ELSEVIER

DRUG DISCOVERY TODAY TECHNOLOGIES

Drug Discovery Today: Technologies

Vol. 7, No. 1 2010

Mechanistic pharmacology, new developments

Use of functional assays to detect and quantify functional selectivity

William P. Clarke*, Kelly A. Berg

Department of Pharmacology, University of Texas Health Science Center at San Antonio[1], 7703 Floyd Curl Drive, San Antonio, TX, 78230, USA

Drug selectivity is arguably a critical concern for drug development. Recently, experimental evidence suggests that drugs have more selectivity than that afforded by differential affinity for different receptor subtypes. Drugs, acting at a single receptor, can selectively and differentially activate each of the multiple signaling pathways coupled to a receptor. This type of selectivity has been termed functional selectivity. Understanding functional selectivity and how to measure it will be important for new drug development.

Introduction

Functional selectivity is a term used to describe the ability of drugs to differentially regulate each of the multiple signaling pathways coupled to a receptor (Fig. 1). Although functional selectivity has been most thoroughly studied for the seven transmembrane-spanning receptor superfamily, it also applies to other receptor families, such as the nuclear receptors (e.g. selective estrogen receptor modulators; SERMs) and theoretically should extend to receptor tyrosine kinases, ligand-gated ion channels and others. It is difficult to overestimate the importance of functional selectivity for drug discovery and development. Functional selectivity extends the concept of drug selectivity beyond that afforded by differential affinity for different receptor subtypes. Not only can drugs have receptor selectivity but also drugs acting at a single receptor subtype can have selectivity for distinct signaling pathways. With the assumption that regulation of a specific signaling pathway has therapeutic benefit (and/or that adverse effects may be linked to certain pathways), quantifying the functional selectivity of drugs would be expected to result in the development of drugs with improved therapeutic efficacy and reduced adverse effects.

Intrinsic efficacy

Functional selectivity is based upon a drug's ability to activate a receptor, thus it deals with the pharmacological property of intrinsic efficacy (see Glossary). Since the term was first introduced by Furchgott 45 years ago [1], intrinsic efficacy was considered to be a constant, unique for each drug-receptor pair and, importantly, independent of the signaling system to which the receptor is coupled. In molecular terms, Furchgott's intrinsic efficacy is the ability of a drug to promote an active receptor conformation capable of regulating cellular signaling mechanisms. Within this framework, drugs can have high, moderate or low levels of intrinsic efficacy or may have zero intrinsic efficacy (the ability to bind, but not to activate a receptor; i.e. an antagonist [see Glossary]).

The magnitude of a ligand's intrinsic efficacy is one, but not the only, factor that influences the magnitude of the response elicited by the ligand. The ability of a drug to produce a response (drug efficacy, see Glossary) depends not only upon intrinsic efficacy, but also on other system-dependent parameters, such as the density of receptors expressed and the efficiency of receptor–effector coupling. Thus, although in Furchgott's world the ability of a drug to

*Corresponding author.: W.P. Clarke (clarkew@uthscsa.edu)
[1] http://www.uthscsa.edu.

