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Chemical genetics approach to drug discovery by diversity-oriented synthesis (DOS) of peptidomimetics*

Andrea Trabocchi[1,‡], Duccio Cavalieri[2], and Antonio Guarna[1,‡]

[1]Department of Chemistry "Ugo Schiff", University of Florence, Polo Scientifico e Tecnologico, Via della Lastruccia 13, I-50019, Sesto Fiorentino, Florence, Italy; [2]Department of Pharmacology "Mario Aiazzi Mancini", University of Florence, Viale Pieraccini 6, 50139, Florence, Italy

Abstract: Chemical genetics, which relies on selecting small molecules for their ability to induce a biological phenotype or to interact with a particular gene product, is a new powerful tool for lead generation in drug discovery. Accordingly, diversity-oriented synthesis (DOS) of small-molecule peptidomimetics gives access to collections of new chemotypes bearing high structural diversity. Biological evaluation using cell growth as a phenotypic screening on *Saccharomyces cerevisiae* deletant strains is a powerful tool to identify new chemotypes as hit compounds in the discovery of new antifungal and anticancer agents, and also in the dissection of their mode of action. Our contribution in this field focused on the screening of morpholine-based peptidomimetic collections toward yeast deletant strains, which provided the identification of new chemotypes involved in mitochondria metabolism and respiration.

Keywords: combinatorial chemistry; genetics; organic synthesis; peptides; yeast.

INTRODUCTION

An important topic in chemical biology is the elucidation of processes involved in cell division and metabolism, because of the potential for anticancer applications, and much effort has been oriented in this area. Also, membrane-bound receptors represent an attractive target for small-molecule-mediated control of cellular activity because of their accessibility. In this context, chemical genetics [1] is one of the best examples of a methodological development in lead generation, as it relies on selecting small molecules for their ability to induce a biological phenotype or to interact with a particular gene product.

Chemical genetics can be divided similarly to genetics, and specifically into forward and reverse chemical genetics. The idea is that small molecules can phenocopy the effects of precise gene deletion when inactivating a target protein, or gene overexpression if activating transcription of a gene or activity of a protein. The former approach involves the use of small molecules to screen for phenotypic effects on the biological system under investigation, and the latter involves the use of small molecules against a protein of interest. Thus, forward chemical genetics is from molecule to phenotype to protein,

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‡Corresponding authors: E-mail: antonio.guarna@unifi.it; andrea.trabocchi@unifi.it

and in the reverse direction it is from protein to phenotype. In both cases, the identification of a selective small molecule followed by detailed biological investigation is needed.

The application of chemical genetics concepts to biomedical problems gives access to the study of complex biological issues by combining medicinal chemistry, biological screening, and combinatorial synthesis techniques. Its interdisciplinary profile is striking, as it consists of harmonizing synthetic methodology and library development with new analytical and biological methods applied to screening systems.

Forward chemical genetics is of particular interest for drug discovery issues, as it allows for the identification of both new chemical probes and dissection of biological mechanisms at the same time. The advantages of this approach are several: the biological effect of small molecules is reversible and usually rapid, thus allowing early effects to be characterized; small-molecule effects can be used to produce "dose–response" data, where the level of phenotype effects are dependent on the concentration of the small molecule, thus giving greater confidence in the apparent biological effect of a molecular probe.

Nevertheless, there is no technology enabling a chemical genetic approach to be applied generally, as it requires a selective small-molecule ligand to the protein of study. A chemical genetic study requires the involvement of a selective small molecule, its protein partner, and biological screening.

Since the early reports of Schreiber et al. [2], diversity-oriented synthesis (DOS) has become a new paradigm for developing large collections of structurally diverse small molecules as probes to investigate biological pathways and to provide a larger array of the chemical space in drug discovery issues [3]. The principles of DOS have evolved from the concept of generating structurally diverse compounds from a divergent approach consisting in a complexity-generating reaction followed by cyclization steps and appendage diversity, to the development of different cyclic structures through the build/couple/pair approach [4]. The building stage of the overall DOS process involves the generation of suitable building blocks for subsequent coupling reactions, and it is generally achieved using compounds from the chiral pool or achiral moieties as substrates for stereoselective reactions.

