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Letter

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From dynamic combinatorial chemistry to *in vivo* evaluation of reversible and irreversible myeloperoxidase inhibitors

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KEYWORDS: Myeloperoxidase, reversible and irreversible inhibitors, dynamic combinatorial chemistry, molecular docking, kinetic study.

ABSTRACT: The implementation of dynamic combinatorial libraries allowed the determination of highly active reversible and irreversible inhibitors of myeloperoxidase (MPO) at the nM level. Docking experiments highlighted the interaction between the most active ligands and MPO and further kinetic studies defined the mode of inhibition of these compounds. Finally, *in vivo* evaluation showed that one dose of irreversible inhibitors is able to suppress the activity of MPO after inducing inflammation.

Neutrophils represent the first line of the human innate immune defense system by phagocytosing and killing invading pathogens.[1] Optimal antimicrobial action in neutrophils relies on the action of hypochlorous acid (HOCl), the product of the myeloperoxidase (MPO, EC 1.11.2.2)-hydrogen peroxide-chloride system.[2]

In certain inflammatory events, MPO and/or HOCl are released from neutrophils causing oxidative damage of host tissue and modification of biomolecules.[2,3,4] Consequently, MPO has become a new target for designing anti-inflammatory drugs.[5,6,7]

From a general point of view, the development of pharmacophores typically proceeds according to a conventional pathway, namely the structural design and synthesis of analogues from a "hit" molecule followed by the evaluation of structure-activity relationships.[6,8] However, this classical method is particularly costly and time consuming. Another innovative strategy consists in the generation and screening of a dynamic combinatorial library (DCL).[9] In the realm of dynamic combinatorial chemistry (DCC), DCL constitutes a rational alternative in drug discovery, opening thus new horizons for medicinal chemists. Indeed, the *in situ* reaction of simple building blocks is able to give rise to a wide range of new molecules through reversible covalent bond formation. In the last 10 years, this strategy allowed for the creation and the identification of ligands that specifically recognize targets such as proteins and nucleic acids.[10]

With this in mind, we decided to apply this approach in order to develop new irreversible inhibitors of MPO. Recently, we evaluated a new family of scaffolds, *i.e.* hydralazine[11] and isoniazid, endowed with the ability to inhibit MPO irreversibly but with high IC_{50} values (0.9 and 5 µM, respectively) (Figure 1). Keen to improve these substrates, we decided to take advantage of the high reactivity of hydrazine and hydrazide functionalities toward aldehyde partners in order to prepare and evaluate a library of ligands by a dynamic combinatorial strategy.

A set of aldehydes and hydrazine derivatives was selected to compose the building blocks as follows: group A contained aromatic aldehydes **1A-24A**, group B was comprised of aliphatic aldehydes **1B-15B** and group C consisted of hydralazine, isoniazid and some other hydrazines **1C-6C** (Figure 1). The selected aldehydes have a molecular weight (Mw) lower than 160 g/mol in order to achieve ligands with Mw < 320 g/mol since the active site of MPO

is located at the end of a narrow tunnel.[12] At first, the inhibitory ability of groups A and B was assessed against MPO, but none of the aldehydes had an activity at a 1 µM concentration. In contrast, hydrazines of group C were capable of inhibiting 61% of MPO activity at 1 µM. Next, more efficient ligands were designed according to a dynamic combinatorial approach. In substance, MPO was incubated with two mixtures A-C and B-C composed of 1 µM of each building block A/C and B/C, respectively. From this, the complete suppression of activity of MPO (>96%) using both libraries A/C and B/C (Figure 2, step 1) was observed.

