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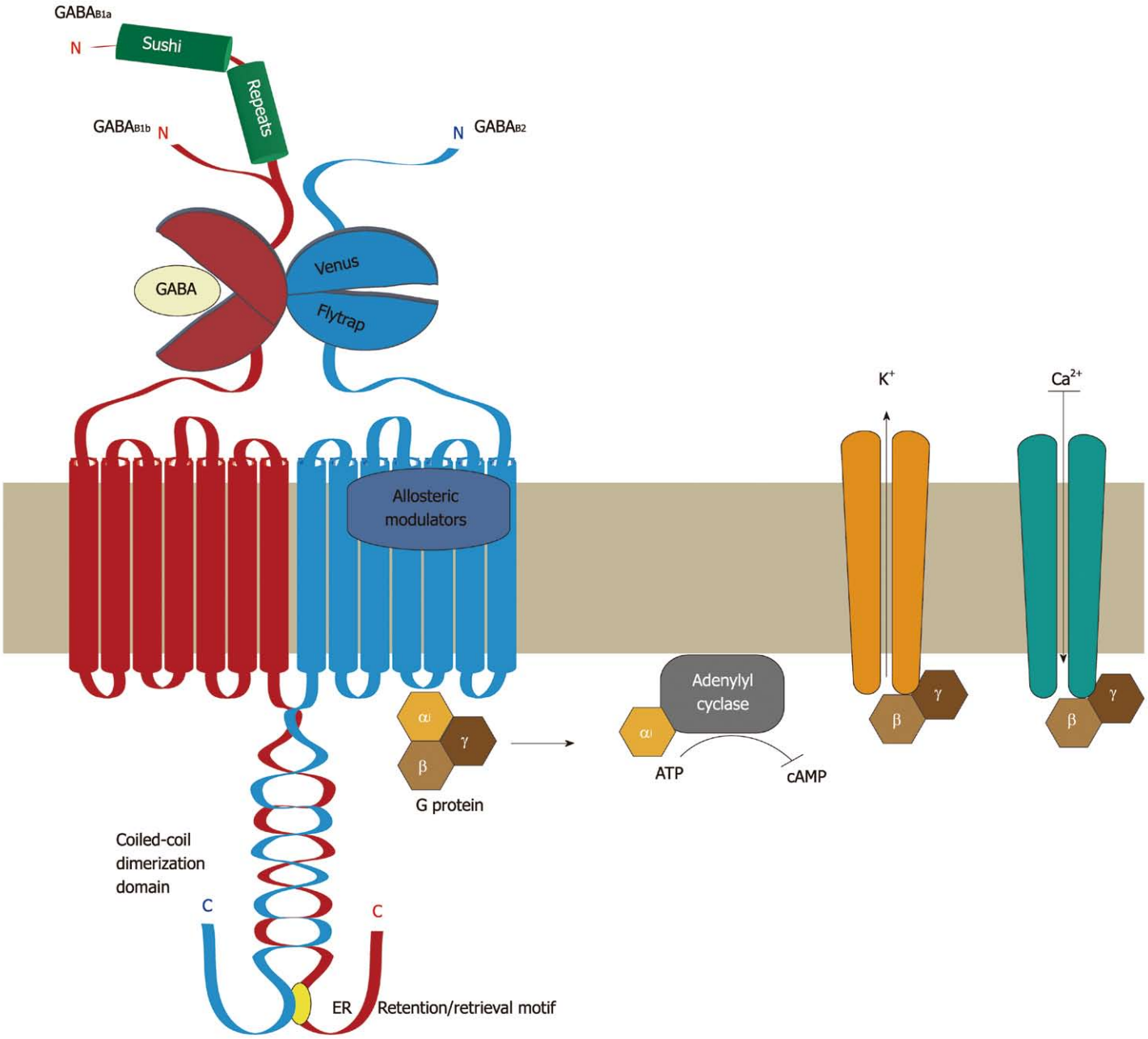

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EDITORIAL

Modulation of cell surface GABAB receptors by desensitization, trafficking and regulated degradation

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Abstract

Inhibitory neurotransmission ensures normal brain function by counteracting and integrating excitatory activity. γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system, and mediates its effects *via* two classes of receptors: the GABAA and GABAB receptors. GABAA receptors are heteropentameric GABA-gated chloride channels and responsible for fast inhibitory neurotransmission. GABAB receptors are heterodimeric G protein coupled receptors (GPCR) that mediate slow and prolonged inhibitory transmission. The extent of inhibitory neurotransmission is determined by a variety of factors, such as the degree of transmitter release and changes in receptor activity by posttranslational modifications (e.g., phosphorylation), as well as by the number of receptors present in the plasma membrane available for signal transduction. The level of GABAB receptors at the cell surface critically depends on the residence time at the cell surface and finally the rates of endocytosis and degradation. In this review we focus primarily on recent advances in the understanding of trafficking mechanisms that determine the expression level of GABAB receptors in the plasma membrane, and thereby signaling strength.

Key words: GABAB receptors; Neuron; Trafficking; Endocytosis; Recycling; Degradation

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FUNCTIONS OF GABAB RECEPTORS

Metabotropic GABAB receptors are widely distributed throughout the central nervous system where they mediate slow, prolonged inhibition to control neuronal excitation, and contribute to synaptic plasticity[1].

GABAB receptors are present at pre- and postsynaptic sites of both inhibitory and excitatory neurons. Electron microscopy revealed that GABAB receptors are located predominantly at areas close to neurotransmitter release sites and at peri- and extrasynaptic areas of spines and dendrites, but only rarely directly at active zones or postsynaptic densities[2-7]. This location of GABAB receptors implies that they are not directly activated by synaptically released GABA. One mechanism to activate GABAB receptors requires intense neuronal activity, resulting in

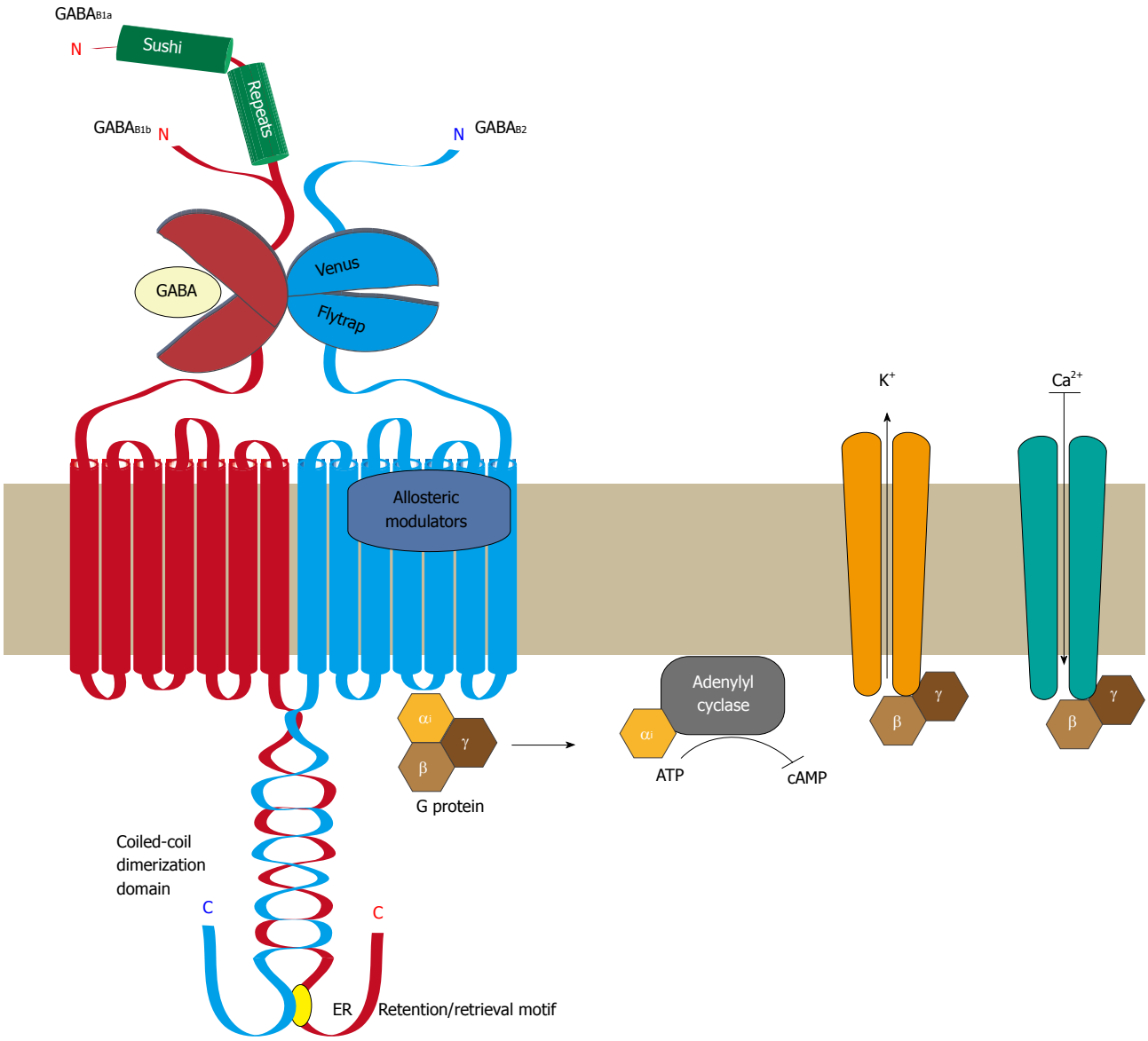

Figure 1 Structural organization of GABA$_B$ receptors. Functional GABA$_B$ receptors are heterodimers composed of the two subunits GABA$_{B1}$ and GABA$_{B2}$. Both subunits are heptahelical membrane proteins with a large extracellular located N-terminal domain containing a "Venus flytrap" module and a large intracellular C-terminal domain containing a coiled-coil protein-protein interaction module. GABA$_{B1}$ and GABA$_{B2}$ heterodimerize *via* their "Venus flytrap" and coiled-coiled domains. An endoplasmic reticulum (ER) retention/retrieval signal is present distal to the coiled-coil domain in GABA$_{B1}$ and prevents ER exit of GABA$_{B1}$ unless it is masked by heterodimerization with GABA$_{B2}$. The "Venus flytrap" module of GABA$_{B1}$ constitutes the GABA binding site, whereas that of GABA$_{B2}$ is inactive and not involved in ligand binding. Instead, the heptahelical domain of GABA$_{B2}$ contains a binding site for allosteric modulators, which affects the affinity of ligands binding to the GABA site. Binding of GABA results in the recruitment and activation of Gαi/o proteins *via* GABA$_{B2}$. The activated Gαi/o subunit inhibits the adenylyl cyclase, resulting in lowered cAMP levels, while the Gβγ dimer activates K^+ channels and inhibits Ca^{2+} channels, leading in either case to neuronal inhibition. There exist two isoforms of GABA$_{B1}$, named GABA$_{B1a}$ and GABA$_{B1b}$, which are generated by alternative promoter usage. They only differ by the additional presence of two so-called "sushi repeats" (protein-protein interaction modules) in the N-terminal domain of GABA$_{B1a}$. GABA: γ-Aminobutyric acid; ATP: Adenosine-5'-triphosphate; cAMP: 3'-5'-cyclic adenosine monophosphate.

a spill-over of synaptically released GABA[8]. However, there are also other sources that may increase the ambient level of GABA, such as activity-dependent release of GABA from dendrites and glia cells[9-11]. Recently, it has been shown that basal synaptic activity generates a sufficient concentration of ambient GABA to tonically induce a low level of presynaptic GABA$_B$ receptor activation, which results in the control of transmitter release[12].

