-regulated genes annotated to that pathway and the result of the FET, false discovery rate-corrected, for the same pathway. In our analysis, we included yeast pathways from the KEGG (31), Reactome (32), and YOUNG (lists of target genes for known transcription factors) (33) databases. Transcriptional data of deletion mutant strains were obtained from the Rosetta compendium (34). Clustering of pathway-based analysis of microarray data was performed according to the procedure described in Ref. 35.

**Fluorescence Microscopy**—After 4 h of treatment with 089 at 0.2 mM concentration, or without it as a control, cultured cells were resuspended at 1 × 10⁶ cells/ml in 10 mM HEPES buffer, pH 7.4, containing 5% glucose. Rhodamine B hexyl ester (Molecular Probes) was added to a final concentration of 100 nM. After 15–30 min of incubation, the mitochondrial membrane potential was visualized by fluorescence microscopy (excitation λ at 555 nm, green; emission λ at 579 nm, red). An equal aliquot of cells was treated with dihydrorhodamine 123 (Molecular Probes) to analyze endogenous reactive oxygen species (ROS) production, observed after 60 min of incubation (excitation λ at 505 nm, blue; emission λ at 534 nm, green). Each aliquot was treated with Calcofluor White (M2R) (blue) to show the cell wall in order to count total cells.

**RESULTS**

**Selection of the Panel of Strains**—Several easily detectable phenotypes in the yeast cell are related to growth rate, respiratory metabolism, and cell wall-multidrug resistance. The panel of strains of the budding yeast we have selected encompasses *S. cerevisiae* wild type for respiratory function and multidrug resistance, two laboratory strains, W303 and FY2609, and one wild-type strain (Fig. 1). Five of the compounds tested, 011, 012, 018, 022 and 028, decreased the growth rate of W303, whereas they exerted the minimum concentration that induced an effect on the wild-type strain (Fig. 1). Five of the compounds tested, 011, 012, 018, 022 and 028, decreased the growth rate of W303, whereas they showed no effects on BY4742 and an increase in BY4742 Δsnq2 and BY4742Δpdr3 strains at a concentration of 0.3 mM, which is the minimum concentration that induced an effect on the wild-type strain (Fig. 1). Five of the compounds tested, 011, 012, 018, 022 and 028, decreased the growth rate of W303, whereas they showed no effects on BY4742 and an increase in BY4742 Δsnq2 and BY4742Δerg6 mutant strains. This effect can be attributed to an increase in the intracellular/cytoplasmic concentration of the molecule, due to a defect in the efflux activity (deletion of SNQ2) or to a higher membrane permeability (deletion of *ERG6*). Four of the molecules tested were able to increase the growth rate of BY4742. Specifically, 066 and 067 exerted the same effect even on Pdr3p and Snq2p; 038 and 053 induced an increase in growth rate even on Pdr3p, Snq2p, and Erg6p. Further assays on 089, which was identified as the molecule inducing the most intense cell growth decrease (more than 80%), showed that after 18 h of treatment at 0.3 mM concentration, the variation in OD₆₅₀ value of the culture of BY4742Δerg6 became negative due to cell death, confirmed by microscopic observation with LIVE/DEAD (live cells converting the green fluorescent intracellular staining FUN1 dye into red fluorescent intravacuolar structures) and methylene blue (dead cells turning blue) assays as well as the presence of cellular debris.

**Relationship between Molecular Structure and Biological Effect**—The screening conducted in this study was carried out by treating a whole organism with small molecules. Analysis of the observed effects provided information on the connection between the molecular structure and the ability to perturb the growth rate of treated cells, regardless of the target receptor of each molecule. The class of molecules having the general structure III, as shown in Table 1 (106, 107, 108, 110, 111, 112, 113, and 114) induced a growth rate decrease only if the molecule...
had a secondary amine as the R^4 group. The molecules containing a leucine isostere as the R^1 group (119, 120, 123, and 124) did not induce effects in any case. Phenotypic effects of compounds having an ethyl group at R^4, (020, 024, 025, 042, 049, 057, 072, 075, 076, 089, and 090) suggested that 1) the presence of a prolic donor group (resulting in R^4 hydroxyethyl residue), which in some cases was connected to higher cell growth rate, and 2) the substitution of the methyl group with CF_3 at the second position of the ethylamine was associated with a higher growth decrease, not attributable to the steric hindrance of fluoro (similar to hydrogen) but to the ability to form polar interactions. The ability of molecules having the general structure I, as shown in Table 1 (056, 057, 058, 059, 060, 061, 066, 067, 068, 069, 070, and 071), to induce a biological effect was associated with the steric hindrance of the R^4 group; the lower the steric hindrance, the higher the biological effect, decreasing up to 50% in the presence of a bulkier group. On the contrary, the opposite correlation was evident in molecules possessing the scaffold II, as shown in Table 1 (001, 002, 004, 005, 008, 009, 010, 011, 012, 013, 014, 015, 016, and 018), thus suggesting two different targets for the two compound classes. By comparing 089 with other molecules of the library that differed in some functional groups, a drastic decrease in the ability to induce a biological effect was seen to be due to the lack of the carbonyl group embedded in the bicycle, the benzhydryl group substitution with a benzyl group, and the lipophilic R^4 chain elongation.