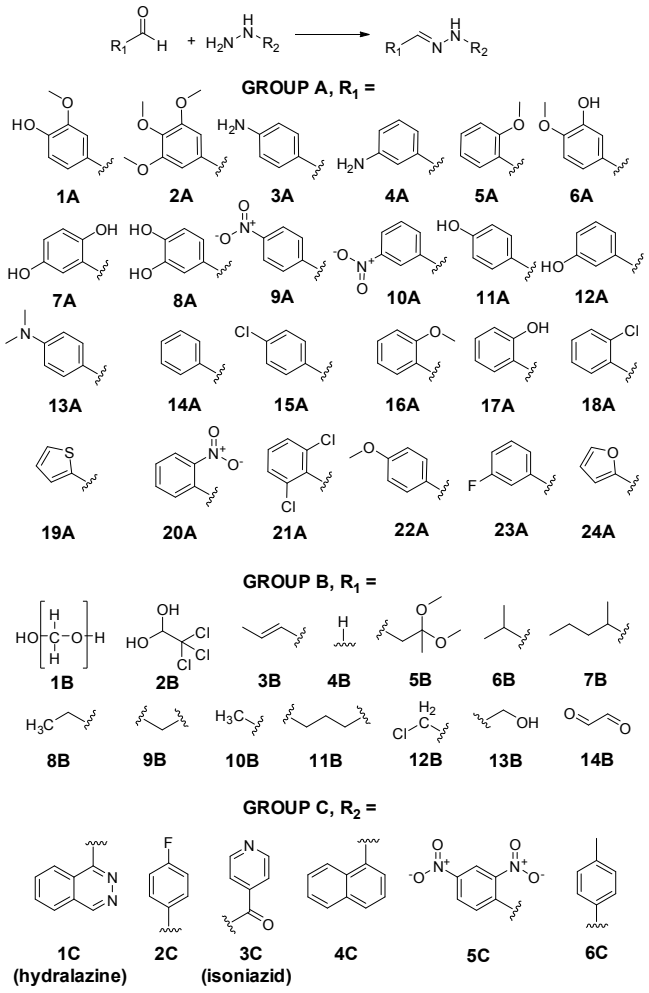


Figure 1. Structures of aromatic aldehydes **1A-24A**, aliphatic aldehydes **1B-14B** and hydrazine/hydrazide derivatives **1C-6C**.

The results clearly indicated that new scaffolds can be formed and that the resulting inhibitors have a good affinity toward MPO, even better than the hydrazines of group C (Figure 2). A step further, a new experiment was set up in order to determine the best aldehyde/hydrazine partners which cause the highest inhibitory effect. First, in a 96-well plate, each aldehyde A and B (1 µM each) was challenged with all hydrazines of group C through DCC in the presence of MPO. The resulting DCLs highlighted an increased inhibitory activity in most cases, but ligands obtained from vanilline **1A**, 3-hydroxy-4-methoxybenzaldehyde **6A**, 4-dimethylaminobenzaldehyde **13A** and glycolaldehyde **13B** provoked a high inhibition of the enzyme (>82%). Therefore, potent inhibitors of MPO were formed from these building blocks. Subsequently, the remaining experiments have focused on the determination of the best aldehyde/hydrazine couple by the reaction of each hydrazine of group C (1µM) with each aldehyde **1A**, **6A**, **13A** and **13B**. It could be demonstrated that hydralazine **1C**, 4-fluorophenylhydrazine **2C** and isoniazid **3C** (Figure 2) gave rise to scaffolds with a high inhibitory effect toward MPO (>82%), but the hydrazone derivative **13A-1C** was able to suppress the activity of MPO at 100% (Figure 2, step 2).