Binding of GABA to the GABA$_B$ receptor activates Gi/o-type G proteins[13-18], which in turn modulate three major effector systems: adenylyl cyclases, voltage-sensitive Ca^{2+} channels and inwardly-rectifying K^+ channels (Figure 1).

The α subunit of the activated G protein inhibits adenylyl cyclase activity, which decreases cellular 3'-5'-cyclic adenosine monophosphate (cAMP) levels and affects the activity of cAMP-dependent processes. Unfortunately, the contribution of GABA$_B$ receptor-induced lowering of cAMP levels to physiological processes is poorly investigated. So far it has been shown that it retards synaptic vesicle recruitment during sustained activity, which reduces transmitter release[19]. In addition, GABA$_B$ receptor-mediated Gαi/o effects may be important for long-term

adaptations involving regulation of protein kinase activity and gene transcription[20-22].

However, the most well established GABA$_B$ receptor actions are mediated *via* the βγ dimer of the activated G protein. At presynaptic sites, voltage-sensitive P/Q- and N-type Ca^{2+} channels are the predominant effectors of GABA$_B$ receptors[23-27]. GABA$_B$ receptor activated Gβγ inhibits Ca^{2+} channel activity by slowing their current activation kinetics[28], which eventually results in reduced transmitter release. Postsynaptically, GABA$_B$ receptor effects are mainly mediated by the family of G protein-gated inwardly rectifying K^+ channels (GIRK1-4 also called Kir3.1-3.4)[29,30]. Gβγ directly binds to GIRK channels[31,32] and activates them[33,34], resulting in an outward K^+ current. This hyperpolarizes the membrane and consequently inhibits neuronal activity. However, there is no strict mechanistic segregation of pre- (Ca^{2+} channels) and postsynaptic (K^+ channels) effector systems. There is accumulating evidence that GABA$_B$ receptors also activate K^+ channels at presynaptic sites, which assists inhibition of transmitter release[35-37]. Conversely, there is also data for GABA$_B$ receptor mediated inhibition of postsynaptic Ca^{2+} channels[38-41]. This provides an additional mechanism for controlling the excitability of dendrites and spines. Thus, the current data is consistent with a complex pattern of regulating the activity of multiple G protein-gated inwardly rectifying K^+ channels and voltage-sensitive Ca^{2+} channels, both at pre- and postsynaptic sites, resulting in the inhibition of neuronal activity.

To ensure efficient activation of the effector system, GABA$_B$ receptors are localized in close proximity to their effector channels[36,42] and may even constitute signaling complexes by physical interaction[36,43].

MOLECULAR ORGANIZATION OF GABA$_B$ RECEPTORS

Although the GABA$_B$ receptor was discovered in 1980[44], its molecular identity and characterization was delayed for almost 20 years until the first constituent of the receptor was cloned. This delay was due to the fact that all biochemical attempts to purify the receptor failed and expression cloning proved unsuccessful. The development of high-affinity antagonists eventually permitted the successful screening of expression libraries yielding two cDNAs derived from a single gene, GABA$_{B1a}$ and GABA$_{B1b}$[45]. GABA$_{B1a}$ and GABA$_{B1b}$ are generated by differential promoter usage[46] and differ solely by the presence of an additional N-terminal sequence in GABA$_{B1a}$ coding for two protein-protein interaction domains, so-called "sushi domains". GABA$_{B1a}$ and GABA$_{B1b}$ show all the characteristics of class Ⅲ G protein-coupled receptors (e.g., a very large extracellular domain, seven transmembrane-spanning (heptahelical) sequences and a large intracellular located C-terminal domain) (Figure 1). So far, no functional differences among GABA$_B$ receptors containing GABA$_{B1a}$ and GABA$_{B1b}$ have been detected. The cloning of these first GABA$_B$ receptor constituents provided the basis for numerous research efforts analyzing the molecular characterization and function of GABA$_B$ receptors. It soon became clear that functional GABA$_B$ receptors are obligatory heterodimers composed of GABA$_{B1}$ (either GABA$_{B1a}$ or GABA$_{B1b}$) and a second heptahelical membrane protein named GABA$_{B2}$, sharing about 35% sequence identity with GABA$_{B1}$[47-51]. Both subunits serve distinct functions within the heterodimeric receptor complex. GABA$_{B1}$ contains the agonist and antagonist binding site in the large N-terminal extracellular domain, which is most likely arranged in a Venus flytrap-like structure[52-54]. Association with GABA$_{B2}$ is necessary to keep the GABA binding site in a high affinity state[55,56]. On the other hand, GABA$_{B2}$ contains a binding site for allosteric modulators, which is not however associated with the N-terminal Venus flytrap domain, but is located in the heptahelical domain[57]. Binding of ligands to this site does not directly activate the GABA$_B$ receptor but instead affects the affinity of orthosteric agonists and antagonists to GABA$_{B1}$[58]. Finally, GABA$_{B2}$ is responsible for G protein activation[56,59-63] and plays an important role in cell surface trafficking of the heterodimerized receptor complex by masking an arginine-based endoplasmic reticulum (ER) retention/retrieval (RXR) signal present in the C-terminal domain of GABA$_{B1}$[64-68].

THE ROLE OF DESENSITIZATION AND PHOSPHORYLATION ON THE AVAILABILITY OF FUNCTIONAL GABA$_B$ RECEPTORS

Prolonged exposure of G protein coupled receptors (GPCR) to agonists generally leads to a complex series of events in order to attenuate or terminate signal transduction, protecting the cell from overstimulation. Signal transduction is often attenuated by desensitization of the receptors (i.e., abrogating signaling), although the agonist is still present[69,70]. Desensitization of many GPCRs involves phosphorylation-dependent uncoupling of the receptor from the G proteins, followed by internalization of the receptor. Activated GPCRs are usually phosphorylated by G protein-coupled receptor kinase (GRKs) at serine and/or threonine residues residing in the carboxyl-terminal tail- or intracellular loop regions, which rapidly attenuates receptor responses. Phosphorylation leads to the recruitment of arrestins, which is thought to sterically prohibit signaling to G proteins and induces internalization of the receptor by linking it to components (clathrin, AP2 complex) of the endocytosis machinery[69,70]. Internalized receptors are then either degraded in lysosomes or are dephosphorylated and subsequently recycled to the plasma membrane, where they are again available for signaling.

It is well known that prolonged activation of GABA$_B$ receptors commonly leads to their desensitization. Recent studies suggest that there might be more than one mechanism for desensitization of GABA$_B$ receptors[71-75],

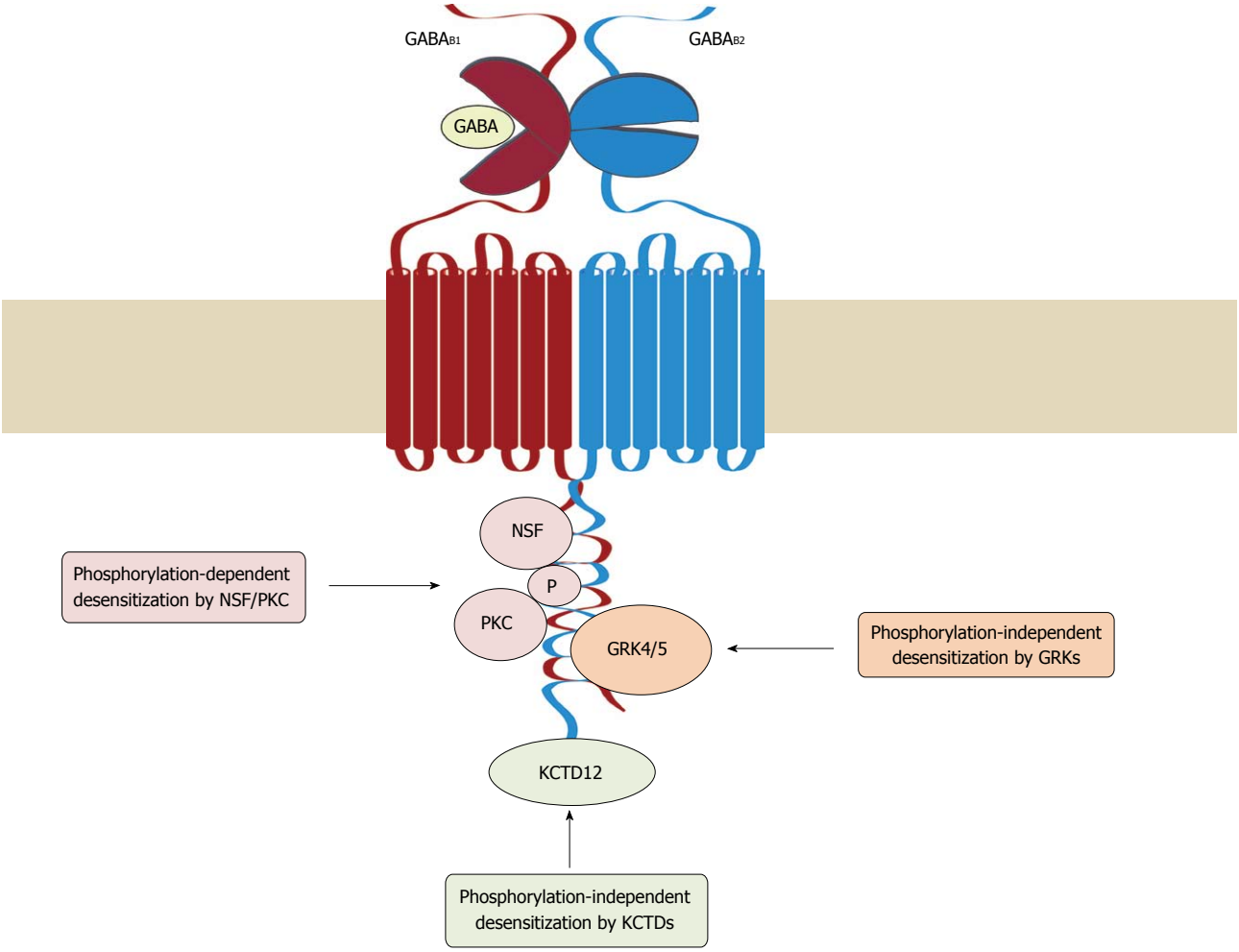

Figure 2 Mechanisms of GABA$_B$ receptor desensitization. Three distinct mechanisms have been so far implicated in the desensitization of GABA$_B$ receptors. In cerebellar granule cells, G protein receptor kinase (GRK) 4 and 5 associate with GABA$_B$ receptors and induce desensitization of the receptors in a phosphorylation-independent manner. In cortical and hippocampal neurons, desensitization of the receptors involves the interaction of NEM-sensitive fusion protein (NSF) with GABA$_{B1}$ and GABA$_{B2}$, which is thought to prime the receptor for phosphorylation by protein kinase C (PKC). Association of potassium channel tetramerization domain-containing (KCTD) proteins 12 and 12b with the C-terminus of GABA$_{B2}$ appears to render the receptor complex competent for desensitization. GABA: γ-Aminobutyric acid.

which does not follow the classical desensitization pattern of GPCRs described above (Figure 2). Distinct desensitization mechanisms for GABA$_B$ receptors may be operative in different neuronal populations.