Secondly, biological assays are required to recognize and characterize the small molecule–protein interaction. In forward chemical genetics, a collection of small molecules is screened in a phenotypic assay; for example, the assay for antibiotic discovery could be looking for compounds that killed bacterial but not mammalian cells.

The approach followed by our group for forward chemical genetics programs consisted of the use of *Saccharomyces cerevisiae*. The set-up of easy, rapid, and economical chemical synthesis and biological screening processes is essential to make new chemical genetics approaches time- and money-saving [5]. The budding yeast *S. cerevisiae*, which has been defined as an "honorary mammal" [6], is a suitable model organism to this aim, mainly because of the high degree of conservation with human cells concerning main biological processes. *S. cerevisiae* is particularly suited for the high-throughput screening of new chemical entities on whole cell systems due to key features connected to easy manipulation and the versatility of its genome [7]. The life cycle is particularly rapid, as cells divide in about two hours, it is possible to manipulate both aploid and diploid yeast systems. Moreover, it shows a high degree of conservation with mammalian cells concerning fundamental biological processes from cell cycle [8] to oleate metabolism [9]. Thus, it can be successfully used to identify molecules of pharmacological interest [10]. In addition to the advantage of conservation and growth pace, *S. cerevisiae* is a goldmine of phenotypes. Several easily detectable phenotypes in the yeast cell are related to growth rate, respiratory metabolism, and cell wall-multidrug resistance (MDR). 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 hyper-resistance to each molecule, thus identifying pathways that influence the cellular response to tested compounds [11]. Specifically, the identification of a compound providing a gain of function to a mutant harboring a specific gene deletion or a loss of function in the wild type that resembles phenotypically a known gene deletion allows association of a small molecule to a gene and a protein, and is of paramount importance

for target identification as the deletant strain that is not perturbed by the presence of such a compound is the one that lacks the gene encoding for the target of that molecule. Moreover, the correlation between a specific mutation and a specific substitution on the molecule may suggest connections between specific groups and biological effects.

In addition to the growth rate phenotype, gene expression has proven a valuable phenotype to assess the mechanism of action of small molecules, from the early experiments on rapamycin [12] and neocarzinostatin [13], to the more recent large toxicogenomics screens. Yet even when using gene expression to classify a small molecule, the use of clonal isogenic cultures of yeast provides a selective advantage improving the standardization of the genetic background, and the environmental conditions.

Among various classes of small molecules suitable for biomedical applications, peptidomimetics have shown great interest both in organic and medicinal chemistry. They 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.

The generation of peptidomimetics is basically focused on the knowledge of electronic and conformational features of the native peptide and its receptor or active site of an enzyme. Thus, the development of peptidomimetics as compounds with potential biological activity must take account of some basic principles [14]:

- replacement of peptide backbone with a non-peptide framework, if an amide bond does not change the biological activity or amide bonds are not exposed to the active site, then the template may be designed in order to eliminate peptide bonds;
- maintenance of peptide side chain involved in biological activity as they constitute the pharmacophore. In the development of second-generation mimetics, several modifications may be introduced in order to improve biological activity, i.e., chain length modification, introduction of constraints, cyclopeptide bond replacement with a covalent one, introduction of isosteric replacements, and so on [15];
- maintenance of flexibility in first-generation peptidomimetics: if a biological activity is observed for a flexible mimetic, then the introduction of elements of rigidity to side chains is a rational approach to improve the preliminary activity observed;
- selection of proper targets based on a pharmacophore hypothesis. In other words, the knowledge of the structure–activity relationship or the tridimensional structure of bioactive conformation is a promising route to rapidly achieve the best compound, without generating an enormous number of compounds with poor biological activity.