 DOI: 10.1016/j.ddtec.2010.07.001

Glossary

Agonist: A ligand which increases the activity of a receptor, leading to increased receptor-mediated response. Agonists increase the proportion of the receptor population in an active conformation. Agonists have positive values of intrinsic efficacy.
Antagonist: A ligand which does not change the activity of a receptor, thus there is no change in receptor-mediated response. However, because the antagonist occupies the receptor, its presence will interfere with occupancy of the receptor by agonists and inverse agonists. Antagonists have an intrinsic efficacy value of zero.
Constitutive receptor activity: Spontaneous activity of a receptor system in the absence of an activating ligand (agonist). In the absence of an activating ligand, a percentage of the receptor population is in an active conformation. Contributes to basal response.
EC_{50}: Concentration of drug which produces half-maximal response; often used as a measure of potency; midpoint of the position of the drug concentration–response curve.
Efficacy: The ability of a drug to produce a response.
Ensemble: Collection of functionally similar receptor conformations (see [13,15]).
Full agonist: An agonist that can produce a maximal response by occupying less than 100% of the receptor population.
Intrinsic efficacy: The capacity of a drug to activate a receptor, to increase the proportion of receptors in an active conformation. Negative intrinsic efficacy is a property of inverse agonists and reflects the capacity of a drug to inactive a receptor, to decrease the proportion of receptors in an active conformation.
Inverse agonist: A ligand which decreases the activity of a receptor, decreases the proportion of the receptor population in an active conformation, leading to decreased receptor-mediated response. Inverse agonists have negative values of intrinsic efficacy.
K_e: Operationally defined as the concentration of the drug-receptor complex which produces half-maximal response [4]; a measure of the efficiency of signal transduction. K_e incorporates a drug's intrinsic efficacy as well as receptor–effector coupling efficiency.
Partial agonist: A agonist that must occupy 100% of the receptor population to produce a maximal response, which is typically less than that of a full agonist.
Protean ligand: A ligand which can behave as an agonist toward one response and an inverse agonist toward another response at the same time and mediated by the same receptor. The intrinsic efficacy value of a protean ligand may be positive or negative depending upon the response measured.
Receptor system: A receptor and its associated signal transduction and effector molecules.
Relative efficacy: Comparison of the efficacy of a test drug to that of a reference drug measured in the same system thereby nullifying the contribution of system-dependent parameters to the response produced by the test drug; an indirect measure of the intrinsic efficacy of the test drug.
R_T: Total receptor density.
τ (tau): An efficacy-related parameter from the Black and Leff operational model of agonism [4]. τ is the ratio of R_T to K_e.

activate a receptor may be a constant, the response produced by receptor activation will differ when the drug is tested in different systems. For example, the efficacy of the β1-adrenergic ligand, prenalterol, differs from full agonism (see Glossary) to partial agonism (see Glossary) to antagonism, depending upon the tissue to which it is applied [2].

Because of its system-independence, values of ligand intrinsic efficacy were considered to be especially valuable

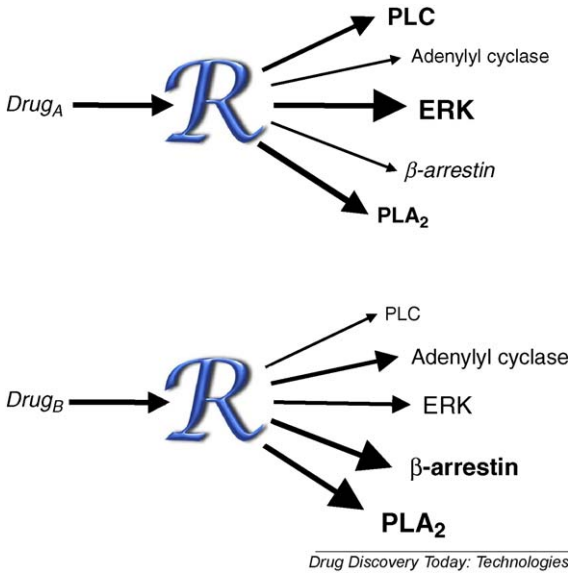

Figure 1. Functional selectivity is the term used to describe the ability of drugs to selectively and differentially activate certain signaling pathways coupled to a single receptor. In this example, when drug A interacts with a receptor (R), extracellular signal-regulated kinase (ERK) is activated strongly, phospholipase C (PLC) and PLA_2 are activated moderately, and adenylyl cyclase and β-arrestin is weakly activated. However, the pattern of efficacy of effector activation is different when Drug B interacts with the same receptor. Drug B leads to strong activation of PLA_2 and β-arrestin, moderate activation of adenylyl cyclase and ERK, and weak activation of PLC.

for drug development. If the intrinsic efficacy of a drug is determined in one system, it should be the same across all systems. This allows for the use of cell model systems expressing the receptor of interest to measure drug intrinsic efficacy and permits rigorous structure-activity studies to be done to guide chemists in the synthesis of new drugs. If intrinsic efficacy is quantified from one system, the response to the drug can be predicted in others.