Second Level Assays—The MIC of 089 on W303 and BY4742 wild-type strains was determined (supplemental Fig. 1). Both strains were characterized by a MIC of 1 mM, but the EC_{50} values, which were 0.25 mM for W303 strain and 0.38 mM for BY4742 strain, were more interesting. The survival rate of the strains W303, BY4742, BY4742\_erg6, BY4742\_snq2, and BY4742\_dpr3 after a 2- and 4-h treatment with different concentrations of 089 (1, 0.5, 0.2, and 0.1 mM and control) was evaluated. The results of this assay (Fig. 2) showed that at higher concentrations, the molecule induced steady effects in each strain even at the early time points. Lower concentrations (up to 0.1 mM in the 2-h treatments and up to 0.2 mM in the 4-h treatments) induced a survival percentage decrease only for W303, thus confirming the MIC results. Each strain showed almost the same higher survival decrease at 0.5 mM concentration of 089 (nearly 30% in the 2-h treatment, around 10% in the 4-h treatment). By contrast, BY4742 revealed itself to be the most resistant to the treatment, as previously observed. The 4-h exposure to 089 with a concentration of 1 mM triggered the same effects on all of the tested strains, including BY4742. The concentration of 089 inhibiting the growth of deletion mutant strains induced a minor effect on BY4742, probably due to different respiratory ability.

Because the two wild-type tested strains, BY4742 and W303, had a different genetic background, we further investigated the role of HAP1 using FY2609 (HAPI) and FY2607 (hap1T) as two
additional strains having similar alterations of \( \text{HAP1} \) in the same genetic background. The FY strains (36) have a similar pedigree and genetic background as the BY series strains, all being derivatives of S288c; therefore, the results observed in FY2607 can be reasonably generalized to BY4742. We treated the FY strains with \( \text{089} \) at 0.3 mM concentration. We observed an inhibition rate of 7% for FY2607 \( \text{hap1T} \) and of 8.9% for FY2609 \( \text{HAP1} \), and this difference was statistically significant (\( p < 0.05 \)). These results confirmed the \( \text{HAP1} \)-associated trend highlighted in the comparison between BY4742 and W303.

Specifically, compound \( \text{089} \) elicits stronger inhibiting effects in the presence of the wild-type \( \text{HAP1} \) gene. To further investigate the role of the \( \text{HAP1} \) gene in combination with the other mutations studied, we are planning to introduce the wild-type allele into the BY4742 strain and into the three knock-out strains, BY\( \text{erg6} \), BY\( \text{snq2} \), and BY\( \text{pdr3} \).

**Transcriptional Analysis**—Transcriptional analysis following the treatment with \( \text{089} \) at 0.2 mM concentration was carried out on deletion mutant strains that in former assays showed higher sensitivity to the treatment (BY4742\( \text{erg6} \) and BY4742\( \text{snq2} \)) and on the parental strain (BY4742). We chose to perform the transcriptional profiling in the presence of a molecule concentration having some effects on cell viability but allowing survival of at least 50% of treated populations, as determined by EC\(_{50}\) evaluation. We focused on the laboratory type strains because a large quantity of publicly available data make this genetic background the most suitable for further comparisons of transcriptional profiles obtained in this study with those present in public databases. We analyzed the lists of differentially expressed genes obtained, applying 1.5 as the \( -\text{fold} \) change threshold. The two deletion mutants showed an overexpression of genes coding mitochondrial proteins or genes involved in respiration and in response to oxidative stress, in contrast to BY4742. This strain also showed down-regulation of genes encoding some mitochondrial \( F_1 F_0 \)-ATP synthase subunits (\( \text{ATP1}, \text{ATP2}, \text{ATP3}, \text{ATP4}, \text{and ATP16} \)). The deleted strains showed increased expression
same genetic background as BY4742, the observation of overexpression of genes involved in respiration and response to oxidative stress only in the deleted strains suggests that the intracellular concentration of the molecule in the wild-type strain is reduced by active export mechanisms. As a consequence, the effects are more pronounced in deletant strains. Every strain showed an up-regulation of genes involved in telomere maintenance and organization and in homeostatic processes (YRF1-1, YRF1-2, YRF1-3, YRF1-4, YRF1-5, YRF1-6, and YRF1-7), showing instead a down-regulation of genes involved in protein folding and protein targeting to membrane and to endoplasmic reticulum (SSAI, SSA4, SSE1, SSE2, YDJ1, HSP82, and KAR2), thus indicating that 089 induces cellular damage, as indicated also by the up-regulation of telomere maintenance and organization.