A study conducted in mouse cerebellar granule cells showed that GRK4, which is mainly expressed in testes and cerebellum[69,76,77], promotes agonist-induced desensitization of GABA$_B$ receptors *via* direct association, but does not involve GABA$_B$ receptor phosphorylation[78]. These findings were confirmed by Kainaide *et al*[75], who demonstrated that the association of GABA$_{B2}$ with GRK4 or GRK5, but not GRK2, -3 or -6, leads to agonist-induced receptor desensitization in *Xenopus* oocytes and baby hamster kidney cells. Interestingly, GRK4 and -5-mediated desensitization was partially suppressed by application of S(+)-ketamine, which leads to inhibition of the GABA$_B$ receptors/GRK complex formation by an as yet unidentified mechanism[79]. It is currently not understood how GRK4 and -5 mediate desensitization of GABA$_B$ receptors. However, it might well be that the binding of GRK4 and -5 disrupts GABA$_B$ receptor/G-protein interaction.

On the other hand, for cortical and hippocampal neurons, a phosphorylation-dependent desensitization mechanism of GABA$_B$ receptors was reported[74]. This mechanism is based on the direct interaction of NEM-sensitive fusion (NSF) protein with the C-terminal domains of GABA$_{B1}$ and GABA$_{B2}$, which primes the receptor for recruitment of protein kinase C (PKC). The data indicate that the association of GABA$_B$ receptors with NSF is a prerequisite for recruiting PKC to the receptor upon agonist activation. PKC phosphorylates the receptor leading to its desensitization, and induces dissociation of NSF from the receptors. The precise roles of NSF and PKC in this complex process remain to be determined. NSF might be required for unmasking phosphorylation sites of the receptor or involved in PKC activation. In addition, it is unclear whether NSF dissociates from the receptor before desensitization occurs or whether releasing NSF initiates recovery of the receptor from desensitization.

Another factor determining desensitization of GABA$_B$ receptors was recently discovered by functional proteomics[80]. Members of the potassium channel tetramer-

ization domain-containing (KCTD) protein family were found to interact as tetramers with the C-terminus of GABA$_{B2}$, generating high-molecular mass protein complexes. Depending on the co-expressed KCTD subtype, distinct parameters of GABA$_B$ receptor function were affected, such as agonist potency, signaling onset or desensitization. Interestingly, only when GABA$_B$ receptors were co-expressed with KCTD-12 or -12b desensitization of GABA$_B$ receptors was observed, whereas in the presence of KCTD-8 or -16 the receptors displayed no desensitization[80]. This finding may explain the observation that GABA$_B$ receptor desensitization varies among different neuronal populations. One striking example is the ventral tegmental area (VTA). GABA$_B$ receptors expressed in GABAergic neurons of the VTA display baclofen-induced, largely non-desensitizing, currents, whereas in dopaminergic neurons of the VTA baclofen elicited desensitizing currents[81]. These findings suggest that the general ability of GABA$_B$ receptors to desensitize may be determined by the associated KCTD subtype, which may then recruit distinct desensitization mechanisms depending on the neuronal population.

Interestingly, PKA-dependent phosphorylation appears to counteract desensitization of GABA$_B$ receptors. Couve *et al*[71] showed that PKA exclusively phosphorylates serine 892 (S892) in the C-terminal domain of GABA$_{B2}$, resulting in reduced receptor desensitization. This effect on desensitization can be overcome by activation of the receptors, which results in inhibition of adenylyl cyclases, reduced cAMP levels and consequently diminished PKA activity and GABA$_{B2}$-S892 phosphorylation. The precise mechanism as to how PKA phosphorylation of GABA$_{B2}$-S892 affects desensitization of GABA$_B$ receptors remains unclear. There is an indication that it stabilizes cell surface GABA$_B$ receptors and thereby increases effector coupling[71,82]. This is, however, unlikely because it is now well accepted that prolonged agonist exposure does not trigger increased internalization of cell surface receptors[78,82-85]. However, GABA$_{B2}$-S892 phosphorylation provides a mechanism for regulating the extent of GABA$_B$ receptor desensitization by the activity of Gα_s-coupled GCPRs that enhance PKA activity.

Another kinase that is involved in regulating GABA$_B$ receptor activity is the 5'AMP-dependent protein kinase (AMPK). AMPK directly binds to the C-terminus of GABA$_{B1}$ and phosphorylates S917 and S783 in the C-terminal domains of GABA$_{B1}$ and GABA$_{B2}$, respectively[86]. Functional analysis revealed that phosphorylation of S783 resulted in a stabilization of baclofen-induced K^+ currents[86]. This effect has been shown to be of particular relevance in limiting neuronal cell death in experimental ischemia. Anoxic or ischemic conditions are associated with neuronal over-excitation, a decline in cellular adenosine-5'-triphosphate (ATP) and a rise in Ca^{2+} and AMP levels, which are all factors activating AMPK[87,88]. Under such conditions, increased phosphorylation of GABA$_{B2}$-S783 was detected along with an over-expression of a GABA$_{B2}$ mutant that cannot be phosphorylated at this site associated with increased neuronal death[86]. These findings support a mechanism in which AMPK functions as a metabolic sensor that detects severe cellular stress and phosphorylates, amongst others, GABA$_B$ receptors. This is thought to result in enhanced GABA$_B$ receptor signaling that counteracts over-excitation of the neuron and limits neuronal death.

REGULATION OF GABA$_B$ RECEPTORS BY TRAFFICKING

The lifecycle of a plasma membrane protein like the GABA$_B$ receptor starts with its synthesis at the rough ER where the nascent protein is co-translationally incorporated into the ER membrane. After folding, initial posttranslational processing, and assembly, the receptor is exported to the Golgi apparatus where it is further processed and finally transported *via* the trans-Golgi network to the plasma membrane. After a certain time span of function, the receptor is internalized and recycled back into the plasma membrane for another cycle of function, or is eventually degraded into lysosomes. To ensure a constant number of receptors in the plasma membrane for signaling, these trafficking events need to be precisely coordinated. On the other hand, regulation of each of the different trafficking steps permits adjusting the number of cell surface receptors, and thus signaling strength, according to the physiological requirements.

ER export of GABA$_B$ receptors

Little is known about the early stages in the lifecycle of GABA$_B$ receptors. So far it is clear that exit of heterodimeric GABA$_B$ receptors from the ER is controlled by an arginine-based ER retention/retrieval signal (RXR) present in the C-terminal domain of GABA$_{B1}$[64-66]. The mechanism that prevents cell surface trafficking of GABA$_{B1}$ appears to involve the coat protein complex I (COP I), which plays a central role in the retrograde transport of proteins from the Golgi apparatus back to the ER[89]. COP I binds to the ER retention/retrieval signal of GABA$_{B1}$ and shuttles monomeric GABA$_{B1}$ that reached the cis-Golgi apparatus back to the ER. Heterodimerization with GABA$_{B2}$ masks the ER retention/retrieval signal and permits forward transport[64-68]. In contrast to GABA$_{B1}$, monomeric GABA$_{B2}$ can leave the ER and reach the cell surface. However, it is assumed that the GABA$_{B2}$ expression level in the ER is a limiting factor for ER exit of the heterodimeric GABA$_B$ receptors. This mechanism is thought to ensure that only correctly folded and assembled (i.e., functional) receptors are exported to the cell surface.

Endocytosis of GABA$_B$ receptors

There are two principal mechanisms by which GPCRs are internalized from the plasma membrane, constitutive endocytosis and agonist-induced endocytosis. Constitutive endocytosis constantly removes receptors from the

cell surface, whereas agonist-induced endocytosis initiates removal of receptors from the plasma membrane upon activation of the receptors and ensures fast termination of signaling. It is now well established that GABA$_B$ receptors undergo constitutive endocytosis, whereas the presence of agonist-induced internalization of the receptors is less clear.

Heterologously expressed, as well as neuronal, GABA$_B$ receptors display fast constitutive internalization, as evidenced by distinct experimental approaches including immunofluorescence staining and microscopy, live cell imaging and cell surface biotinylation methods[83,84,90-93]. Constitutive internalization of GABA$_B$ receptors is a fast process, as shown by the rapid loss of labeled receptors from the cell surface, which reaches a plateau after 10-30 min (40% of labeled receptors remain at the cell surface), with rates of internalization of ranging from 2-10 min[92-94]. GABA$_B$ receptors internalize as heterodimers and are not dissociated into its subunits prior to endocytosis[84,90,92,95]. The rate of internalization appears to be determined by GABA$_{B2}$. GABA$_{B1}$, which contains an inactivated ER retention signal so that it is exported to the plasma membrane, displays a considerably faster rate of internalization than the GABA$_{B1,2}$ heterodimer[92]. This is due to a dileucine motif within the coiled-coil domain of GABA$_{B1}$, which gets masked upon assembly with GABA$_{B2}$.