MORPHOLINE-BASED PEPTIDOMIMETICS

Our contribution to the development of methods for the identification of small-molecule modulators of protein function focused on the generation of peptidomimetic libraries from amino acid and sugar derivatives in a diversity-oriented fashion, and on the screening of such compound collections to yeast wild-type and deletant strains as a suitable model of the eukariotic cell. Specifically, we developed a high-throughput screening method based on the use of the model organism *S. cerevisiae*, and carried out a first round of screening on wild-type to select hit compounds having an effect toward cell growth (as

the phenotype) of wild-type yeast strain. Successively, the screening on selected yeast deletant strains to assess preliminary evidences about MDR and membrane permeability was addressed.

The generation of heterocycles using amino acid and sugar derivatives as building blocks is a powerful approach to access chemical and geometrical diversity thanks to the high number of stereocenters and the polyfunctionality of such compounds, and numerous examples in the literature report the use of such substrates to access combinatorial libraries of heterocyclic compounds [16]. We have been interested in the synthesis of heterocyclic compounds containing the morpholine ring, as among the various structures employed by medicinal chemists, this heterocycle represents a common motif [17]. For example, the morpholine moiety can be found in several bioactive molecules, such as MMP and TNF inhibitors [18], and it has been embedded in the structure of tricyclic benzodiazepines [19], and of 8,6-fused bicyclic peptidomimetic compounds as interleukin-1β converting enzyme inhibitors [20]. Thus, we conceived the application of selected building blocks from the chiral pool to develop a DOS strategy to access stereochemically rich and rigid morpholine-based heterocycles according to the build/couple/pair approach.

The strategic approach to the generation of morpholine scaffolds utilizes some α-amino acid derivatives as amino acetals (*path i*) or as β-amino alcohols (*path ii*) to be coupled with suitable building blocks possessing complementary functionalities in order to achieve structurally diverse heterocycles A–D after the cyclization step.

Bicyclic peptidomimetics

During the past few years, we reported the generation of bicyclic compounds from the combination of sugar or tartaric acid and amino acid derivatives through two key steps consisting of the coupling of two components from the chiral pool followed by an intramolecular cyclization step to achieve the bicyclic structure (Fig. 2, right), according to *path i* of Fig. 1 [21].

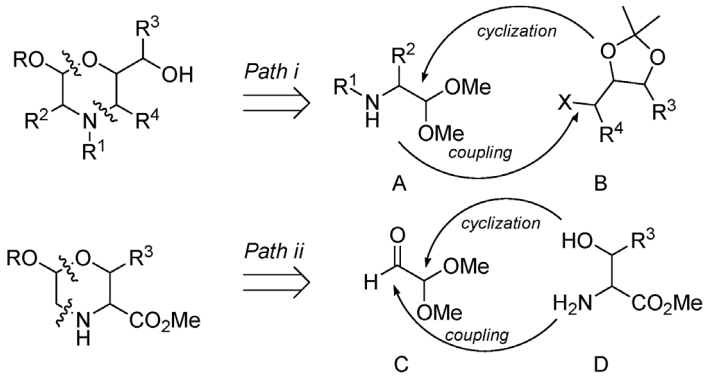


Fig. 1 Strategic approaches for morpholine scaffolds using selected building blocks for the coupling step.

This class of bicyclic compounds based on the 6,8-dioxa-3-azabicyclo[3.2.1]octane core showed structural similarity to dipeptides through an atom-by-atom correlation, as shown in Fig. 2, left. Such features are allowed for their application as suitable molecular platforms for peptidomimetic chemistry. Moreover, by tuning the starting compounds from the chiral pool and the cyclization process, it was possible to generate a wide array of molecules bearing such bicyclic moiety, also as constrained amino acids to be embedded in peptide structures (Fig. 3).