Pathway Analysis—Pathway analysis was used to assign a probability of alteration in selected pathways by using the FET \( p \) value as an indication of the likelihood of the involvement of a cellular compartment or of a set of biological reactions. Pathway analysis over our selected set of pathways was carried out using the FET (see “Experimental Procedures”). The Fisher’s test \( p \) value, along with the state of regulation of each pathway (described considering the relative number of up- or down-regulated genes annotated to that pathway) indicated the likelihood of its involvement. The pathway analysis of transcriptional data confirmed the increase in respiratory deficiency, highly evident in the BY4742Δerg6 strain, and of anaerobic respiration, especially of the electron acceptor reaction list and electron transport chain, with an opposite trend in the wild-type strain. This result confirmed the involvement of respiratory metabolism and HAPI function in response to 089, confirming HAPI as one of the targets.

We further used an advanced pathway approach to compare the three experiments of this study with a collection of publicly available experiments describing the effect on transcription of selective gene deletions. This approach uses pathway signatures to compare experiments at the pathway level (35). We used this approach with a chemical genetic mindset, asking which other deletion was phenocopied at a transcriptional level. The Fisher’s test \( p \) value was used to assign a probability of alteration in selected pathways (by using the FET \( p \) value as an indication of the likelihood of the involvement of a cellular compartment or of a set of biological reactions). Pathway analysis over our selected set of pathways was carried out using the FET (see “Experimental Procedures”). The Fisher’s test \( p \) value, along with the state of regulation of each pathway (described considering the relative number of up- or down-regulated genes annotated to that pathway) indicated the likelihood of its involvement. The pathway analysis of transcriptional data confirmed the increase in respiratory deficiency, highly evident in the BY4742Δerg6 strain, and of anaerobic respiration, especially of the electron acceptor reaction list and electron transport chain, with an opposite trend in the wild-type strain. This result confirmed the involvement of respiratory metabolism and HAPI function in response to 089, confirming HAPI as one of the targets.
FIGURE 3. Pathway signatures visualized with T-Mev 4.4. Pathway signatures were obtained with Eu.Gene Analyzer 5.1, using FET and then visualized with T-MEV 4.4. This is a significant selection of the pathway analysis and the relative sample cluster of transcriptional data of the treatment of yeast with 089. Up-regulated pathways are visualized in red, and down-regulated pathways are shown in green. Yeast pathways from the KEGG, Reactome, and YOUNG databases were included in the analysis. The YOUNG list is composed of target genes for known transcription factors.

FIGURE 4. Quantitative evaluation of ROS accumulation and mitochondrial activation. Wild-type strain cells (BY4742) and deletant strain cells (BY4742Δerg6 and BY4742Δsnq2) were treated for 4 h with 089 at 0.2 mM concentration, and then the mitochondrial membrane potential was detected by rhodamine B hexyl ester staining, and ROS accumulation was evaluated by dihydrorhodamine 123 staining. White, the percentage values corresponding to cells treated with 089; black, the percentage values of untreated cells. Percentages were calculated as the mean of the number of stained cells over total cells in the optical field evaluated in triplicate. Student’s t test was used to evaluate the data significance. ∗, p < 0.05. Error bars, S.D.

Chemical Genetics of S. cerevisiae

The profile observed in ROS accumulation was confirmed when evaluating mitochondrial activation. Cells of the deletant strains stained with rhodamine B hexyl ester showed a 3-fold increase in mitochondrial membrane potential activation (12 and 9%), compared with untreated cells (4 and 2%), whereas the wild-type strain mitochondrial activation level did not change in either treated or untreated cells (Fig. 4).