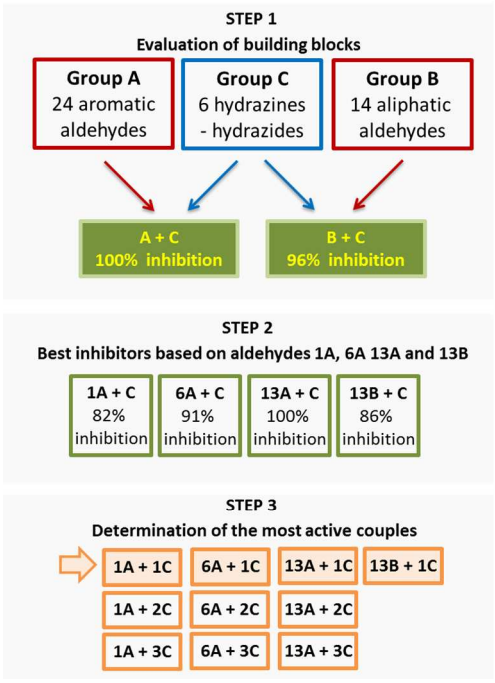


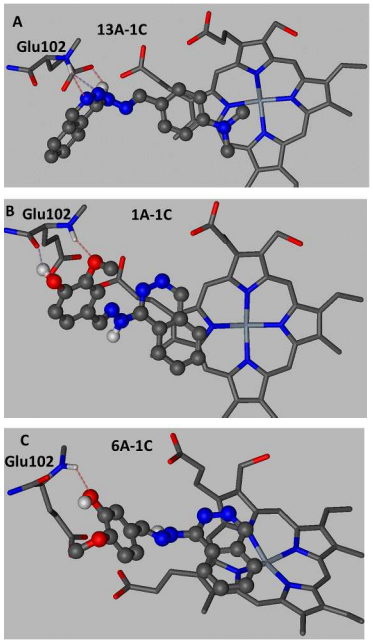
Figure 2. Determination of the most active inhibitors of MPO by dynamic combinatorial chemistry using aromatic aldehydes (group A), aliphatic aldehydes (group B) and hydrazine derivatives (group C).[13]

Table 1. Values of IC_{50}, free energies of binding ΔG predicted from docking experiments and residual activity of MPO after diluting 100 times the active hydrazone compounds. IC_{50} values are given as mean ± SD, n = 3.

Code	IC_{50} (µM)	ΔG (kcal/mol)	Residual activity of MPO without H_2O_2	Residual activity of MPO with H_2O_2
1C	0.90±0.2	-9.3	92%	19%
1A-1C	0.34±0.07	-17.1	87%	22%
6A-1C	0.15±0.04	-18.4	93%	25%

13A-1C	0.08±0.03	-23.2	93%	92%
13B-1C	0.11±0.06	-19.0	82%	18%
2C	>5	-7.6	103%	98%
1A-2C	1.2±0.1	-15.6	89%	98%
6A-2C	1.13±0.05	-17.5	81%	92%
13A-2C	3.1±1.0	-12.9	87%	84%
13B-2C	>5	-8.9	107%	97%
3C	4.7±1.1	-9.4	98%	64%
1A-3C	0.46±0.12	-15.4	96%	91%
6A-3C	0.43±0.16	-15.6	88%	87%
13A-3C	1.62±0.4	-11.2	91%	93%
13B-3C	>5	-12.4	100%	94%

In order to prevent any bias in the previous DCL results, the correlation between the increased inhibitory activity and the hydrazone content was investigated by ^{1}H NMR. Hence, equimolar mixtures of complementary randomly-chosen active (**13A-1C**) and inactive (**10A-1C** and **17A-4C**) building blocks, were incubated in the presence of MPO. After 15 min, the disappearance of the aldehyde peak (C*H*O) and the increase of hydrazone proton (C*H*=N-) clearly suggested the formation of hydrazones in variable amounts (5-13%), this being observed both for active and inactive couples. More importantly, the activity appeared independent of the hydrazone quantity. Note that in all instances, the low conversion rate in hydrazone provides a sufficient amount of compound for a complete suppression of activity. As a consequence, this experiment confirmed the formation of active and inactive pairs, consistent with those obtained by DCC.



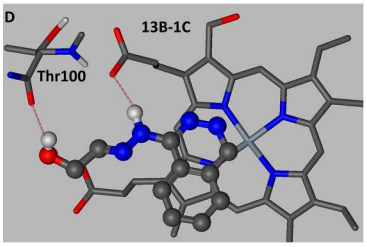
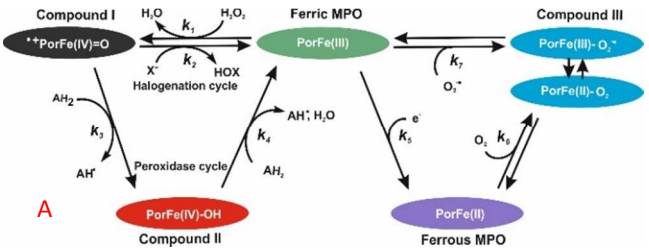


Figure 3. Comparison of the best-scored docking poses of hydrazones **13A-1C** (A), **1A-1C** (B), **6A-1C** (C) and **13B-1C** (D) derived from hydralazine **1C** (left). Structures of ligands **13A-1C**, **1A-1C**, **6A-1C** and **13B-1C** (right).