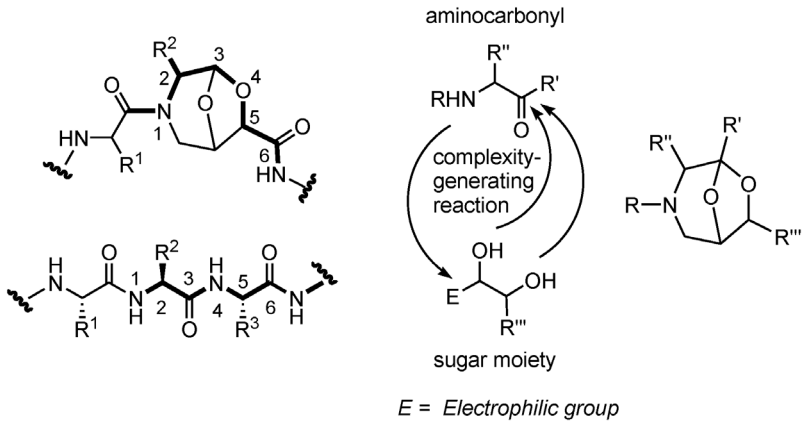


Fig. 2 Morpholine-derived peptidomimetic scaffolds.

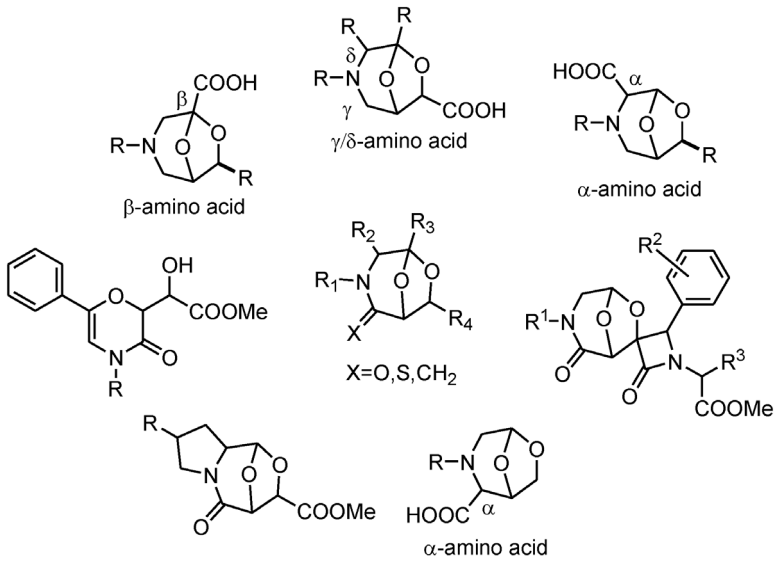
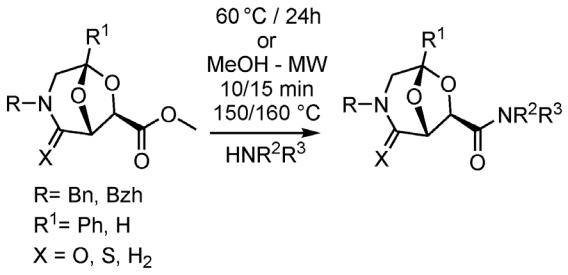


Fig. 3 Array of bicyclic amino acids and other rigid scaffolds.

Additionally, the facile functionalization of the activated carbomethoxy group at C-7 of the bicyclic structure enabled the generation of a large library of dipeptide isosteres by reaction with neat amines under conventional or microwave heating (Scheme 1) [22].

The collection of these dipeptide isosteres was applied as probes to dissect their effects on a panel of *S. cerevisiae* strains [23]. Specifically, we conceived the screening toward wild-type strains and selected deletants involved in MDR, and using cell growth as the phenotype of study. We selected a panel of strains of the budding yeast encompassing wild-type for respiratory function and MDR, two lab strains, W303, FY2609, and one wine strain, 0117. We probed the effect of the molecules on respiration by using strains altered in the respiratory function, BY4742 and FY2607, that harbor a mutation caused by a transposon in the *HAP1* coding sequence that reduces Hap1p function by 75 % [24]. This mutation was studied alone and in combination with other deficiencies in genes involved in cell wall and MDR (*Δerg6*, *Δsnq2*, *Δpdr3*).