The data so far suggest that for endocytosis, GABA$_B$ receptors are recruited to clathrin-coated pits and internalized in a dynamin-dependent manner[83,90,95]. Clathrin-coated pits are composed of clathrin heavy and light chains that form a polymeric lattice and contain numerous adaptor and endocytic accessory proteins. For endocytosis, the cargo-loaded clathrin-coated pit invaginates and is eventually released from the plasma membrane in a GTP-dependent reaction mediated by dynamin[96]. There is evidence based on colocalization and immunoprecipitation data that GABA$_B$ receptors interact with the AP2 adaptor, which is one of the adaptor complexes that recruit membrane proteins to clathrin-coated pits[83,84,90].

Colocalization studies with marker proteins for various endosomal compartments revealed that endocytosed GABA$_B$ receptors first enter early endosomes and are then either sorted to Rab4 or Rab11-positive recycling endosomes, or to Rab7-positive late endosomes, and finally to lysosomes for degradation[84,90,92,95,97,98].

In addition to the colocalization data, there is also functional evidence that endocytosed GABA$_B$ receptors constitutively recycle back to the cell surface. Using immunofluorescence staining and tagged GABA$_B$ receptors transfected into hippocampal neurons Vargas *et al*[90] showed that a significant fraction of endocytosed receptors recycle back to the cell surface. Quantitative cell surface biotinylation and immunofluorescence-based methods indicate that the vast majority of native GABA$_B$ receptors in cortical neurons are rapidly recycled to the plasma membrane. After 15 min, about half of the internalized receptors have recycled back to the cell surface, and after 30 min this has increased to the majority of the receptors[84,94].

In summary, the current data indicate that GABA$_B$ receptors constitutively internalize at a high rate *via* the classical clathrin-dependent pathway and rapidly recycle back to the cell surface. Since endocytosis and recycling are highly energy-consuming processes, this mechanism is most likely of significant physiological relevance. The most obvious explanation is that a high rate of constitutive internalization and recycling generates a pool of intracellular receptors that can be immediately inserted into the plasma membrane to increase the cell surface number of receptors by increasing the rate of recycling while leaving the rate of internalization constant. In the case of synaptic AMPA receptors, such a mechanism has been proposed to contribute to increasing the level of the receptors during the early phase of long-term potentiation, which is thought to underlie learning and memory formation[99].

Degradation of GABA$_B$ receptors

Most cell surface receptors are eventually degraded in lysosomes, the major catabolic cellular compartment. After endocytosis, the endocytic vesicles carrying the receptors fuse with early endosomes, which then mature to late endosomes containing the material destined for degradation. Mature late endosomes are competent to fuse with lysosomes that contain a variety of hydrolases for the breakdown of all kinds of macromolecules[100].

There is now solid data that, at the end of their lifetime, GABA$_B$ receptors are endocytosed and degraded in lysosomes. This is evidenced by the intracellular accumulation of internalized GABA$_B$ receptors upon inhibition of lysosomal function[83,84,101] and the colocalization of intracellular GABA$_B$ receptors with marker proteins for late endosomes and lysosomes[84,92]. GABA$_B$ receptors are most likely sorted by the ESCRT (endosomal sorting complex required for transport) machinery to lysosomes, because the knockdown of tumor susceptibility gene 101 (TSG101), an integral component of the ESCRT machinery, prevents degradation of the receptors[101]. Three distinct ESCRT complexes sequentially target mono- and K63-linked polyubiquitinated membrane proteins to late endosomes[102]. However, it remains to be shown whether GABA$_B$ receptors are ubiquitinated and whether ubiquitination serves as a lysosomal sorting signal.

Another unresolved issue is how the decision is made as to whether a receptor is sorted to the degradation pathway. As discussed above, the vast majority of endocytosed GABA$_B$ receptors recycle back to the plasma membrane and only few are degraded. However, pharmacological inhibition of recycling leads to rapid lysosomal degradation of the receptors (about 50% of the total receptor population within 30 min)[84]. This indicates that recycling and degradation of GABA$_B$ receptors is tightly controlled, and decreasing the rate of recycling constitutes a mechanism to rapidly reduce the receptor number (discussed below).

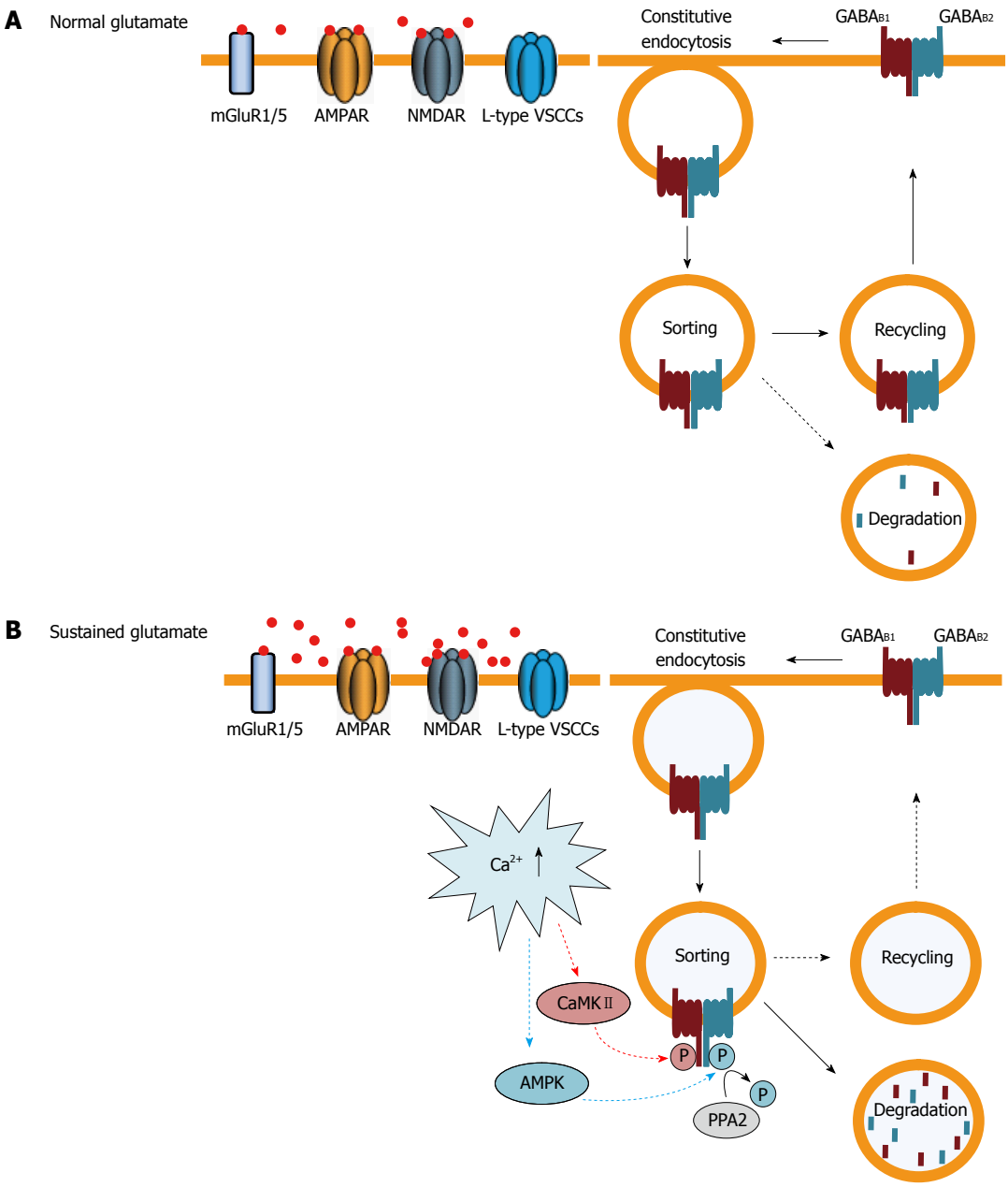

Figure 3 Regulation of cell surface GABA$_B$ receptors by trafficking. A: Under normal conditions GABA$_B$ receptors are constitutively internalized and recycled back to the plasma membrane. Only a small fraction of receptors are sorted to lysosomes for degradation; B: Sustained activation of glutamate receptors [primarily 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors] and L-type voltage-gated Ca^{2+} channels raises intracellular Ca^{2+} levels. This induces phosphorylation of GABA$_{B1}$ at serine 867 by calmodulin-dependent protein kinase II (CaMK II) and of GABA$_{B2}$ at serine 783 by adenosine monophosphate (AMP) kinase, followed by slow dephosphorylation, by protein phosphatase 2 (PPA2). These events shift the recycling/degradation equilibrium towards degradation so that the majority of GABA$_B$ receptors are no longer recycled, but instead degraded in lysosomes. Since constitutive endocytosis of the receptors remains unaffected, this mechanism results in a rapid down-regulation of GABA$_B$ receptors. AMPK: 5'AMP-dependent protein kinase; GABA: γ-Aminobutyric acid; VSCCs: Voltage-sensitive calcium channels.

Regulation of cell surface GABA$_B$ receptors by glutamatergic excitatory activity

GABA$_B$ receptors control glutamate signaling *via* presynaptic and postsynaptic mechanisms. They are abundantly expressed at glutamatergic synapses[2-5,103] where they are activated by GABA spillover from adjacent GABAergic terminals and inhibit glutamate release[8,104-106]. This limits activation of postsynaptically located excitatory glutamate receptors (AMPA/kainate and NMDA receptors). Although GABA$_B$ receptors are also located in close proximity to AMPA and NMDA receptors they do not appear to directly modulate AMPA and NMDA receptor excitatory postsynaptic currents (EPSCs)[105]. However, activation of postsynaptic GABA$_B$ receptors seem to limit Ca^{2+}-influx through NMDA receptors by inhibition of the cAMP/PKA signaling pathway, which normally enhance NMDA receptor Ca^{2+} conductance[105].

