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# The development of molecular clamps as drugs

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**Some enzymes catalyze the modification of an ensemble of substrates *in vivo* and, as a consequence, are not ideal targets for active-site-directed drugs. One solution to inhibiting such multisubstrate enzymes would be a drug that binds tightly to only one substrate, which prevents the binding of that substrate to the enzyme. Ideally, such a drug (called a molecular clamp, a molecular forcep or a molecular tweezer) would prevent the enzymatic processing of only the targeted substrate. This would enable the enzyme to function normally on all other substrates. Here, we review the unique steady-state kinetic features of molecular clamp inhibition, identify potential targets for molecular clamp inhibition, and discuss problems for the therapeutic use of molecular clamps.**

The canonical strategy for the development of drugs that inhibit enzyme activity is to design a molecule that binds to the targeted enzyme to form a binary enzyme•inhibitor complex of decreased activity. The inhibition mediated by such enzyme-targeted inhibitors can be either competitive, targeting the enzyme active site, or noncompetitive, targeting an allosteric site. A conceptually similar approach has been used to develop receptor-targeted drugs that inhibit the function of targeted receptors. This strategy has led to multiple successes in drug discovery and continues to do so, as exemplified by recent reports of inhibitors of the cyclin-dependent kinases [1] and the retinoic acid receptors [2].

However, the development of enzyme-targeted inhibitors is not appropriate in all cases because some enzymes have more than one substrate *in vivo*. The broad inhibition of multisubstrate enzymes is likely to have undesirable effect side effects as a consequence of blocking reactions not involved in the disease being treated. One example is the inhibition of K-Ras prenylation. Inhibition of K-Ras prenylation is an attractive anticancer therapy [3]; however, the use of prenylation enzyme inhibitors is toxic to the point of lethality [4], most likely resulting from the nonprenylation of other proteins.

One possible solution to this problem would be the design of a substrate-targeted inhibitor that binds to the substrate rather than the enzyme. Rather than having the inhibitor compete with the substrate for the enzyme active site (or an activator binding to an allosteric site), the inhibitor competes with the enzyme for the substrate – analogous to antibodies binding to their antigens. The inhibitor binding to substrate would prevent the targeted substrate from binding to the enzyme, leaving the enzyme free to act upon all other substrates (Figure 1).

## Precedence for the use of molecular clamps as drugs

One class of well known and clinically useful substrate-targeted molecular clamps is the glycopeptide antibiotics, the prototypical example of which is vancomycin [5,6]. Vancomycin was first identified in the 1950s [7] and is used to treat serious Gram-positive bacterial infections that are resistant to other antibiotics [8]. Research carried out in the 1960s indicated that vancomycin most likely functioned by binding the D-Ala-D-Ala terminus of Lipid II [9,10] and, thus, prevented the final maturation of the peptidoglycan layer in the bacterial cell wall. The recent availability of adequate amounts of Lipid II and the requisite peptidoglycan biosynthetic enzymes have clearly demonstrated that vancomycin and several the other lantibiotic, glycopeptide and lipoglycopeptide antibiotics are all Lipid II binders [11–16]. However, it is also clear that the activity of some of these antibiotics might be related to their direct interaction with the peptidoglycan biosynthetic enzymes [6,12]. Bacitracin, a cyclic peptide antibiotic, also shuts down bacterial cell wall biosynthesis as a substrate binder. In this case, bacitracin binds tightly to $C_{55}$-isoprenyl

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1359-6446/06/$ - see front matter.  doi:10.1016/j.drudis.2006.07.011

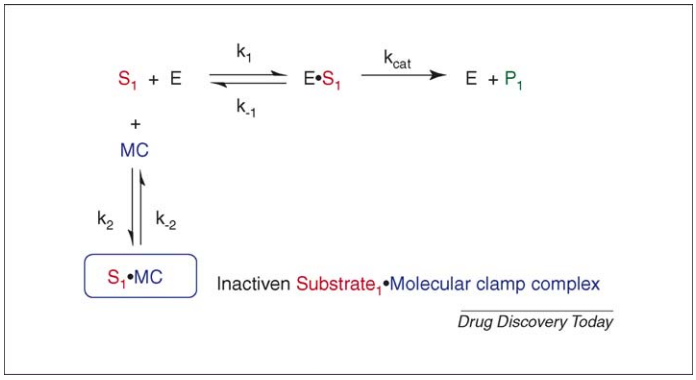


**FIGURE 1**

**The molecular-clamp-mediated inhibition of the enzymatic processing of one specific substrate ($S_1$).** The binding of the molecular clamp (MC) to $S_1$ only inhibits the formation of $P_1$. All other substrates processed by the enzyme ($S_2$, $S_3$, $S_4$, etc) are converted without any inhibition by the molecular clamp.

pyrophosphate and prevents its enzymatic dephosphorylation to $C_{55}$-isoprenyl phosphate [17].