Scheme 1

The effect of 16 compounds that induced a decrease between 10 and 20 % in wild-type genetic context was further tested on BY4742*Δerg6*, BY4742*Δsnq2*, and BY4742*Δpdr3* strains at a concentration of 0.3 mM, which is the minimum concentration that induced an effect on the wild-type strain. Molecules selected because of their significant effects on the wild-type strains at the concentration of 0.3 mM were tested on BY4742*Δerg6*, BY4742*Δsnq2*, and BY4742*Δpdr3* strains. This selection process through phenotype screening on yeast strains enabled the selection of library member **89**, which was identified as the molecule inducing the most intense cell growth decrease (more than 80 %). This peptidomimetic showed a negative variation in $O.D._{650}$ value of the culture of BY4742*Δerg6* negative due to cell death after 18 h of treatment at 0.3 mM concentration (Fig. 4).

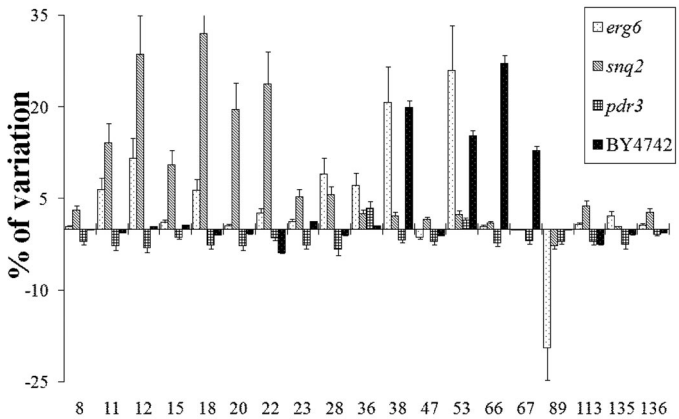


Fig. 4 Phenotype screening on yeast strains of selected peptidomimetic compounds.

By comparing **89** with other molecules of the library which differed in scaffold decoration at positions 3, 4, 5, and 7, 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 chain elongation at position 7. In fact, compounds **8–22** produced a growth increase in BY4742*Δsnq2* strain, compounds **38–67** of BY4742 wild-type strain, and compound **89** a marked inhibition of cell growth (Fig. 5). This behavior could be correlated to some structural features, as the first group lacked the carbonyl group at position 2 of the scaffold, whereas the second one possessed such moiety. Compound **89** possessed both the carbonyl function at position 2 and three aromatic rings around the scaffold, possibly conferring such marked activity toward yeast cell growth (Fig. 5).

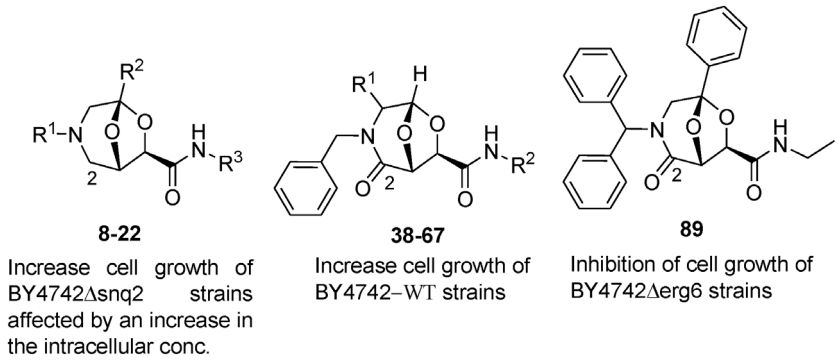


Fig. 5 Selected compounds showing significant modulation of yeast cell growth.