Measurement of intrinsic efficacy

As described above, Furchgott's intrinsic efficacy reflects the capacity of a drug to activate a receptor; to promote an active receptor conformation capable of regulating a cellular response. Consequently, it seems reasonable that methods which permit measurement of ligand-induced changes in receptor conformation (e.g. X-ray crystallography, NMR spectroscopy, conformationally-sensitive fluorescent probes) would be ideal methods to measure the intrinsic efficacy of a drug. However, while these methods do permit highly precise measurement of receptor conformational changes produced by the application of a ligand, they currently do not allow for quantification of the active conformation. In large part, this is because we do not know, precisely and fully, the changes in receptor conformation that are important for regulating interactions with signaling molecules. For

example, structural studies have shown that activation of a seven-transmembrane-spanning receptor involves rearrangement of transmembrane helices V and VI [3]. Although this change in receptor conformation is believed to be important in receptor activation (it may cause the opening of a crevice on the cytoplasmic side of the receptor to allow G protein binding), it is not yet possible to associate the magnitude of the conformational change with the extent of receptor activation and the ability of the receptor to regulate signaling (i.e. assignment of a value for intrinsic efficacy). It is also clear that there are many other conformational changes that occur upon receptor activation. Furthermore, there are probably many ligand-induced changes in the three-dimensional positions of atoms within a receptor that are not involved in (or are secondary to) the formation of an active receptor conformation. Until we learn much more about the molecular nature of receptor–effector interactions, the measurement of ligand-induced changes in receptor structure will not be a viable approach for quantification of intrinsic efficacy.

Currently, the only way to quantify intrinsic efficacy of a drug involves measurement of a response. However, as mentioned above, production of a response from receptor activation by a drug is not solely dependent upon intrinsic efficacy. The magnitude of response also depends upon system-dependent parameters, such as receptor density and receptor–effector coupling efficiency (which depends upon the number, type and location of signal transduction molecules in the cell). Thus, to isolate the ability of a drug to activate a receptor (intrinsic efficacy) when a response is measured, system-dependent properties must be nullified.

The approach to remove the system-dependent parameters from the measurement of a response is to measure ligand *relative* efficacy (see Glossary). Measurement of relative efficacy involves comparison of the same cellular responses produced by test ligands with that of a reference ligand obtained in the same system. Because the responses to the test ligands and reference ligand are obtained in the same system (same receptor density, same quantity and type of signaling molecules, among others), differences in relative efficacy between test ligands must be due to differences in their ability to activate the receptor (i.e. intrinsic efficacy).

For test ligands with low intrinsic efficacy which do not promote sufficient receptor activation to saturate the cellular signaling mechanisms that lead to the response measured (partial agonists), relative efficacy can be measured as the ratio of the maximal response (the response produced at concentrations of the agonist that produce full receptor occupancy) of the test ligand to that of the reference ligand ($E_{maxtest}/E_{maxref}$). Although measurement of relative efficacy does not allow for quantification of an absolute value for a test drug's intrinsic efficacy, it does allow for quantification of differences in the intrinsic efficacy of test drugs. For example, if the relative efficacy of test drug 'A' is 0.8 with respect to the reference drug and that of test drug 'B' is 0.4, then the intrinsic efficacy of drug A is twice that of drug B.

The measurement of relative efficacy becomes a bit more complex for test drugs with high intrinsic efficacy values – where the degree of receptor activation is sufficient to saturate some components of the cellular response-generating mechanisms. Even though these drugs may have different intrinsic efficacies, they produce the same maximal response and are called 'full' agonists. For full agonists the $E_{maxtest}/E_{maxref}$ ratios all equal 1 and therefore do not reflect the differences in the capacity of the drugs to activate the receptor. For full agonists, there are two ways to measure relative efficacy. The first is to calculate the ratio of occupancy (K_A) and response (EC_{50}) parameters of the test and reference ligands $(K_{A(test)}/EC_{50(text)})/(K_{A(ref)}/EC_{50(ref)})$. This is a laborious method which involves receptor binding studies to determine the agonist K_A as well as performing concentration–response curves to obtain the agonist EC_{50}. Thus, for two full agonists that produce the same maximal response, the agonist that occupies a smaller percentage of the receptor population (lower K_A value) has the greater intrinsic efficacy.