The clinical use of antibodies [18] also provides some precedence in considering molecular clamp as drugs. The most common clinical use of antibodies is to bind the ligand of a receptor, such as the anti-TNF-α antibodies, Remicade® and Humira® [19,20]. Although therapeutic use of antibodies has multiple advantages, including high specificity, long half-life and lack of resistance, antibodies also have serious disadvantages in that they can be immunogenic [21,22] or toxic, and they have limited ability to cross the blood–brain barrier or to penetrate solid tumors [18,23].

## Potential targets for molecular clamps as drugs

The best targets for the development of molecular clamps as drugs would be receptors, enzymes and regulatory proteins that have a diversity of ligands, substrates, and/or binding partners *in vivo*. In addition, molecular clamps would be most useful to inhibit substrates that are not present in high concentrations. Some potential targets for molecular clamp drugs could include kinases, phosphatases, proteases, hydrolases, protein-modifying enzymes and protein–protein interactions. Another consideration would be the affinity of the protein for the ligand of interest. Because the protein and the molecular clamp are competing for the same ligand, a molecular-clamp-based drug is probably best targeted against ligands that bind to their protein partners with $K_d$ or $K_M$ values >1–10 μM. For example, the $K_M$ for Lipid II binding to *Escherichia coli* transglycosylase (PBP1b) is 2 μM, whereas the $K_d$ for the binding of the vancomycin dimer to Lipid II is 0.8 μM [24]. The formation of vancomycin dimers is important for its antibiotic activity [25].

Proteases and enzymes catalyzing either the cleavage or post-translational modification of peptides and proteins are particularly attractive targets for molecular clamps [26]. Defects in these reactions correlate to serious human health problems, such as cancer, arthritis, inflammation and cardiovascular disease [3,27–31]. However, because protein- and peptide-modifying enzymes usually have a family of substrates *in vivo*, the general inhibition of this class of enzymes with a protein-targeted inhibitor is likely to be toxic. As explained previously, the toxicity of protein-targeted inhibitors against farnesyl transferase [4] and protein kinases [32] illustrate the problem. The specific inhibition of the cleavage or modification of a disease-linked peptide or protein with a molecular clamp could provide an exciting new clinically useful therapeutic.

There are reports of molecular clamp inhibitors for enzymes catalyzing protein modification. One is a substrate-targeted inhibitor that blocked the farnesylation of H-Ras by farnesyl transferase [33], and a second inhibited the proteolysis of human pro-interleukin-1β by interleukin-converting enzyme [34]. Both of these molecular clamps were peptide-based and bound relatively weakly to the targeted substrates, with $IC_{50}$ values of 100–150 μM. These reports of successful molecular clamps are exciting, despite the relatively low affinity for their target substrates, because they demonstrate the feasibility of inhibiting the cleavage and/or modification of a specific protein. Furthermore, they relied on combinatorial strategies to select for peptide-based molecular clamps. These reports, in combination with other methods for the development of amino acid sequence selective binding molecules [35–38] (some that exhibit $K_d$ values of 100 nM [36]), strongly suggest that molecular clamp inhibitors could prove useful for the inhibition of disease-related aberrations in protein cleavage or modification.

Molecular clamps could also prove useful as inhibitors of protein–protein interactions, particularly in cell signaling pathways where one regulatory protein can interact with a set of binding partners. Regulator of G-protein-signaling (RGS) proteins negatively regulate G-protein-receptor signaling by activating the GTPase activity of the $G_\alpha$ subunits [39,40]. Most RGS proteins can bind multiple $G_\alpha$ isoforms, have functions that are independent of their ability to stimulate GTPase activity, and might have a role in cardiovascular disease [40,41]. A molecular clamp targeted against a particular RGS-binding partner that leaves the RGS protein free to bind to other partners could be a valuable therapeutic. In a recent review, Yin and Hamilton [42] discuss the strategies necessary to develop small molecule and peptidomimetics to create inhibitors of protein–protein interactions. Given the combinatorial methods for the generation of compounds that will bind to specific amino acid sequences and other work on the creation of artificial receptors for selective peptide binding [35–38,43–47], it seems likely that molecular clamps to inhibit a specific protein–protein interaction will be produced.