Further experiments demonstrated the influence of a functional *HAP1* gene in the performance of *S. cerevisiae* as a model system. The selected peptidomimetic **89** induces more severe effects on the wild-type W303 strain, having a functional *HAP1* gene, than on the laboratory BY4742 strain, with 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 **89** perturbation. The use of a pool of strains bearing deletions of genes involved in membrane assembly or function enabled the identification of effects that could be concealed by the membrane permeation incapability of the molecule. This is suggested by the opposite effect of **89** on yeast growing rate, which decreases for deletant strains, but increases for the wild-type strain. Transcriptional analysis indicated 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 **89** in inducing an increase in respiratory deficiency and anaerobic respiration was 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 was confirmed by an increase in ROS accumulation and mitochondrial membrane activation level. The pathway signature-based approach is a powerful tool for 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. The cell wall is a peculiar yeast structure, hence it is an excellent target for selective antifungal drugs. By taking advantage of all these findings, it was possible to identify **89** as a potential selective antifungal.

DOS library from morpholine acetals

In a second application of chemical genetics to drug discovery, we generated a new set of morpholine-based compounds to be screened toward yeast strains. We recently reported on the DOS of morpholine scaffolds taking advantage of a two-step process involving the combination of amino acid derivatives as building blocks, thus enabling a high degree of chemical diversity around such a heterocycle (Fig. 6) [25]. Also, the conformational analysis of peptides containing morpholine-3-carboxylic acid (Mor) suggested this molecule acts as an unexplored proline surrogate [26].

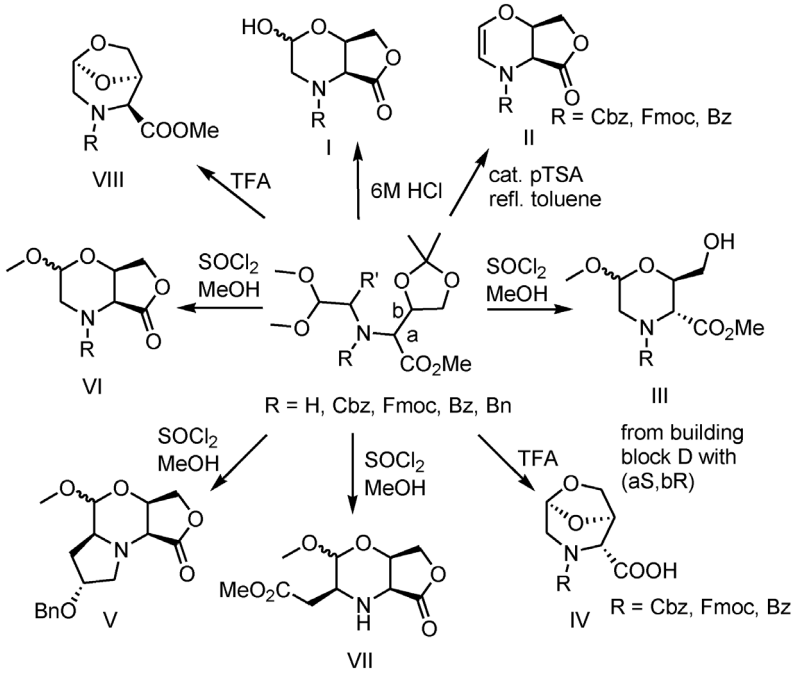


Fig. 6 Diversity-oriented synthesis of morpholine scaffolds.

We applied the reactivity of some morpholine acetals to give access to a library of morpholine-derived molecules, some of which also contained the 2,5-diketopiperazine nucleus, which in turn is considered a privileged scaffold in medicinal chemistry owing to the wide number of bioactive natural products and drugs containing such chemical entity [9]. A library of new chemical entities was successively applied for the systematic exploration of yeast deletants, so as to classify deletants as a function of bioactive molecules. Accordingly, further diversity was accessed by applying morpholine acetals in a one-pot multistep process giving skeletal diversity as a function of the reaction methodology (Fig. 7).