Besides the prominent regulation of glutamate signaling by GABA$_B$ receptors, there is now evidence that glutamatergic activity, in return, may affect GABA$_B$ receptor expression to attenuate inhibitory control (Figure 3). Application of glutamate to cultured neurons dra-

matically down-regulates cell surface GABA$_B$ receptors and GABA$_B$ receptor-activated currents[90,94,97,98]. Specific activation of AMPA receptors[94] or NMDA receptors[97,98] was sufficient to induce the down-regulation of GABA$_B$ receptors. Interestingly, the kinetics of AMPA-induced down-regulation of GABA$_B$ receptors was significantly slower than that induced by glutamate and was accelerated upon co-activation of group I metabotropic glutamate receptors[94]. These findings indicate that beside the ionotropic AMPA and NMDA receptors, metabotropic glutamate receptors also contribute to the glutamate-induced down-regulation of GABA$_B$ receptors. The underlying mechanism of this rapid down-regulation of GABA$_B$ receptors is a shift of the recycling/degradation equilibrium towards lysosomal degradation[94,97]. Glutamate application reduced the rate of GABA$_B$ receptor recycling without altering the rate of their internalization and was fully restored after inhibition of lysosomal degradation. The precise intracellular signaling cascade leading to the glutamate-induced shift in sorting the GABA$_B$ receptors preferentially to the degradation pathway is currently not fully resolved. It is clear that the down-regulation of GABA$_B$ receptors depends on the influx of Ca^{2+}[94,98], which is most likely mediated by L-type voltage-gated Ca^{2+} channels[94]. Two downstream effector systems were identified to be involved in the down-regulation of GABA$_B$ receptors (Figure 3). One depends on phosphorylation of serine 867 (S867) in GABA$_{B1}$ by Ca^{2+}/calmodulin-dependent protein kinase Ⅱ (CaMK Ⅱ)[98]. The other involves phosphorylation of serine 783 (S783) in GABA$_{B2}$ by AMP kinase and subsequent dephosphorylation by protein phosphatase 2A (PP2A)[97]. Mutational inactivation of each phosphorylation site prevented glutamate-induced down-regulation of GABA$_B$ receptors. However, while the cell surface expression of the receptors containing the mutant GABA$_{B1}$(S867A) was normal[98], the mutant GABA$_{B2}$(S783A) was expressed to a significantly lesser level in the plasma membrane[97]. This suggests that phosphorylation of S783 in GABA$_{B2}$ is involved in sorting the receptors to the recycling pathway, while phosphorylation of S867 in GABA$_{B1}$ may constitute a direct signal for sorting the receptors to lysosomal degradation. Alternatively, phosphorylation of GABA$_{B1}$(S867) may be required for dephosphorylation of S783 in GABA$_{B2}$, for instance by recruiting PPA2 to the receptor. In this respect it would be very interesting to test whether phosphorylation of GABA$_{B1}$(S867) by CaMK Ⅱ is required for dephosphorylation of GABA$_{B2}$(S783) by PPA2.

What is the physiological relevance of this mechanism? Since glutamate-induced down-regulation of GABA$_B$ receptors has so far only been studied in cultured neurons, the role of this process *in vivo* remains to be shown. However, there are physiological, as well as pathological, conditions involving sustained activity of glutamate receptors where this mechanism might be operative. Under pathological conditions associated with excessive activation of glutamate receptors, such as ischemia, down-regulation of GABA$_B$ receptors results in diminished inhibitory control and may further enhance excitotoxicity and neuronal cell death. This view is supported by an *in vitro* model of ischemia where total GABA$_{B2}$ protein levels were found to be strongly reduced 60 min after the ischemic insult[107]. Likewise, in an *in vivo* model of hypoxia/ischemia, significantly reduced levels of GABA$_B$ receptors were detected[108].

Under normal physiological conditions, glutamate-induced down-regulation of GABA$_B$ receptors may contribute to the process of long-term potentiation, which is thought to be the molecular basis for learning and memory formation, as long-term potentiation is associated with sustained activity of glutamate receptors[109]. In this scenario, enhanced glutamatergic activity would induce the down-regulation of GABA$_B$ receptors and consequently relieve the synapses from inhibition, resulting in a further increase of synaptic excitability.

CONCLUSION

Trafficking events play a pivotal role in the cell surface availability of receptors and largely determine their signaling strength. Currently, we are only beginning to identify and understand the trafficking mechanisms of GABA$_B$ receptors and how cell surface expression of the receptors is regulated. In particular, we almost completely lack knowledge on forward trafficking of GABA$_B$ receptors from the ER *via* the Golgi network to the plasma membrane. In addition, mechanisms on the targeting of the receptors to specific sites in the neuron are unknown. There is an initial indication that GABA$_{B1}$ may be transported independent of GABA$_{B2}$ within the ER into dendrites and are then assembled and exported to the plasma membrane[110]. This finding implies that heterodimerization of GABA$_B$ receptors is a spatially and temporally controlled mechanism, and would provide an additional level to regulate cell surface expression of the receptors. It is now clear that GABA$_B$ receptors are constitutively endocytosed *via* the clathrin and dynamin-dependent pathway, and are predominantly recycled back to the plasma membrane with only a minor fraction being degraded in lysosomes. The equilibrium of sorting the receptors to the recycling and degradation pathway appears to be controlled by phosphorylation/dephosphorylation events and regulated by changes in neuronal activity associated with increased influx of Ca^{2+}. It will be a major future effort to unravel the mechanisms involved in trafficking, sorting and degradation of GABA$_B$ receptors and how they are regulated by physiological and pathological stimuli. It is now well established that receptor trafficking regulates signal transduction and that disturbances in these mechanisms may contribute to disease states[111]. Since GABA$_B$ receptors have been implicated in a variety of neurological disorders-ranging from epilepsy, addiction, schizophrenia, depression, anxiety to chronic pain-it is likely that altered GABA$_B$ receptor trafficking is involved, at least to some extent, in these diseases. We expect that a deeper knowl-

edge of the trafficking mechanisms of GABA$_B$ receptors under physiological and pathological conditions will provide the basis for the development of novel and highly selective future therapeutic interventions.

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TOPIC HIGHLIGHT

Xiaotian Zhong, PhD, MPH, *Series Editor*

Pharmacokinetics and toxicology of therapeutic proteins: Advances and challenges

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Abstract

Significant progress has been made in understanding pharmacokinetics (PK), pharmacodynamics (PD), as well as toxicity profiles of therapeutic proteins in animals and humans, which have been in commercial development for more than three decades. However, in the PK arena, many fundamental questions remain to be resolved. Investigative and bioanalytical tools need to be established to improve the translation of PK data from animals to humans, and from *in vitro* assays to *in vivo* readouts, which would ultimately lead to a higher success rate in drug development. In toxicology, it is known, in general, what studies are needed to safely develop therapeutic proteins, and what studies do not provide relevant information. One of the major complicating factors in nonclinical and clinical programs for therapeutic proteins is the impact of immunogenicity. In this review, we will highlight the emerging science and technology, as well as the challenges around the pharmacokinetic- and safety-related issues in drug development of mAbs and other therapeutic proteins.

Key words: Pharmacokinetics; Toxicology; Therapeutic proteins; Biotherapeutics; Monoclonal antibodies

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INTRODUCTION

Biotherapeutics are therapeutic agents that are produced from living organisms or their products (including recombinant DNA technology, biotechnological manufacturing, and chemical synthesis using nucleotides or amino acids) and include monoclonal antibodies (mAbs), antibody fragments, peptides, replacement factors, fusion proteins, oligonucleotides and DNA preparations for gene therapy, as well as vaccines. This is a rapidly growing class of therapeutics for a broad spectrum of indications, ranging from oncology and autoimmunity to orphan and genetic diseases.

Pharmacokinetics (PK) refers to the biological processes determining absorption, distribution, metabolism and excretion (ADME) of a drug in an organism. Phar-

macodynamics (PD) refers to drug action on a living organism, including the pharmacologic response and the duration and magnitude of response observed relative to the concentration of the drug at an active site in an organism. Significant progress has been made in understanding PK, PD, as well as toxicity profiles of biotherapeutics in animals and humans, especially for proteins and mAbs, which have been in commercial development for more than three decades.

However, many fundamental ADME questions remain to be resolved. Investigative tools need to be established to improve the translation of PK data from animals to humans and from *in vitro* assays to *in vivo* readouts, which would ultimately lead to a higher success rate in drug development and provide safer and more effective drugs. In addition, commercial considerations, such as cost of goods and convenience (including less frequent dosing and self-administration), drive the need for a continuous advancement of mechanistic ADME evaluations and structure activity relations (SAR) for protein therapeutics in order to enable rational protein engineering of desired ADME profiles.

The goal of this review is to highlight emerging science and technology, as well as challenges around the pharmacokinetic- and safety-related issues in drug development of mAbs and other therapeutic proteins.

WHAT IS KNOWN

Absorption, distribution, metabolism, and excretion

Absorption: Unlike small molecules, which are frequently delivered *via* oral administration, therapeutic proteins are almost exclusively administered by parenteral routes, such as intravenous (IV), subcutaneous (SC) or intramuscular (IM) injection. Molecular size, hydrophilicity, and gastric degradation are the main factors that preclude gastrointestinal (GI) absorption of therapeutic proteins[1]. Pulmonary delivery with aerosol formulations or dry powder inhalers has been used for selected proteins, e.g., exubera (TM)[2,3]. Intravitreal injections have been used for peptides and proteins that require only local activity[4], as well as for antisense oligonucleotides[5].

From the convenience standpoint, SC administration of therapeutic proteins is often a preferred route. In particular, the suitability of SC dosing for self-administration translates into significantly reduced treatment costs. Absorption of therapeutic proteins from the SC injection site tends to be slow compared to small molecules, and the absorption rates depend on the size of the molecule. For example, following SC administration, the time to reach the maximum systemic concentration (T_{max}) in humans for peptides is in the range of hours, while the T_{max} for mAbs is generally several days[6-8]. For mAbs, SC bioavailability for currently marketed products is in the range of 24% to 95% in humans[1,9,10] (Table 1).

In general, factors influencing SC absorption parameters are believed to include intrinsic subject characteristics for a given species (such as body weight, sex, age, activity level); species characteristics with regard to skin morphology and physiology (such as the presence or absence of the *panniculus carnosus* muscle in the skin, maximum SC injection volume which varies by species, catabolic capacity at injection site and/or in the lymphatic system, SC blood flow); drug substance and product characteristics [presence of an Fc (see below), target interactions, charge, formulation, dose concentration, total dose]; and mode of administration (injection site, injection time, depth of injection, anesthesia status), as discussed in references[1,9-14]. However, surprisingly little is known about the mechanisms and pathways of SC absorption and which pathways are affected by a particular factor described above. The emerging science and issues around the mechanisms and factors involved in SC absorption that are not known are further discussed in the "WHAT IS NOT KNOWN" section.

Distribution: Tissue distribution of therapeutic proteins usually is limited because of the size of the molecules, which is in contrast to small molecule drugs that tend to have higher tissue penetration. In addition to size, other factors that influence the tissue distribution of a therapeutic protein include the physical and chemical properties (e.g., shape and charge), binding properties (e.g., receptor-mediated uptake), the route of administration (e.g., IV *vs* SC, formulation), and the production process (which may affect post-translational modifications, such as glycosylation). These factors can be modulated *via* rational design to modulate tissue penetration properties of a biotherapeutic molecule. For example, a modeling analysis of the effects of molecular size and binding affinity on tumor targeting was conducted to guide the design of new therapeutic protein drugs[15,16]. A similar approach was used to engineer a novel human IL-2 analog that antagonizes the IL-2 receptor[17]. Tissue- or target-specific delivery of therapeutic biologics is a challenging, yet a very attractive area for pharmaceutical research.