## Steady-state kinetic evaluation of molecular clamp inhibitors

Molecular clamp binding to substrate to form the binary inhibitor•substrate complex, S•MC, will decrease the concentration of substrate available to the enzyme-of-interest, resulting in a decrease in the rate of product formation [48] (Figure 1). In the absence of the molecular clamp, the dependence of the initial velocity of product formation, v, on the initial concentration of substrate, [S], is given by Equation 1.

$$v = (V_{MAX}[S])/(K_M + [S]) \quad \text{[Eqn 1]}$$

The presence of the molecular clamp will decrease the concentration of free substrate, $[S]_{free}$, in a manner that is dependent on the concentration of the molecular clamp, [MC], as given in

Equation 2 [48]. The total substrate concentration, $[S]_{total} = [S]_{free} + [S{\bullet}MC]$.

$$[S]_{free} = 1/2[\{([MC]_{total} - [S]_{total} + K_{MC})^2 + 4K_{MC}[S]_{total}\}^{0.5} - ([MC]_{total} - [S]_{total} + K_{MC})] \quad \text{[Eqn 2]}$$

The dissociation constant for the molecular clamp•substrate complex is $K_{MC} = k_{-2}/k_2$ (Figure 1). The rate expression for a protein-targeted competitive inhibitor, I, is given in Equation 3 with the $K_{i,s}$ representing the dissociation constant for the enzyme•inhibitor complex, E•I.

$$v = (V_{MAX}[S])/(K_M + [I]\{K_M/K_{i,s}\} + [S]) \quad \text{[Eqn 3]}$$

The sigmoidal relationship between v and [S], as a function of the molecular clamp concentration, [MC], is evident in Figure 2. Sigmoidal v versus [S] curves have been reported for the inhibition of transglycosylase by the glycopeptide [15] and lipoglycopeptide [12] antibiotics. Dixon plots of v versus [MC] at a fixed concentration of substrate are also sigmoidal (Figure 3); however, the sigmoidal nature of the curve is subtle if the molecular clamp binds to the substrate with a relatively low affinity (see the orange curve in Figure 3 when $K_{i,s}$ = 2.0 μM). For comparison, the Dixon plot for a protein-targeted competitive inhibitor with a $K_{i,s}$ = 0.8 μM = $K_{MC}$ is included in Figure 3 (dashed green line). The shape of the two curves is approximately the same, but the degree of inhibition caused by the molecular clamp at any given concentration is less than that observed for the protein-targeted competitive inhibitor.

Enzyme inhibition data are often depicted using a linear form of Equation 3, the most common representation being a double-reciprocal plot of 1/velocity versus 1/[substrate]. The inhibition resulting from a protein-targeted competitive inhibitor yields a set of lines that intersect on the 1/v intercept, with the slope increasing as [I] increases [48]. A 1/v versus 1/[substrate] plot for a substrate-targeted molecular clamp is shown in Figure 4. This plot appears deceptively like a plot for a protein-targeted competitive inhibitor; however, deviation from linearity is apparent at high [substrate] (or low 1/[substrate]), as indicated in the inset to Figure 4.