DISCUSSION

Yeast genetics, cell biology, and molecular genetics have benefited enormously from the presence of a well-characterized set of strains derived from S288c genetic background. S288c is profoundly different from other strains of the S. cerevisiae genus (37–39). When using yeast in chemical genetics studies, the extent to which a phenotype induced by a molecule is dependent on the genetic background of the S288c derivatives is yet to be fully understood. The results presented herein demonstrate
the influence of a functional HAP1 gene in the performance of *S. cerevisiae* as a model system and the importance of using a panel of strains to investigate the effects of a library of bioactive molecules. The set of strains used in this work significantly broadens the ability to dissect the biological effects of a selected family of molecules, thus circumventing the limitations of the laboratory strain S288c. Our results show how a combined use of the proposed panel of gene deletion strains with alterations in the respiratory function or multidrug resistance—cell wall function can ascribe the biological function of a molecule to a specific set of biological processes, paving the way to more insightful investigations. The selected peptidomimetic 089 induces more severe effects on the wild-type W303 strain, having a functional HAP1 gene, than on the laboratory BY4742 strain, having almost the same genetic background as W303 but the mutated HAP1 gene, thus giving an indication of an involvement of the respiratory metabolism in response to 089 perturbation. This hypothesis is confirmed even by the HAP1-associated trend of the molecule effects on FY strains. The strain FY2609 (HAP1) showed greater inhibition than FY2607 (hap1T). Nevertheless, the intensity effect is weaker on FY strains than on the W303 strain, indicating that a HAP1-mediated increase in the respiratory metabolism alters the effects of 089 in a genetic background-associated function. It is evident that a number of other genetic differences between the strains could have epistatic effects on this phenotype. Overall, considering the wide range and differences of effects exerted by the treatment with 089 on different strains, the genetic background of the strain used for the screening again turns out to be of extreme relevance when evaluating the biological effects of small molecules (Table 3).

The use of a pool of strains bearing deletions of genes involved in membrane assembly or function enables the identification of effects that could be concealed by the membrane permeation incapability of the molecule. This is suggested by the opposite effect of 089 on yeast growing rate, which decreases for deletant strains but increases for the wild-type strain. Transcriptional analysis indicates the presence of an important stress condition that occurs more prominently in the deletion mutant strains, a variation that can be ascribed to a higher ability of the molecule to permeate the cellular or mitochondrial membrane or to remain in the cell cytoplasm. The activity of 089 in inducing an increase in respiratory deficiency and anaerobic respiration is confirmed by pathway analysis of transcriptional data, thus corroborating the HAP1 as one of the targets. The induction of oxidative stress at the mitochondrial level is confirmed by an increase in ROS accumulation and mitochondrial membrane activation level.

The application of the novel pathway-based approach (35) to compare 089 effects at the transcriptional level with those of a pool of gene deletions is a powerful in silico simulation that identifies response trends to different stimuli (the former a molecule, the latter a gene function loss). Accordingly, we identified a similar trend between the treatment with 089 and the deletion of GAS1 and ERG4, the former stricter and the latter weaker, leading to localization of the first molecular target at the cell wall level, with oxidative stress being a secondary effect. The cell wall is a peculiar yeast structure; hence, it is an excellent target for selective antifungal drugs.

The pathway signature-based approach used here holds the promise of enabling the classification of bioactive molecules based on their transcriptional pathway signatures and the assessment of similarity of the effects of different molecules based on the pathway profile. By taking advantage of all of these findings, it was possible to identify 089 as a potential selective antifungal.

Model organisms that have evolved in permissive laboratory conditions may introduce a bias into the assessment of biological functions of a molecule, derived from the accumulation of genetic defects in laboratory strains (such as the improper respiratory metabolism). When using yeast to learn fundamental lessons about cell biology or the mechanism of action of a molecule, often to be generalized to mammalian cells, it is fundamental to assess to what extent the observed phenotype can be generalized to other yeasts of the same species. In this study, the use of a panel of different strains has been fundamental to the analysis of the relationship between the activity and the structure of the whole peptidomimetic library and cell function, thus providing important insights for optimization of its activity.

Acknowledgments—We are grateful to Dan Spatt for supplying the FY strains, to Luca Beltrame for helpful discussion on pathways analysis, and to Mary Forrest for linguistic editing. We also thank Fondazione Giuseppe Tommasello Organizzazione Non Lucrativa di Utilità Sociale.