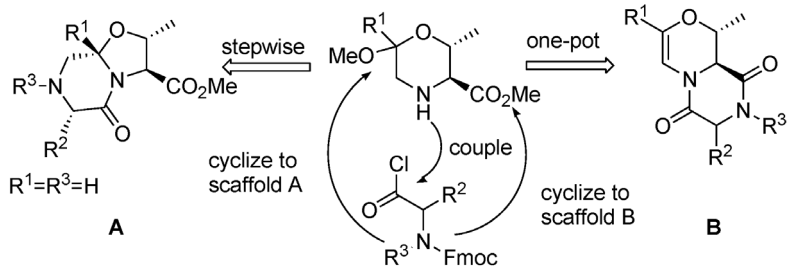


Fig. 7 Skeletal diversity by one-pot and stepwise routes.

In order to improve the profile of phenotype screening, the effect of the molecules toward yeast cell growth in both the exponential and stationary phases was assessed as a criterion for hit selection (Fig. 8) [27]. The effects of 48 morpholine-based library members were initially tested at 0.3 mM concentration on the BY4742 wild-type strain. Compounds responsible for the variation in cell generation

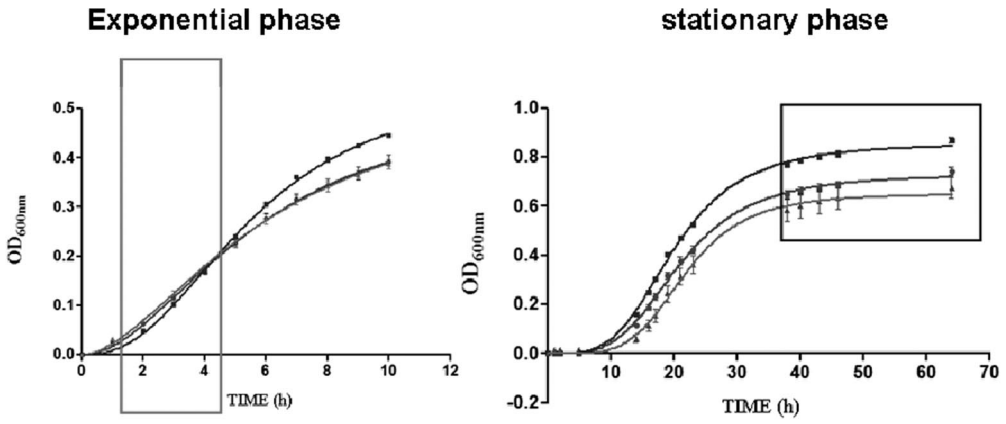


Fig. 8 Evaluation of effect of library members toward yeast cell growth in both the exponential and stationary phases.

time, which is the time interval required for a yeast cell to divide, or of the $O.D._{650}$ value of the stationary phase ($O.D._{st}$), or both, were selected for further characterization.

The screening on the wild-type strain allowed for the selection of 21 molecules inducing an $O.D._{st}$ and/or GenT variation in absolute value higher than 10 % or other interesting effects (e.g., decrease of the yeast fitness). Such compounds were thus tested for effects on the BY4742*Δerg6*, BY4742*Δsnq2*, and BY4742*Δpdr3* deletion strains bearing deficiencies in genes involved in cell wall and MDR, to allow for the identification of pathways that influence the cellular response to tested compounds. The bidimensional scatter plots proved to rapidly identify the library members displaying the most prominent activity toward selected deletant strains (Fig. 9).