The second method to measure the relative efficacy of full agonists is to alter the system such that the full agonists become partial agonists. Often this can be accomplished by irreversible inactivation of a portion of the receptor population with an irreversible antagonist or with a receptor alkylating agent. This reduces the maximal level of receptor activation to below that which saturates the cellular response-generating mechanisms. Because the test ligand is now a partial agonist, the ratio of the test agonist maximal response to that of the reference agonist reflects intrinsic efficacy.

Another strategy to measure relative efficacy which applies to both partial and full agonists involves measurement of agonist efficacy using the Black and Leff model of operational agonism [4].

$$\text{Response} = \frac{[A] \cdot \tau \cdot E_{max}}{[A](\tau + 1) + K_A}$$

where $[A]$ is the agonist concentration, E_{max} is the maximal response of the system, K_A is the equilibrium dissociation constant of the ligand and τ (see Glossary) is an efficacy term (transducer ratio) equal to the ratio of receptor density (R_T) to K_e (R_T/K_e), where K_e (see Glossary) represents the efficiency of signal transduction by the ligand–receptor complex. K_e incorporates both ligand intrinsic efficacy and system-dependent parameters such as the efficiency of receptor–effector coupling. The values of these parameters can be obtained with non-linear regression analysis of agonist concentration–response curves. Relative efficacy then becomes the ratio of τ of the test agonist to that of the reference agonist (τ_{test}/τ_{ref}). The system-dependent parameters associated with efficacy are removed when both the test agonists and reference agonists are measured for the same response in the same system.

Thus, differences in relative efficacy of two drugs must be due to differences in the ability of the drugs to activate the receptor.

Negative intrinsic efficacy

The pioneering work of Cerione *et al.* [5] with purified β-adrenergic receptors and Gαs proteins followed by that of Costa and Herz [6] studying opioid receptors expressed natively in intact cells lead to the realization that receptors in a population were not necessarily quiescent, but could spontaneously adopt an active conformation capable of regulating cellular responses, in the absence of an activating ligand. The realization that receptors could be constitutively active led to the discovery of ligands that decrease constitutive receptor activity (inverse agonists) in addition to ligands which increase receptor activation (agonists). Just as an agonist has intrinsic efficacy, defined as the ability of the ligand to increase receptor activation, inverse agonists also have intrinsic efficacy defined as the ability to decrease receptor activation. For inverse agonists, the sign of intrinsic efficacy is negative (negative intrinsic efficacy) to indicate a reduction in receptor activation. Inverse agonists, like agonists, can differ in the magnitude of negative intrinsic efficacy (partial and full inverse agonists). As for agonists, intrinsic efficacy of inverse agonists cannot be measured directly. However, measurement of relative efficacy (as described above), with respect to a reference inverse agonist, provides an indirect measure of the intrinsic efficacy of inverse agonists.

The discovery of constitutive receptor activity and inverse agonism also led to a change in our view of the molecular nature of drug intrinsic efficacy. Rather than producing receptor activation by inducing a conformational change in a receptor from a quiescent conformation, the active conformation could occur spontaneously and the proportion of active receptors in a population enriched or depleted by the presence of a ligand. Thus, intrinsic efficacy could be considered as the capacity of a ligand to enrich (for agonists) or deplete (for inverse agonists) the proportion of receptors in the active conformation (see induction versus selection, [7]).

Multiple intrinsic efficacies – functional selectivity

It is now generally accepted that an individual receptor subtype can regulate the activity of more than one effector response in a cell. In fact, individual receptor subtypes typically regulate the activity of several distinct cellular signaling pathways. Traditional receptor theory held that a receptor could adopt a single active conformational state which could regulate cell signaling. As mentioned above, intrinsic efficacy is the property of a drug to increase (agonists) or decrease (inverse agonists) the proportion of a receptor population in the active conformational state. Since the term was introduced 45 years ago, it was believed that intrinsic efficacy was a constant, unique for each drug-receptor pair and independent of the cellular signaling systems coupled to the receptor [1]. With a single active conformational state, the degree to which a drug enriched (or depleted) the active state receptors would be reflected similarly in all signaling pathways regulated by that active state. Thus, the relative efficacy of a ligand must be independent of the cellular response measured.