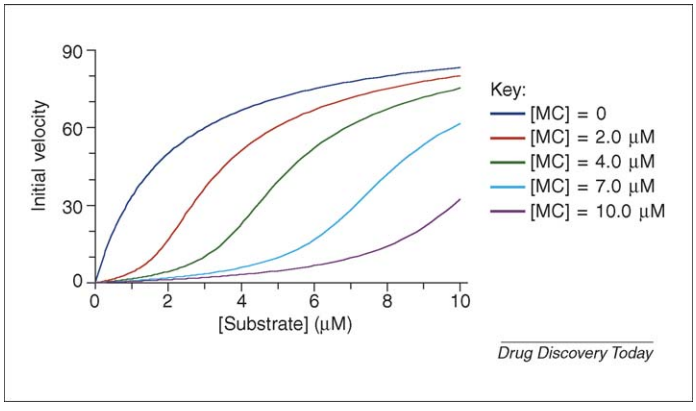


**FIGURE 2**

**A plot of initial velocity versus substrate concentration at increasing concentrations of a molecular clamp**. The curve at [MC] = 0 was generated using Eqn 1 with the $K_M$ = 2.0 μM and the $V_{MAX}$ = 100 arbitrary units. The curves at [MC] = 2.0 to 10 μM were generated by first solving for $[S]_{free}$ using Eqn 2 with $K_{MC}$ = 0.1 μM and then using the $[S]_{free}$ concentration in Eqn 1 to calculate the initial velocity with the same values for $K_M$ and $V_{MAX}$ as when [MC] = 0.

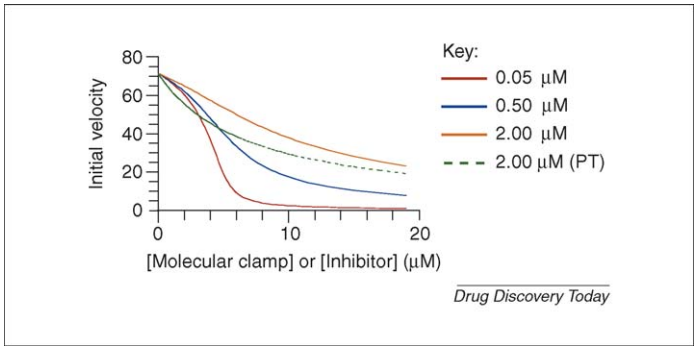


**FIGURE 3**

**Dixon plots for the inhibition mediated by a molecular clamp**. Initial velocity values were calculated at one fixed substrate concentration, $[S]_{total}$ = 5.0 μM, at different concentrations of the molecular clamp, [MC] = 0.05–2.00 μM and at increasing values of the $K_{MC}$ = 0.05 to 2.0 μM. The concentration of $[S]_{free}$ was calculated at specific values for [MC] and $K_{MC}$ according to Eqn 2 and the resulting $[S]_{free}$ used in Eqn 1 to generate initial velocity value with $K_M$ = 2.0 μM and $V_{MAX}$ = 100 arbitrary units. For comparison, a Dixon plot for a protein-targeted competitive inhibitor is also shown (dashed green line labeled PT Comp). This line was calculated using Eqn 3 using the following values: $K_{i,s} = K_M$ = 2.0 μM and $V_{MAX}$ = 100 arbitrary units. The box in the figure shows the values of the $K_{MC}$ or $K_{i,s}$ used to construct the Dixon plots.

The double reciprocal plot of Figure 4 also indicates that the extent of inhibition caused by the substrate-targeted molecular clamp is less than that expected for a protein-targeted competitive inhibitor of equal potency. This is manifested in Figure 4 by the relatively high 1/v values at high 1/[substrate] as molecular concentration clamp increases, but is best illustrated in Figure 5 by comparing the residual velocity at increasing molecular clamp concentration or competitive inhibitor concentration at fixed substrate concentration (= $1/2K_M$) with $K_{MC} = K_{i,s}$ = 0.1 μM. The molecular clamp is less effective at inhibiting the enzyme at inhibitor concentrations below 1.0 μM inhibitor (= 10 × $K_{MC}$ or $K_{i,s}$). Even at 10 μM inhibitor or molecular clamp, 100 × $K_{MC}$