For mAbs and other large therapeutic proteins, the reported volume of distribution after IV administration is close to the plasma volume, suggesting limited distribution into tissues[18]. However, tissue distribution studies with radiolabeled mAbs indicate that many tissues are exposed to mAbs, but at lower concentrations than usually seen in systemic circulation[19]. Despite the limited tissue penetration, large biotherapeutics, such as mAbs, often do have efficacy even in cases when the site of action is believed to be the tissue, indicating that it is possible to design a therapeutic regimen such that the tissue exposure is adequate to modulate the target at the site of action. The therapeutic areas for tissue-acting biotherapeutics are diverse and examples for autoimmunity and oncology are presented in recent reviews[20,21].

Once in the tissue vasculature, the common transport mechanisms for proteins from systemic circulation across capillary endothelial cells and into tissues are listed in Table 2[22]. The uptake of therapeutic proteins into cells may be carried out *via* receptor-mediated transporters (e.g.,

Table 1 Examples of proteins and peptides administered subcutaneously[10]

INN/BAN (description)	Trade name	MW (kDa)	Absolute bioavailability[1]	SC animal models used in drug development
Buserelin acetate (LH-releasing hormone analog)	Suprefact	1.30	Human: 70%	Pharm: rat, hamster, guinea pig, rabbit, dog and monkey Tox: mouse, rat, rabbit and dog
Pramlintide acetate (amylin analog)	Symlin	3.95	Human: 30 to 40%	Pharm: rat and dog PK: mouse, rat, rabbit and dog Tox: mouse, rat, rabbit and dog
Insulin lispro (insulin analog)	Humalog	5.81	Human: 55 to 77%	Pharm: rat, rabbit, dog and pig PK: rat and dog Tox: rat, rabbit and dog
Insulin glulisine (insulin analog)	Apidra	5.82	Human: about 70% Dog: 42% Rat: 96%[2]	Pharm: rat and dog PK: rat and dog Tox: mouse, rat, rabbit and dog
Insulin glargine (insulin analog)	Lantus	6.06	Precipitates in skin-slow uptake in human, dog and rat	Pharm: mouse, rat, guinea pig, rabbit and dog PK: rat and dog Tox: mouse, rat, guinea pig, rabbit and dog
Mecasermin (IGF-1)	Increlex	7.65	Human: about 100% Rabbit: 47% Rat: 38 to 57%	Pharm: mouse, rat, rabbit and monkey PK: rat, rabbit, dog and monkey Tox: rat, dog, rabbit and monkey
IFNβ-1b (cytokine)	Betaseron	18.5	Human: 50% Monkey: 31 to 44%	Pharm: monkey PK: monkey Tox: rabbit and monkey
Somatropin (GH)	Nutropin	22	Human: 81%	Pharm: rat PK: rat and monkey Tox: mouse, rat, dog and monkey
IFNβ-1a (cytokine)	Rebif	22.5	Human: 6 to 62% Monkey: 12 to 38% Rat: 16%	Pharm: mouse and monkey PK: rat and monkey Tox: monkey
PEG-IFNα-2b (cytokine variant)	PEG-Intron	31	Monkey: 57 to 89% Rat: 43 to 51%	Pharm: rat and monkey PK: rat and monkey Tox: mouse, rat, rabbit and monkey
Pegfilgrastim (PEG-G-CSF)	Neulasta	39	Monkey: 49 to 68% Rat: < 10% to 30%	Pharm: mouse, rat and dog PK: mouse, rat and monkey Tox: rat and monkey
Pegvisomant (PEG-GH)	Somavert	42, 47 and 52[3]	Human: 49 to 65% Monkey: 70 to 81% Mouse: 45 to 73%	Pharm: mouse and monkey PK: mouse, rat, rabbit and monkey Tox: mouse, rat, rabbit and monkey
PEG-IFNα-2a (cytokine variant)	Pegasys	60	Human: 61 to 80%	Pharm: mouse PK: rat and monkey Tox: mouse, rat and monkey
Certolizumab pegol (PEG-anti-TNFα Fab' fragment)	Cimzia	91	Human: 76 to 88% Rat: 24 to 34%	PK: rat and monkey Tox: monkey
Canakinumab (anti-IL-1β mAb)	Ilaris	145	Human: 63 to 67% Monkey: 60%	Pharm: mouse, rat and monkey (marmoset) PK: mouse and monkey Tox: mouse and monkey
Adalimumab (anti-TNF mAb)	Humira	148	Human: 64% Monkey: 96%	PK: monkey Tox: rabbit and monkey
Omalizumab (anti-IgE mAb)	Xolair	149	Human: 53 to 71% Monkey: 64 to 104% Mouse: 90%	Pharm: monkey PK: mouse and monkey Tox: monkey
Golimumab (anti-TNF mAb)	Simponi	150	Human: 53% Monkey: 77%	PK: monkey Tox: mouse and monkey
Ustekinumab (anti-p40 mAb)	Stelara	150	Human: 24 to 95% Monkey: 97%	Pharm: monkey PK: monkey Tox: monkey
Etanercept (TNF receptor-Fc-IgG1 fusion protein)	Enbrel	150	Human: 76% Monkey: 73% Mouse: 58%	Pharm: mouse PK: mouse, rat and monkey Tox: mouse, rat, rabbit and monkey
Rilonacept (IL-1 inhibitor, fusion protein)	Arcalyst	251	Human: 43% Monkey: 70% Rat: 60% Mouse: 78%	Pharm: mouse and monkey PK: mouse, rat and monkey Tox: monkey

[1]Systemic dose following subcutaneous (SC) injection relative to systemic dose following intravenous injection; [2]Assumes linearity of AUC/dose; [3]Product is a mixture of three distinct protein variants. GH: Growth hormone; LH: Luteinizing hormone; MW: Molecular weight; Pharm: Pharmacology; PK: Pharmacokinetics; Tox: Toxicology (including safety pharmacology); INN: International nonproprietary name; BAN: British approved name; SC: Subcutaneous; PEG: Polyethylene glycol; IFN: Interferon; TNF: Tumor necrosis factor; IL: Interleukin.

Table 2 Transport mechanisms for proteins from systemic circulation across capillary endothelia[22]

Type of capillary endothelium	Barrier/transport mechanism	Particle size subject to passage	Typical tissues
Continuous (non-fenestrated)	Basal lamina membrane supported by collagen	50-110 nm	Muscle, central nervous system, bone, skin, cardiac muscle
Fenestrated	Large pores, open fenestrae, intracellular junctions, basal lamina	50-800 nm	Renal glomeruli, intestinal villi, synovial tissue, endocrine glands, choroid plexus (brain)
Discontinuous (sinusoidal)	Large pores (fenestrae), pinocytotic vesicles	1000-10 000 nm	Liver, spleen, bone marrow, postcapillary venules of lymph nodes

Fc receptors, often leading to recycling of the molecule) or other internalization processes, such as endocytosis or pinocytosis (often leading to degradation of the molecule). Target-mediated tissue distribution has also been reported for some mAbs[23,24]. High drug concentrations in kidney and liver have been reported for peptides, low molecular proteins, and oligonucleotides[25,26]. Upon tissue uptake, metabolism/catabolism of protein drugs will occur in tissues before the remnants of the molecules are excreted from the body as smaller peptides and amino acid degradants, or they are recycled for synthesis into other proteins in the body.

The high vascular concentrations of the test article provide a potential source for interference of tissue drug concentrations, and should be considered when interpreting biodistribution data for therapeutic proteins. To minimize vascular interference, whole body perfusion is often performed before tissue analysis in biodistribution studies of therapeutic proteins, especially for rodents[19]. Other methods to correct for the contribution of residual drug in tissue blood vessels, such as the use of radiolabeled erythrocytes or the use of dual isotopes of ^{125}I- and/or ^{131}I-labeled proteins, have also been applied[27,28].

Metabolism/Catabolism: Therapeutic proteins are removed from circulation or interstitial fluid *via* several pathways: degradation by proteolysis, Fcγ receptor-mediated clearance, target-mediated clearance, nonspecific endocytosis, and formation of immune-complexes (ICs) followed by complement- or Fc receptor-mediated clearance mechanisms. While proteolysis occurs widely in the body, its kinetics and mechanistic details are poorly understood, especially for large therapeutic proteins such as mAbs. *In vitro* incubations with plasma, liver and kidney homogenates have been used for peptides to facilitate the selection of leads in discovery research; however the *in vitro* in *vivo* correlations for such an approach remain to be established (see additional discussions in the "WHAT IS NOT KNOWN" section). Once taken up into cells, a biotherapeutic may be metabolized to peptides or amino acids. This may occur in circulation by circulating phagocytic cells or by their target antigen-containing cells, or may occur in tissues by various cells. For molecules with an Fc (including therapeutic mAbs, endogenous Abs, and fusion proteins), binding of the Fc domain to Fc gamma-receptors may result into the internalization and subsequent degradation by lysosomes in the reticuloendothelial system (e.g., macrophages and monocytes)[1,29,30].

Alternatively, molecules with an Fc may be protected from degradation by binding to protective receptors [i.e., the neonatal Fc-receptor (FcRn)] in endothelial cells, explaining the long half-lives (up to 4 wk) of these proteins. The following references provide excellent reviews on the scholarship in this field[1,31-33]. The FcRn receptor is a 52-kDa membrane-bound heterodimeric glycoprotein comprising a heavy chain and a light chain (beta2-microglobulin). Structurally, the FcRn receptor varies only subtly from conventional major histocompatibility complex (MHC) class I proteins protein. Its physiological function and expression in different tissues have been described[1,31-33]. In particular, the FcRn receptor, located in endosomes of endothelial cells, is known to bind to the Fc domain of IgG at pH 6.0-6.5, but only weakly or not at all at pH 7.0-7.5. This unique property allows FcRn to protect Fc-containing molecules from degradation by binding to them in acidic endosomes after uptake into endothelial cells *via* nonspecific endocytosis or fluid-phase pinocytosis. The IgG-FcRn complex is then transported back to the cell surface and disassociated at physiological pH, releasing the intact Fc-containing molecule back to the circulation. In contrast, Fc-containing molecules that are not bound to FcRn are degraded to amino acids by lysosomes in the cells. The correlation between FcRn binding affinity and systemic half-live has been investigated for a number of mAbs[33-43]. While the contribution of FcRn in prolonging half-lives of Fc-containing proteins is well recognized, other factors may also play a role in determining the elimination rate of these proteins, because the binding affinity to FcRn alone could not explain the variation of half-lives observed for all approved Fc-containing therapeutic proteins (see additional discussions in the "WHAT IS NOT KNOWN" section).