Next, the active hydrazones were synthesized in order to determine their level of activity. Under a classical condition, the reaction between equimolar amounts of hydrazine and aldehyde derivatives proceeded in refluxing ethanol for 4 hours. After treatment, the resulting hydrazones were recovered in excellent yields (>90%) as solids. Subjecting MPO to these ligands allowed the confirmation of the previous results, *i.e.* the best inhibitors were derived from hydralazine **1C** and the lowest IC_{50} value (79 nM) was obtained with compound **13A-1C** (Table 1). Although couples **13A-1C** and **13B-1C** are endowed with good inhibitory effects toward the enzyme, the association of aldehydes **13A** and **13B** with hydrazines **2C** and **3C** gave rise to moderate and weak inhibitors (IC_{50} value > 1.60 µM).

These encouraging results have convinced us to implement a comprehensive study of the inhibitory activity by molecular docking experiments. A comparison of binding prediction for active hydrazones **1A-1C**, **6A-1C**, **13A-1C** and **13B-1C** and starting hydrazine **1C** highlighted additional interactions assigned to the structural features of the aldehydes (table 1). Hence, methoxy and hydroxy functions of **1A** and **6A**, respectively, made hydrogen bonds with Glu102 which plays a pivotal role in the interaction with the inhibitor (see ESI). Moreover, **13A-1C** is doubly bonded to Glu102 through phtalazine and NH groups of **1C**.



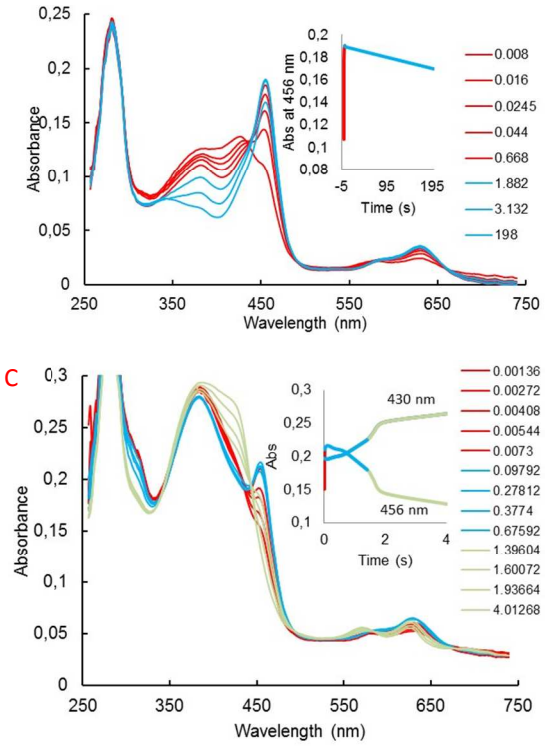


Figure 4. Reactions catalyzed by human myeloperoxidase. In the halogenation cycle **Compound I** is reduced by halides (X^-) directly to the ferric state [thereby releasing hypohalous acids (HOX)], whereas in the peroxidase cycle Compound I is reduced in two one-electron steps via **Compound II** to the resting state (A). Reaction of MPO Compound I with 20 µM **13B-1C** (B). Reaction of MPO Compound I with 500 µM **13B-1C** (C). Red spectra correspond to Compound II formation; light blue spectra correspond to Compound III formation and light green spectra show the decay to incomplete ferric MPO. Insets show the time traces at 430 and 456 nm.

Compounds **1A-1C**, **6A-1C** and **13B-1C** were predicted to stack on the active site of MPO through the aromatic ring of hydralazine, as seen on figure 3. In contrast, the docking pose with ligand **13A-1C** emphasized an interaction involving the aromatic group of **13A** (Figure 3A).

For the sake of comparison, additional docking experiments were carried out with ligands based on pharmacophores **2C** and **3C**. Interestingly, all ligands derived from isoniazid **3C** make a π-π stacking with the heme of the enzyme through the pyridyl group of **3C**. In opposite, hydrazones formed from **2C** showed stacking poses with the π-system of the aldehydes, except for **13A-2C** since no interaction was observed (see ESI). According to the predicted models, molecules bearing both aromatic and polar functionalities have a greater binding affinity toward MPO and therefore a higher inhibition ability.