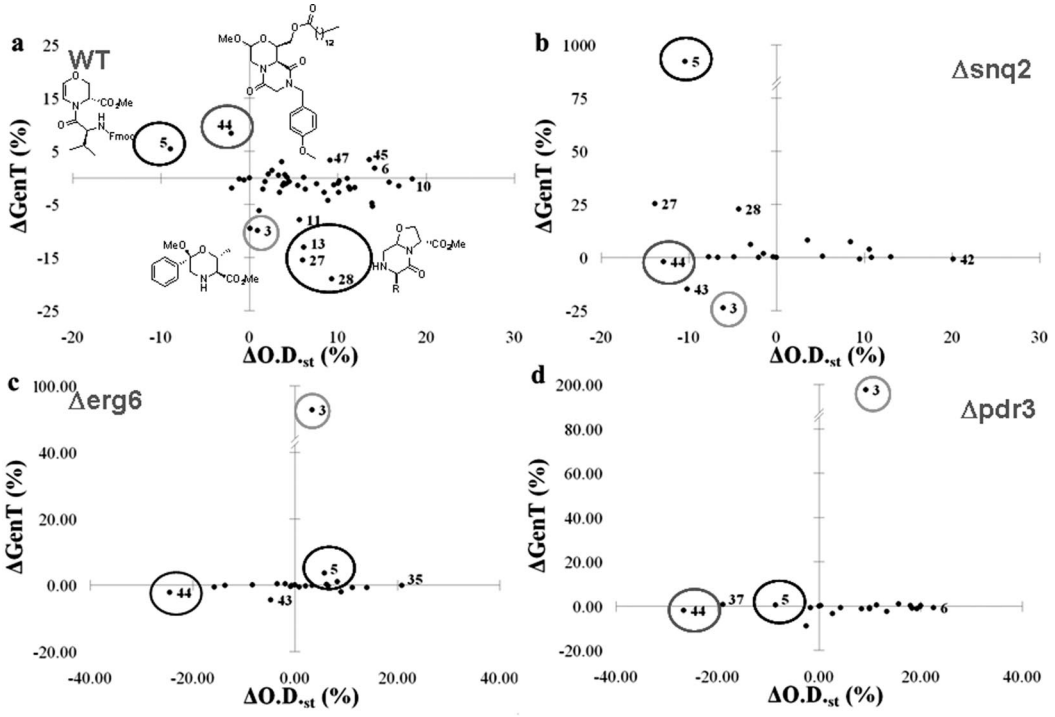


Fig. 9 Bidimensional plots showing effects of library members on cell growth of selected deletant strains.

Indeed, this second round of phenotype screening enabled the selection of eight molecules inducing $O.D._{st}$ and/or GenT variations higher than 10 % on at least two of the deletant strains were tested for mitochondrial membrane potential activation and peroxisomal proliferation, as both mitochondrial membrane activation and peroxisomal proliferation are indicators of the metabolic state of the cell, disclosing respiratory and fatty acid metabolism, respectively. Our results indicated molecules that show bright activities. One induced the lowest peroxisomal proliferation and a growth increase in *Δsnq2* and *Δpdr3* strains, thus enabling the indentification of a molecular instrument to gain further insight into the drug resistance mechanism and acquisition in mammal and yeast cells. The other hit compound showed killer activity only toward wild-type and *Δerg6* strains, thus paving the way to the use of a new chemical probe for assessing the mechanisms underlying the MDR of pathogenic cells with respect to normal cells.

CONCLUSION

In conclusion, chemical genetics, which relies on selecting small molecules for their ability to induce a biological phenotype or to interact with a particular gene product, is a new powerful tool for lead generation in drug discovery. In order to enable this approach to be effective in target and lead identification at the same time, a suitable phenotype screening and collections of highly diverse small molecules are needed. Specifically, peptidomimetics may represent a privileged set of compounds owing to the importance of peptide–-protein and protein–protein in biological processes. Thus, DOS of small-molecule peptidomimetics gives access to collections of new chemotypes bearing high structural diversity. Biological evaluation using cell growth as a phenotypic screening on *S. cerevisiae* deletant strains is a powerful tool to identify new chemotypes as hit compounds in the discovery of new antifungal and anticancer agents, and also in the dissection of their mode of action. We succeeded in developing a rapid and easy screening protocol on yeast deletant strains focusing on cell growth as the selected phenotype. The application of morpholine-containing peptidomimetic libraries to this drug discovery approach provided the identification of new chemotypes involved in mitochondria metabolism and respiration as hit compounds for more specific investigations. Our results in applying functional genomics to the screen show how the construction of databases enabling the assessment of similarity between gene expression profiles of yeast cells exposed to libraries of molecules holds the promise to build classifiers of small molecules for their biological properties using 6240 phenotypes, the RNA levels of all the yeast genes.

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