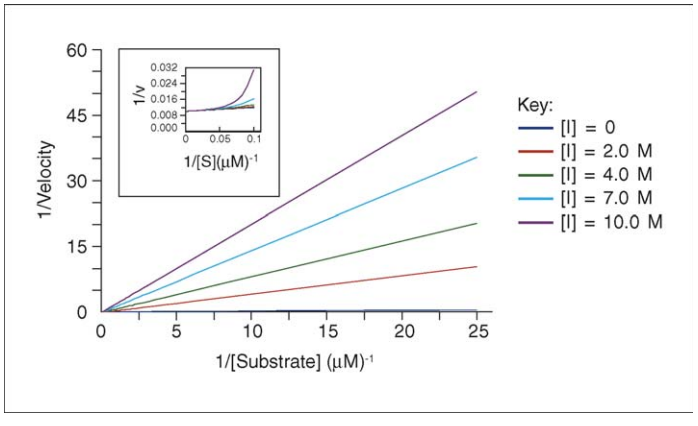


**FIGURE 4**

**Double-reciprocal plot of 1/v versus 1/[substrate] for a molecular clamp inhibitor**. At [MC] = 0, the initial velocity values were calculated at any substrate concentration, [S], according to Eqn 1. At [MC] = 2.0–10.0 μM, $[S]_{free}$ was calculated according to Eqn 2 at the indicated concentration of the molecular clamp with $K_{MC}$ = 0.1 μM. Initial velocity values as a function of a constant [MC] value were then obtained by using the $[S]_{free}$ values in Eqn 2. For all the modeling, the $K_M$ value was 2.0 μM and the $V_{MAX}$ was 100 arbitrary units. The inset shows the deviations from linearity at high [substrate] (or low 1/[substrate]).

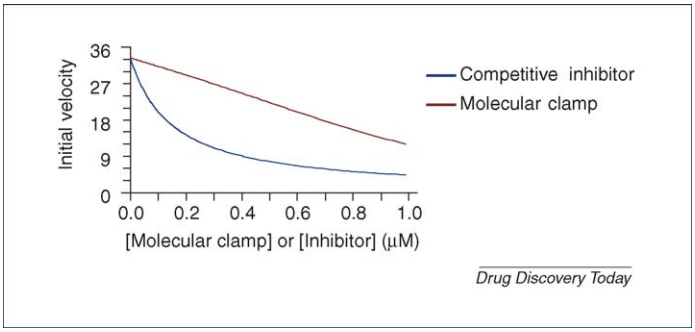


**FIGURE 5**

**Degree of inhibition resulting from a molecular clamp and a protein-targeted competitive inhibitor.** The relevant values used to generate these curves are $K_{MC} = K_{i,s} = 0.1$ μM, $K_M = 2.0$ μM, $V_{MAX} = 100$ arbitrary units, and initial substrate concentration fixed at 1.0 μM. The initial rate values for the protein-targeted competitive inhibitor were calculated using Eqn 3 and those for the molecular clamp were calculated by first calculating $[S]_{free}$ at any [MC] using Eqn 2 and then using the $[S]_{free}$ value in Eqn 1.

or $K_{i,s}$, the protein-targeted competitive inhibitor is a better inhibitor. The ratio of the velocity in the presence of molecular clamp to the velocity in the presence of competitive inhibitor in this case is 1:1.

Figures 2–5 represent the outcomes of the typical steady-state experiments that would be performed in analyzing a substrate-targeted molecular clamp. The goal of the kinetic experiments would be to determine the $K_{MC}$ and to provide experimental evidence to demonstrate that the observed inhibition resulted from the binding of the inhibitor to substrate and not to the enzyme. The $K_{MC}$ value could be obtained by computer fits of the data used to generate figures similar to Figures 2, 3 or 4 to equations 1 and 2. Sigmoidal v versus [S] Dixon plots and non-linear, concave-up 1/v versus 1/[substrate] reciprocal plots would be evidence in favour of substrate binding, but not definitive proof. These plots also depict the kinetic characteristics of allosteric enzymes, meaning enzymes with a Hill number, n, greater than 1.0 in Equation 4.

$$v = (V_{MAX}[S]^n)/(K_{0.5} + [S]^n) \quad \text{[Eqn 4]}$$

Proposed experiments in addition to the steady-state kinetic evaluation that would help demonstrate that the molecular clamp is binding to the substrate include direct binding studies to prove that the molecular clamp binds to the targeted substrate in the absence of enzyme, and kinetic studies to show that the molecular clamp has virtually no inhibitory effect on the enzymatic processing of non-targeted substrates. There is literature precedence for both sets of these experiments. The glycopeptide, lipoglycopeptide, and lantibiotic antibiotics [11–16] as well as bacitracin [17] are well known to bind substrates in bacterial cell wall biosynthesis. In addition, substrate-specific inhibition by a molecular clamp has been reported for substrates of farnesyl transferase [33], interleukin-converting enzyme [34], and RecQ helicase [49].