Finally, we elucidated the mechanism of action of these hydrazones and their capacity to act as irreversible inhibitors. These experiments were performed in the presence and absence of hydrogen peroxide, which is necessary to initiate the catalytic cycle of MPO. In practice, mixtures of ligands and enzymes were diluted 100-fold, followed by measurement of the residual enzymatic activity. In general, in the absence of H_2O_2, inhibitors provoked inactivation up to 19%, whereas hydrogen peroxide alone minimally affected the activity (table 1). In the presence of hydrogen peroxide **13A-1C**, and **2C**-, **3C**-based ligands the activity decreased by 10-15%. By contrast, **1A-1C**, **6A-1C** and **13B-1C** were able to cause an almost complete inhibition of MPO in the presence of hydrogen peroxide (residual activity in the range 18-25%, table 1). Interestingly, these levels of inhibition correlated well with the predicted models. Indeed, the docking poses of hydralazine- and isoniazid-based hydrazones underline the important role played by the nitrogen heterocycles of **1C** and **3C**, with hydralazine being a better inhibitor than isoniazid. Hence, in most of the molecules, the aromatic groups governed interactions with the heme but additional contacts due to aldehyde moieties seemed to lock the system. This aspect is probably responsible for the higher affinity and inhibition rate induced by ligands derived from hydralazine. Moreover, the variation of inhibitory effect between **1A-1C**, **6A-1C**, **13A-1C** and **13B-1C** might be reflected by the distance between hydralazine and heme groups (Figure 3). Hydrazone **13A-1C** acted as a reversible inhibitor while **1A-1C**, **6A-1C** and **13B-1C** were irreversible.

The mechanism of MPO inhibition was subsequently investigated by the multi-mixing stopped-flow technique. Native ferric MPO [Fe(III)···Por] is oxidized (k_1) by H_2O_2, producing water and **Compound I** {oxoiron(IV) combined with a porphyrin cation radical: [$^{+\bullet}$PorFe(IV)=O]} (Figure 4). In the halogenation cycle, **Compound I** is directly reduced back to the resting state (k_2) by chloride [or other (pseudo)halides], thereby releasing hypochlorous acid. Alternatively, in the presence of one-electron donors, the peroxidase pathway is followed, including **Compound I** reduction to **Compound II** [PorFe(IV)–OH] (k_3), and **Compound II** reduction to the ferric state (k_4) (Figure 4).[14] Here, **Compound I** reduction was evaluated with the following ligands: **1A-1C**, **13A-1C** and **13B-1C**. In all cases, a direct and fast transition of **Compound I** to **Compound II** (Soret maximum at 456 nm) was observed with clear isosbestic points (see Figure 4 middle ESI). The determined values of k_3 were 2.8×10^5, 7.1×10^5, and 3.3×10^5 M^{-1} s^{-1} for **1A-1C**, **13A-1C** and **13B-1C**, respectively. The results clearly indicated that all selected molecules behaved as good one-electron donors of **Compound I** reacting similar to hydralazine alone (k_3= 7.1×10^5 M^{-1} s^{-1}). In contrast, the reaction of the tested molecules with **Compound II** gave variable results. Hydrazone **13A-1C** showed a direct transition of **Compound II** to ferric MPO (Soret maximum at 429 nm) with clear isosbestic points, but this reaction was slow. The apparent bimolecular rate constant (k_4) was found to be 89.3 M^{-1} s^{-1}, indicating a high k_3/k_4 index of 7951. This high index suggested that inhibitor **13A-1C** induced the (reversible) accumulation of **Compound II**, which is outside the halogenation cycle (Figure 4). By contrast, during the reactions of hydralazine and substrates **1A-1C** and **13B-1C** with **Compound II**, a steady-state shift to **Compound III** was observed (Figure 4 bottom). **Compound III** can only be formed from ferric or ferrous MPO with activated oxygen

(k_7) or dioxygen (k_6), respectively (Figure 4). It seems that the substrate radicals ($AH^{\bullet}$, Figure 4) which are generated during **Compound I** and **II** reduction react with ferric MPO and reduce it to the ferrous state (k_5). Alternatively, substrate radicals could activate dioxygen and the resulting superoxide reacts with ferric MPO to generate **Compound III**. Interestingly, only partial recovery of ferric MPO after complete consumption of hydrogen peroxide was observed, suggesting that these molecules might act as suicide inhibitors (see ESI). This kinetic study supports previous findings which demonstrated that ligands **1A-1C**, and **13B-1C** inhibits MPO irreversibly, while substrate **13A-1C** behaves as a reversible inhibitor.