Over the past several years, experimental evidence has accumulated which demonstrates that ligand relative efficacy is not independent of the response measured. In fact, relative efficacy of a ligand, acting at a single receptor subtype, can differ dramatically depending upon the cellular response measured. In extreme cases, a single ligand, acting at a single receptor subtype, can be an agonist (positive intrinsic efficacy) and an inverse agonist (negative intrinsic efficacy) at the same time in the same cell depending upon the response measured. Ligands such as these are called protean ligands [8,9]. The behavior of protean ligands is not compatible with the existence of a single active receptor conformation, but requires multiple active conformations, the relative proportions of which can be differentially regulated by ligands.

Kenakin [10] was the first to formalize an hypothesis that allowed for agonist relative efficacy to differ depending upon the response measured. This hypothesis was originally termed 'agonist-directed trafficking of receptor stimulus' and was based upon the premise that agonist interaction with a receptor can promote the formation/stabilization of *ligand-specific receptor conformational states*. These ligand-dependent receptor conformations would have differential ability (affinity and/or efficacy) to regulate the various signaling molecules that mediate particular cellular responses. Interestingly, the behavior of ligands to differentially regulate signaling pathways coupled to the same receptor has been given a variety of names in the literature, including 'functional selectivity', 'stimulus trafficking', 'differential engagement' and 'biased agonism'. Although the field appears to have settled on 'functional selectivity' as a simple term to describe the phenomenon [11], 'biased agonism' has been used recently, especially with respect to ligands which may have selectivity toward activating β-arrestin versus adenylyl cyclase activity or phospholipase C signaling [12]. The term 'biased' means 'one-sided' and is useful when comparing just two options. However, receptors generally regulate several signaling pathways and it seems likely that ligands may promote multiple sets of receptor conformations ('ensembles', see Glossary and [13] for more information on ensembles) and thus may have selectivity for more than one cellular response.

The ability of ligands to promote (enrich) certain groups of receptor conformations at the expense of others, rather than just single active and inactive conformations, means that ligands have multiple intrinsic efficacy values. By enriching one ensemble of active conformations, a ligand could deplete another active group. In this way, a single ligand acting at a

single receptor subtype could be both an agonist and an inverse agonist at the same time (a protean ligand). It is expected that the spectrum of receptor conformational ensembles that result from the addition of a ligand will be dependent upon ligand chemical structure and be unique for each ligand. Even small differences in molecular structure can lead to significant differences in differential signaling [14].

Response-dependent intrinsic efficacy means that relative efficacy must be measured for each cellular response. Methods of measurement of relative efficacy have been described above for partial and full agonists and inverse agonists. It is important to note that a ligand can be a partial agonist for one response, but a full agonist for a different response coupled to the same receptor in the same system. Consequently different methods (E_{max} ratios or K_A/EC_{50} ratios) may be required for the measurement of relative efficacy for different responses, unless τ ratios are used. Importantly, because relative efficacy is response-dependent, relative efficacy values are not generalizable and must be measured for each ligand–receptor–effector triad.

The use of τ/K_A ratios to quantify functional selectivity

Given the allosteric nature of receptor proteins (binding of a ligand promotes receptor conformations with different capacities to interact with secondary signaling molecules, see [15,16]), the allosteric influence of different signaling molecules on ligand efficacy and affinity should be considered when measuring the response-dependent relative efficacy of an agonist. To address this issue, Kenakin recently proposed the use of τ/K_A ratios as a means to quantify ligand functional selectivity [15,16]. Both τ and K_A can be obtained from non-linear regression of concentration–response curves for each agonist for each response. This method has the additional advantage in that it is applicable to both weak and strong agonists.