Target-mediated clearance is one of main causes of non-linear elimination kinetics. Upon binding to target on cells, the therapeutic proteins are internalized into the cells and subjected to degradation in lysosomes. For targets such as the endothelial growth factor receptor (EGFR), target-mediated clearance is the predominant clearance pathway at clinical doses, as illustrated by the nonlinear kinetic characteristics of cetuximab[44]. Target-mediated clearance could be demonstrated by comparing the disposition kinetics between normal healthy animals *vs* animals over expressing the target[23,45]. PK/PD models are usually established to describe saturable kinetic profiles that are associated with the target-mediated clearance in humans[46-50]. Formation of anti-drug antibodies (ADA) followed by formation of biotherapeutic/ADA ICs, is another main cause for the non-linear elimination

kinetics, including time-dependent clearance, which is often evidenced by a rapid concentration drop in the PK profiles (discussed below).

It should also be noted that many general factors that contribute to inter-subject variability in PK profiles for small molecule compounds may also apply to therapeutic proteins. These factors can be categorized into intrinsic factors (such as age, sex, body weight, activity level, renal and hepatic impairment) and to extrinsic factors (e.g., concomitant drugs, diet) and there are several examples in the literature describe the role of some of these factors for mAbs[51,52].

Excretion: Renal excretion is thought to play an important role in the elimination of protein degradation products and low molecular weight (MW) biologics (MW < 30 kDa). The process of renal filtration, transport, and metabolism of low-MW proteins has been well discussed in literature[26]. Proteins are hindered at the glomerular filter in proportion to their molecular size, structure, and net charge. However, the mechanisms of reabsorption of peptides and proteins in the kidney need further investigation.

When radiolabeled mAbs or Fc fusion proteins were used in animal disposition studies, a majority of the radioactive dose was recovered in the urine[19]. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and trichloroacetic acid (TCA) soluble counts indicated that the radioactive materials in urine were associated with low molecular fragments, suggesting that the excretion of intact parent drug was negligible. Biliary excretion of therapeutic proteins, such as insulin and epidermal growth factor has been reported[53]. It appeared that proteins were subjected to degradation in the liver, and the degradants were subsequently excreted into bile[54].

It has also been reported that plasma protein binding plays an important role in the tissue distribution of several new modalities of biologic therapeutics (e.g., oligomers), resulting in altered excretion profiles. Modification of the lipophilicity of the backbone for oligomers has been used to prolong the *in vivo* half-life by increasing plasma protein binding in order to reduce the renal excretion[5].

Anti-drug antibodies

Immunogenicity, specifically formation of ADAs, is one of the major complicating issues in nonclinical and clinical programs for therapeutic proteins. There are many factors that contribute to the ability of a therapeutic protein to elicit ADA production[55]. Intrinsic factors affecting immunogenicity are protein sequence (including similarity to endogenous proteins and the presence of T and B cell epitopes), post-translational modification (glycosylation, oxidation), and tertiary structure (including aggregation propensity). Extrinsic factors include the route, dose, and type of formulation (that may affect aggregation), production process (that may affect both aggregation and post-translational modifications), impurities, subject characteristic (disease population, inflammation status, concomitant medications), as well as drug pharmacology (specifically related to immunosuppression). All of the above factors are thought to contribute to variability in ADA responses observed across the biologic modalities, species, and subjects.

ADA may affect both the PK and PD profiles of therapeutic proteins by introducing additional (IC-dependent) clearance and distribution pathways and by modulating biological activity, including neutralization of the test article. In the case of replacement proteins, the ADA can result in neutralization of the endogenous protein as well, as has been described with erythropoietin[56,57] and factor Ⅷ[58] replacement factors.

When a drug/ADA immune complex is formed, the clearance of a therapeutic protein within the IC may be much faster compared to unbound drug, explaining a rapid concentration drop in PK profiles. It is believed that the clearance of IgG-containing ICs (which would include a drug bound to ADA) occurs primarily in the liver[59-63]. This can be facilitated by red blood cells, which can bind ICs in the circulation (*via* the complement receptor 1) and deliver them to the tissue macrophages of the mononuclear phagocyte system (such as Kupffer cells) in the liver[60]. Because the extent and rate of IC formation varies among human subjects, the IC-related clearance could be considered as a major contributor to the inter-subject variability in clinical and nonclinical PK profiles for therapeutic proteins.

Under some circumstances, ICs (including ADA-bound therapeutic proteins) might not be transported to the liver and cleared properly[59]. Factors that could influence this phenomenon include the IC characteristics (such as nature and quantity of the antigen and the antibody response, including antibody isotype and antigen/antibody stoichiometry) and the state of the systems involved in IC clearance and transport (for example expression of complement components, complement receptors, liver phagocytic system, red blood cells). In these cases, the deposition of circulating complement-fixing IC in various organs (such as the kidney) is observed, with important consequences for safety assessments of biotherapeutics. The impact of ADA on toxicology and PK-PD of therapeutic proteins is further discussed below in the "WHAT IS NOT KNOWN" section.

Glycosylation

Glycosylation, most frequently at asparagine residues ("N-linked") and at serine or threonine residues ("O-linked"), is the most common, complex, and heterogeneic post-translational modification that occurs on endogenous and therapeutic proteins. Recent reviews by Sola *et al*[64] and Li *et al*[65] summarize the current knowledge in this field. The inter- and intra-product heterogeneity in glycosylation profile can arise from the variability in glycan type and structure (including degree of branching), the site of attachment, and the degree of occupancy and can, in part, be controlled by the production system and conditions (such cell-type, cell culture media, and purification process). The glycosylation of proteins is important from the

ADME and efficacy standpoint, because improperly glycosylated proteins, whether endogenous or exogenously produced biotherapeutics, may be rapidly cleared from the circulation by specific receptor-based mechanisms, such as high mannose receptor or asialoglycoprotein receptor, and because glycosylation may directly affect biological activity of a biotherapeutic. For many approved protein drugs, clinical efficacy depends on proper glycosylation[64,65]. The ongoing research in the field is discussed below in the "WHAT IS NOT KNOWN" section.

Toxicology

In the past three decades of the development of biotherapeutics, the toxicity of the molecules and the methods and studies by which to measure such toxicity have been refined. In some cases (for example, for mAbs and fusion proteins that block cytokine pathways), no effects may be seen. When effects are seen in toxicity studies with biotherapeutics, in almost all cases the findings have been linked to target-mediated effects. In some cases these target-mediated effects may be undesirable, and are considered to be a result of exaggerated pharmacology. In this regard, they may not be considered to represent primary toxicity.

There are many examples of on-target pharmacologic effects that can be undesirable. For example, a variety of tumor necrosis factor-α (TNF) inhibitors are used to treat inflammatory and autoimmune diseases, including rheumatoid arthritis, psoriasis, inflammatory bowel disease, and multiple sclerosis. B cell depletion therapies are used for the treatment of B cell tumors, and for inflammatory and autoimmune diseases. These therapies have proven to provide life-altering benefits to many patients. However, infections related to immunosuppression, which can be considered exaggerated pharmacology, have occurred in a small number of patients[66-70], although not all studies have demonstrated such a risk relative to treatment with non-biologic regimens[71]. When they occur, these infections may be associated with latent viral or bacterial infections that recrudesce following the immunosuppression, or infections by organisms that are normally not pathogens in humans, and include *Mycobactrium tuberculosis*, atypical mycobacterial infections, hepatitis B, and John Cunningham virus [JCV, which causes progressive multifocal leucoencephalopathy (PML)]. It should be noted that because patients often receive multiple immunosuppressive therapies as well as have various diseases, identifying clear casual associations between infections and specific biologics can be challenging[67].

Another example of exaggerated pharmacology comes from the erythropoiesis stimulating agents such as erythropoietins[72]. At higher doses, such as those used in toxicity studies, the animals develop polycythemia, chronic blood hyperviscosity, vascular stasis, thromboses, increased peripheral resistance and hypertension, which can be fatal. Similar adverse effects have been suspected in athletes who are seeking supraphysiological hematocrits[73]. However, in an anemic person or animal, the increased red cell mass can be beneficial. The concept that one scientist's pharmacology is another scientist's toxicity is indeed well represented in the field of biotherapeutics. That said, very recently there has been some concern raised regarding off-target effects with biotherapeutics, and this is currently a topic of discussion within the biopharmaceutical industry (a recent case study is reported by Everds *et al* at the Toxicologic Pathology Annual Symposium, 2011; Abstract 04).

Species selection

Regulations require the use of one rodent and one non-rodent animal species in general toxicity studies to assess the toxicity of biotherapeutics, as long as the species are relevant[74,75]. The selection of species for most biotherapeutics should primarily be based by the presence of pharmacological activity. The specificity and biological activity of the biotherapeutic is typically first evaluated *in vitro*. This can be done using binding assays and cell-based assays. Ideally the biotherapeutic will be specific and bind only to the intended target. However, from a practical standpoint, only a limited number of targets can be evaluated, and there is always a chance for unintended binding to untested targets to occur. The *in vitro* activity of the biotherapeutic on the human target should be compared with the activity in commonly used toxicity species. Ideally, the activity in the animal species is similar to that observed in humans. If so, it suggests these species may be relevant for toxicity studies. However, in many cases the activity in animals is lower, and sometimes absent, especially in rodents. When pharmacologic activity is not present in a species, they should generally not be used for toxicity studies (although they may still have value for PK studies). Whenever possible, the presence of partial or full *in vitro* activity should be followed up with *in vivo* studies, as the activity *in vitro* is not always predictive of the *in vivo* activity. For example, a cell line from a mouse might show only 50% pharmacologic activity *in vitro* compared with a human cell line. However, the activity *in vivo* might be greater in the animal (and more similar to humans) if only partial binding or signaling is necessary for a full PD effect. When the biological activity in animals is less than humans, it can sometimes be possible to dose higher and/or more frequently to produce a greater level of activity in the toxicity species, presumably more similar to what would happen in humans. It should be noted that there is no defined rule stating what level of activity compared with humans is needed to suggest inclusion or exclusion of a given species for *in vivo* toxicity testing, but hopefully full pharmacologic activity can be achieved in at least one toxicity species (see "WHAT IS NOT KNOWN" section).

In most cases, toxicologists prefer to use only certain species for toxicity testing. This is based on having many decades of experience with these species, and having a good understanding of the background findings and diseases that can occur. Specifically, the preferred species for general toxicity testing include the mouse, rat, dog, and

cynomolgus monkey (the rhesus monkey is also sometimes used). In the case of small molecules, the rat and dog are most frequently used, unless data exists suggesting alternative species should be used (which might occur in case of species differences in drug metabolism resulting in unique and/or major metabolites). In contrast to the small molecule situation, in many cases the biotherapeutic under development is pharmacologically active only in primates, and thus the large animal species used for general toxicity testing is the cynomolgus monkey. In some cases, the molecule also has activity in mice and/or rats. When this occurs, general toxicity testing should also be conducted in the rodent[74,75]. Unlike the situation with small molecules, metabolites are not a factor in species selection for biotherapeutics.