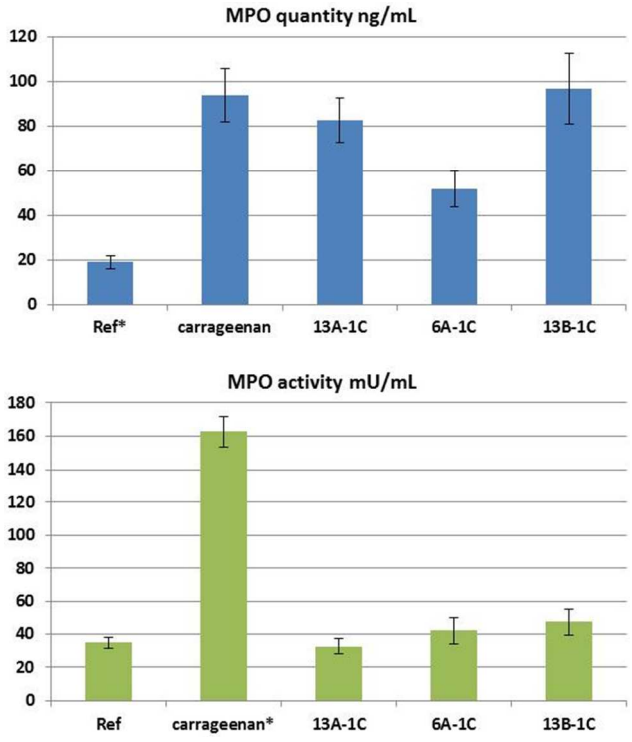


Figure 5. Determination of MPO concentration collected in the peritoneal liquid of rats. (up). Measurement of MPO activity collected in peritoneal liquid after 48h of drug administration (down). (*) MPO concentration in ref group is significantly lower than the other groups, and the activity of MPO in the carrageenan group is significantly higher than in the other groups (P<0.001, Shapiro–Wilk test).

Finally, the inhibition of MPO was tested *in vivo* using hydrazones **6A-1C, 13A-1C** and **13B-1C**. After inducing inflammation in Wistar Han male rats by intraperitoneal injection of carrageenan, 10 mg/kg of the selected ligands were injected intravenously.

24h after drug administration, a slight decrease in enzyme release with molecule **6A-1C** was measured. As concerns **13A-1C** and **13B-1C**, the concentration of MPO was similar to that found in rats treated only with carrageenan (Figure 5).[15] A dramatic decrease of MPO activity was observed with compounds **6A-1C**, **13A-1C,** and **13B-1C**. When **13A-1C** was used, the enzyme activity dropped to the same level as measured for reference rats (untreated with carrageenan). After 48h, MPO was collected in the peritoneal liquid and its activity was determined for all groups of rats. When **13A-1C** was administered, the enzyme recovered its activity, whereas the enzyme activity remained inhibited with **6A-1C** and **13B-1C** to a large extent (Figure 5).

In summary, new potent reversible and irreversible inhibitors of MPO were developed through the implementation of dynamic combinatorial libraries. Starting from a series of aldehydes and hydrazines, a three-steps procedure allowed to select four couples (**1A-1C**, **6A-1C, 13A-1C** and **13B-1C**) which demonstrated high activity as individual ligands, the lowest IC_{50} value (79 nM) being attained with compound **13A-1C**. Docking predictions highlighted the interaction between the hydralazine-based substrates and MPO, and further mechanistic investigations correlated their mode of inhibition with the predicted models. According to the kinetic study, **1A-1C**, **6A-1C,** and **13B-1C** belong to the class of irreversible inhibitors while ligand **13A-1C** suppresses the activity reversibly. Hence, **13B-1C** features the lowest IC_{50} values reported to date for an irreversible MPO inhibitor. At last, *in vivo* evaluation demonstrated that one dose of irreversible inhibitors is able to suppress the activity of MPO released upon proceeding inflammation.