## Pharmacological considerations for the use and design of molecular clamp inhibitors

Based on our modelling of the steady-state kinetics in the previous section, the best target for the development of a molecular clamps would be an enzyme or receptor for which the *in vivo* concentration of the substrate or ligand is low, the affinity of the protein for the substrate or ligand is low (high $K_M$ or $K_D$) and the affinity of the molecular clamp for the substrate is high (low $K_{MC}$).

The major concern about using a molecular clamp targeted against a substrate that satisfies these criteria will be delivering a sufficiently high concentration of the clamp to the site of action. It is necessary for the concentration of a molecular clamp to be high enough to tie up most (>90%), if not all, of the free substrate as the S•MC complex. Limited cellular availability resulting from difficulties in crossing the cell membrane or the blood–brain barrier have caused many drug leads that are highly potent *in vitro* to fail in clinical trails. Strategies developed to address the drug delivery problems of other drugs, such as the use of liposome carriers [50,51], the formation of drug-polymer conjugates [52,53], the production of lipophilic prodrugs [51], the attachment of site-specific 'molecular zip codes' [54] or the attachment to cell-penetrating peptides [55,56], are likely to facilitate the delivery of molecular clamps. Because protein-modifying enzymes are attractive potential targets for molecular clamps, peptide-based molecular clamps are likely to be developed. Fusion to cell-penetrating peptides [55,56], PEGylation [52], incorporation of an arginine-rich sequence [57] or the attachment of a long-chain hydrophobic moiety [51,58] could increase the membrane permeability of peptide-based molecular clamps, thereby facilitating the delivery of this class of drugs to their site of action.

One potential problem with molecular clamp inhibition would be the buildup of the S•MC complex. The accumulation of S•MC complex could prove toxic for various reasons. However, this would become apparent in the testing routinely done for all new drugs: toxicity when applied to cultured cells, toxicity in animal studies and, finally, toxicity in human clinical trials. Enzyme-targeted drugs can lead to the accumulation of the substrate, and the resulting high substrate concentrations could be toxic or could cause metabolic resistance. Metabolic resistance is the establishment of enzymatic flux, despite drug treatment, because high substrate concentrations effectively out-compete the drug for the enzyme's active site [59]. A molecular clamp-based drug could prove to be less prone to metabolic resistance because the substrate would be consumed in forming the S•MC complex.

## Conclusion

Protein-targeted inhibitors have a long and successful history of treating human disease. For example, the statins, which are highly prescribed, clinically efficacious in lowering serum cholesterol and have a low incidence of side effects, are competitive inhibitors of HMG-CoA reductase [60]. Another approach to drug design would be to develop molecular clamps that are not targeted to bind to a protein-of-interest, but instead are targeted to bind a substrate-of-interest. The molecular clamp approach could prove useful not only in inhibiting the conversion of substrate to product, but could inhibiting either protein–protein interactions or the binding of ligand to a receptor. This approach is particularly attractive when the protein-of-interest has a collection of intracellular ligands or substrates. Ideally, a molecular clamp would prevent the binding of only the targeted ligand to the protein, thus allowing the protein to function normally with all other ligands or substrates.

The degree of inhibition resulting from a molecular clamp is dependent on the intracellular concentration of the targeted ligand or substrate, suggesting that a molecular clamp would prove most effective against a ligand or substrate of a low intracellular concentration (<1.0 μM). Steady-state analysis of the molecular-clamp-mediated inhibition of enzymatic catalysis exhibits deviations from standard patterns (Figures 2–4), but the deviations can be subtle and can have explanations other than inhibitor binding to substrate. Steady-state analysis can provide relatively straightforward results pointing toward substrate-binding, but it should be verified with more definitive experiments: either a direct demonstration of the molecular clamp binding to the targeted substrate in the absence of enzyme or additional steady-state kinetic experiments showing that the molecular clamp only inhibits the enzymatic conversion of the targeted substrate to product. In summary, the development of substrate-targeted molecular clamp drugs represents an underused approach to the treatment of diseases and could prove clinically useful against the right targets.

## Acknowledgement

This work was supported, in part, by grants from the National Institutes of Health – General Medical Sciences (R15-GM073659), the Eppley Foundation for Research, and the Shirley W. and William L. Griffin Foundation to D.J.M. and a joint grant from the National Institutes of Health – General Medical Sciences (R21-GM072771) to M.L.M. and D.J.M.



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