Conclusion

Functional selectivity is based upon differential ligand intrinsic efficacy for different cellular signaling pathways coupled to the same receptor subtype. Intrinsic efficacy is defined as the ability of a ligand to promote changes in receptor conformational states (ensembles) and receptors can exist in multiple active conformational ensembles. Ligands, acting at the same receptor, can enrich or deplete different receptor conformational ensembles and therefore can have multiple intrinsic efficacy values that differ depending upon the response measured. As of today, intrinsic efficacy cannot be measured directly. Null methods to remove the system-dependency of ligand-induced responses must be used (relative efficacy). Response-dependent differences in ligand relative efficacy are used to quantify functional selectivity. Although there are a variety of methods available to measure relative efficacy, the most robust one involves fitting ligand concentration–response curves to the Black and Leff operational model of agonism to determine τ ratios. Owing to the allosteric nature of receptor proteins, the interaction of the receptor with different signaling molecules (e.g. G proteins, β-arrestin, among others) may influence ligand affinity. Consequently, τ/K_A ratios, derived from the operational model, provide the most rigorous and complete means of quantifying functional selectivity. Importantly, relative efficacy is not generalizable and must be measured for each ligand–receptor-response triad.

It is difficult to overestimate the importance of understanding and quantifying functional selectivity for drug discovery and development (outstanding issues). By providing medicinal chemists with quantitative information about the functional selectivity of their ligands, structure-activity studies would be expected to provide drugs with improved profiles of therapeutic efficacy and reduced adverse effects. It is therefore important for pharmacologists to measure the relative efficacy of ligands at each of the many signaling pathways that couple to a target receptor subtype. With these relative efficacy values in hand, chemists can modify drugs to increase or decrease ligand signaling selectivity thereby increasing therapeutic efficacy and decreasing the severity of adverse effects.

Outstanding issues

- The therapeutic relevance of functional selectivity is not yet clear.
- The connection between specific cellular signaling pathways and therapeutic benefit or adverse effects needs to be delineated.
- Pathology can alter cellular phenotype. The consequence of differences in cell phenotype with respect to the choices of cell model systems used to assess drug functional selectivity will need to be understood.

Acknowledgements

The authors thank Dr Terry Kenakin for helpful, thought-provoking discussions. The authors are supported by United States Public Health Service grants DA026619 (KAB), NS055835 and DA024865 (WPC).

References

1 Furchgott, R.F. (1966) The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor–agonist complexes. In *Advances in Drug Research*, (Vol. 3) (Harper, N.J. and Simmonds, A.B., eds) pp. 21–55, Academic Press
2 Kenakin, T.P. and Beek, D. (1980) Is prenalterol (H133/80) really a selective beta 1 adrenoceptor agonist? Tissue selectivity resulting from differences in stimulus–response relationships *J. Pharmacol. Exp. Ther.* 213, 406–413
3 Hofmann, K.P. *et al.* (2009) A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem. Sci.* 34, 540–552
4 Black, J.W. and Leff, P. (1983) Operational models of pharmacological agonism. *Proc. R. Soc. Lond. B Biol. Sci.* 220, 141–162
5 Cerione, R.A. *et al.* (1984) The mammalian beta 2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. *Biochemistry* 23, 4519–4525

6 Costa, T. and Herz, A. (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7321–7325
7 Kenakin, T.P. (1996) Receptor conformational induction vs. selection: all part of the same energy landscape. *Trends Pharmacol. Sci.* 17, 190–191
8 Kenakin, T. (2001) Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J.* 15, 598–611
9 Kenakin, T. (2007) Functional selectivity through protean and biased agonism: who steers the ship? *Mol. Pharmacol.* 72, 1393–1401
10 Kenakin, T. (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol. Sci.* 16, 232–238
11 Urban, J.D. *et al.* (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J. Pharmacol. Exp. Ther.* 320, 1–13
12 Violin, J.D. and Lefkowitz, R.J. (2007) Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol. Sci.* 28, 416–422
13 Kenakin, T. (2002) Drug efficacy at G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* 42, 349–379
14 Moya, P.R. *et al.* (2007) Functional selectivity of hallucinogenic phenethylamine and phenylisopropylamine derivatives at human 5-HT2A and 5-HT2C receptors. *J. Pharmacol. Exp. Ther.* 321, 1054–1061
15 Kenakin, T. and Miller, L.J. (2010) Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol. Rev.* 62, 265–304
16 Kenakin, T.P. (2009) 7TM receptor allostery: putting numbers to shapeshifting proteins. *Trends Pharmacol. Sci.* 30, 460–469