ASSOCIATED CONTENT

Supporting Information

Experimental data, kinetic experiments, *in vivo* tests and docking poses.
The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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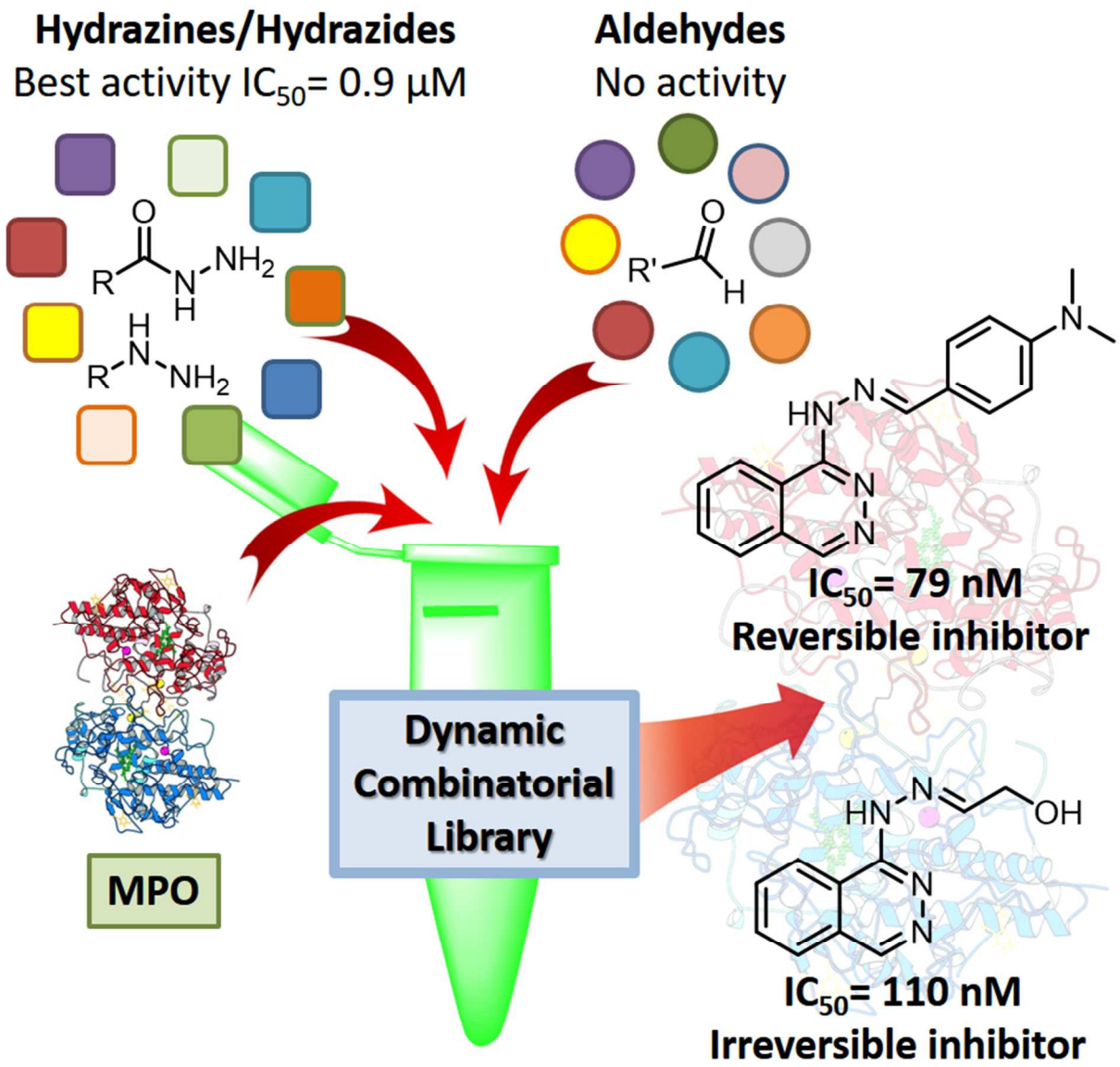
Hydrazines/Hydrazides
Best activity IC50= 0.9 µM
Aldehydes
No activity
MPO
Dynamic Combinatorial Library
IC50= 79 nM
Reversible inhibitor
IC50= 110 nM
Irreversible inhibitor