The need to frequently use the cynomolgus monkey as a primary species for general toxicity testing has highlighted some of the issues with using monkeys in toxicity studies. They are genetically heterogeneous, there are frequently background findings that can mimic test article-related effects, they are expensive and require extensive housing care, their use should be minimized from an animal use perspective, and only small numbers can be used. Nevertheless, there is a good track record in developing biotherapeutics for human use without development of toxicity in humans. In most cases, when effects are seen in humans, they have been related to exaggerated pharmacology, which was considered a possible effect based on known or expected *in vitro* pharmacology, even if not observed *in vivo* in animals.

If there is no activity in any commonly used toxicology species, then it may be necessary to consider other species, such as the common marmoset. In addition, the use of surrogate molecules or transgenic animals expressing the human target can be considered; however, this greatly adds to the complexity of the development programs, and may be a reason to look for other candidate molecules (see "WHAT IS NOT KNOWN" section).

Tissue cross-reactivity studies

For biotherapeutics containing a complementarity-determining region (CDR), a tissue cross-reactivity (TCR) study is recommended[76]. This assay involves an immunohistochemical staining of a broad range of tissues from humans primarily to identify off-target binding, and secondarily to identify previously unknown sites of target expression. If human staining is observed, then similar tissues should be stained in the species planned for toxicity testing. In most cases, the TCR studies in at least one of the species planned for *in vivo* toxicity studies shows a similar pattern of binding. In the unusual cases where no animal staining is observed in human tissues that had staining, other species may need to be considered for the toxicity studies to ensure assessment of the potentially cross-reactive epitope. This topic has recently been reviewed[76].

Cytokine release assays

The implementation of cytokine release assays into nonclinical drug development strategies was driven to a great extent by the unfortunate clinical trial with TGN1412, which resulted in cytokine release in human volunteers and significant morbidity[77-80]. Much effort was put into determining why the nonclinical development studies did not alert the scientists to the cytokine release that occurred in humans. At the present time, *in vitro* cytokine assays have been developed that are believed to be able to detect biotherapeutics that may result in cytokine release. In addition, methods of determining safe starting doses in humans have been implemented across the world. In particular, one approach often used is termed the MABEL approach, which involves determining the Minimal Anticipated Biological Effect Level, and then applying a safety factor to determine the appropriate starting dose[77,81,82]. In addition, it is recommended that dosing in first in human trials be staggered, so that if adverse effects do occur shortly after dosing, only a few subjects would be impacted.

Studies that are not necessary

In the early days of biotherapeutics development, the testing paradigm for development typically followed a small molecule approach. In this regard, genetic toxicity testing was often done. It is now recognized that biotherapeutics alone do not cause direct genetic damage that results in tumor formation, and such testing is not necessary; regulatory guidances specifically state this[74,75]. However, there are occasions where genetic toxicity may be necessary if the biotherapeutic is linked to molecules for which there is a genetic toxicity risk. Examples of this include antibody drug conjugates, where the antibody is attached to a toxin *via* a linker. In this case, both the linker and/or the toxin may be genotoxic, and genetic toxicity testing may be necessary. Many of the other assays often conducted for small molecules, such as *in vitro* toxicity evaluations and the human ether-a-go-go related gene (hERG) assays are typically not conducted with biotherapeutics.

Nonclinical development strategies

A detailed description of possible development strategies for all type of biotherapeutics is beyond the scope of this article. However, some general concepts are applicable to most classes. Once the pharmacologically-relevant species have been identified for the toxicity studies, non-GLP (i.e., not required to follow the good laboratory practice guidelines) exploratory toxicity (dose range finding) studies are typically conducted. These usually use a limited number of animals and usually range from single-dose to two-week studies; the study design should be based on the overall program needs and known pharmacology. In some cases, toxicity evaluations can be conducted in conjunction with efficacy studies, which can provide important information earlier in the program at reduced cost and using fewer animals. It is important, however, that such combination efficacy/toxicity studies do not unduly jeopardize the potential for collecting critical data. For example, if the entire liver needed to be collected for an efficacy evaluation, then it would not be possible to collect it for histopathologic evaluation. In other cases, especially

for monkey studies and in cases where little toxicity is expected, assessments can be included in association with non-terminal single-dose PK studies (for example, clinical observations, clinical pathology assessments), minimizing animal use. If pharmacologically relevant, both a small animal (typically mouse or rat) and a large animal (typically cynomolgus monkey) are evaluated in these early studies. Information from the exploratory studies is used to guide the study design for the first-in-human (FIH)-enabling GLP toxicity studies. These are usually also conducted in a rodent and non-rodent species, if pharmacologically relevant. These studies are usually followed by longer-term studies if they are needed.

The current regulatory guidelines suggest chronic toxicity evaluations only need to be in one relevant species[75]. This can mean only the mouse or rat, if this species is relevant, i.e., the monkey is not necessarily the default species that must be used. Some companies have also used the rabbit as the single species for chronic studies.

Safety pharmacology studies are usually included in the GLP toxicity studies if the only relevant species is the monkey, and usually include central nervous system (CNS), cardiovascular (CV), and respiratory evaluations at a minimum. However, some companies conduct separate studies, especially if there is cause for concern. If the biotherapeutic is active in rodents, these species can be used for safety pharmacology studies.

Reproductive toxicity evaluations for biotherapeutics have evolved significantly over the past decade. If the molecule is active in rodents, then fertility, embryo-fetal development, and peri/postnatal evaluations can be conducted in rodents, if such studies are necessary[75]. It is also important to note that the rabbit needs to be considered as a potential species for use in embryo-fetal development studies[75]. The consideration of the rabbit requires conducting appropriate *in vitro* and/or *in vivo* studies to determine whether the rabbit is a pharmacologically-relevant species. These assays should be done early enough in the development program to ensure the data are available for making appropriate species selection decisions, and for conducting the necessary studies in time to support the clinical program. In some cases, full reproductive toxicity evaluations are not necessary, such as oncology indications, where only developmental toxicity in one species may be necessary[83], or in cases where the indication does not warrant reproductive toxicity evaluations.

If the biotherapeutic is only active in primates, then a relatively new study design is recommended for most indications that require a complete reproductive toxicity evaluation. The design is termed an enhanced pre/postnatal development (ePPND) study, and involves dosing from early gestation (gestation day 20) to the end of gestation, or into the beginning of the post partum period[75,84]. Fertility assessments can be challenging in monkeys. It is generally recommended that at least one longer-term (i.e., > 3 mo) toxicity study be conducted in mature monkeys, if warranted for the indication, to help assess reproductive effects[75]. This paradigm has put increased pressure on the need for mature monkeys, which are more expensive and require increased animal handling capabilities of the larger animals. In addition, the pretest screening to assist in determination of sexual maturity adds time, and must be considered when planning studies.

WHAT IS NOT KNOWN

Absorption, distribution, metabolism, and excretion

Despite the rapid increase in knowledge of mechanisms involved in protein disposition, many fundamental issues in pharmacokinetics and ADME properties of therapeutic proteins remain to be elucidated. Below we highlight some of the major knowledge and technology gaps, as well as emerging science in ADME investigation of protein therapeutics from the standpoint of commercial development.

Impact of bioanalytical assay on PK characterization: Developing a validated bioanalytical assay is critical in determining the PK of therapeutic proteins. At the present time, the majority of bioanalytical methods for determination of drug concentrations, as well as for the determination of ADA, are ligand binding-based assays. Understanding the assay format is important in PK characterization to accurately describe the *in vivo* disposition of a protein molecule, especially when linking the PK data with the PD outcomes[85,86].

The commonly used and emerging bioanalytical approaches for quantification of therapeutic proteins in circulation and tissues are summarized in Table 3. There are currently technical limitations associated with measuring tissue drug concentrations for proteins: in general, the currently available technologies for quantification of tissue concentrations of biotherapeutics in support of ADME studies are relatively labor intensive, of low throughput, and often of low sensitivity (Table 3). For therapeutic proteins with a site of action in tissues, accurate quantifications of drug tissue concentrations are needed to establish the PK/PD correlation and guide design of clinically efficacious dose regimens. Different approaches are often pursued to determine tissue drug concentrations on a case-by-case basis. The advantages and disadvantages of using radiolabeled proteins in tissue distribution studies have been discussed[19]. Noninvasive imaging with radiolabeled or fluorescently-labeled proteins and peptides is a fast growing research field and this diagnostic and/or bioanalytical technology has been widely used in different therapeutic areas[87]. For example, Palframan *et al*[88] examined uptake of several commercially available TNF inhibitors in mouse model of arthritis using a non-invasive biofluorescence imaging method.

The identification and quantification of specific isoforms within the drug product (for example those containing a specific posttranslational modification or bound impurity) in blood and tissues are important because different isoforms may have differential ADME

Table 3 Bioanalytical methods applied to absorption, distribution, metabolism and excretion studies of therapeutic proteins

Methods	Capability to assay for	Current throughput	Currently use	Current sensitivity
Immunoassay	Total, free, intact	High for serum, low for tissues, requires homogenization	Mostly serum/plasma, physiological fluids (e.g., synovial and bronchoalveolar lavage)	Usually high for serum/plasma
Bioassay	Activity of targets, biomarker, ex vivo efficacy	Medium to low, may require fresh samples for certain assays	Serum/plasma and tissues	Varies depending on individual assay
Radioactivity counting	Total, intact and degradants	High, requires probe preparation and characterization	Serum/plasma, tissues, biological fluids, and excreta	Usually high, depending on specific activity of labeled materials
MS	Total, free, intact and degradants	High for peptides in serum/plasma, requires homogenization for tissues	Serum/plasma, tissues, biological fluids, and excreta	Usually high for peptides
		Medium to low for proteins in plasma/serum, requires purification (e.g., immunocapture) and digestion for large MW biologics		Low for large proteins
Imaging	Total, intact and degradants	Medium to low, requires probe preparation and characterization	Live animals, clinical studies in humans, cells and tissues	Varies, depending on probes used and study settings
Auto-radiography	Total, intact and degradants	Low, requires tissue slicing and film developing	Tissues	Varies, depending on specific activity of labeled materials

"Low" and "High" sensitivity is an assessment of likelihood of obtaining a sufficient data-set for quantitative assessment of absorption, distribution, metabolism and excretion properties. MW: Molecular weight; MS: Mass spectrometry.

properties, as demonstrated for several therapeutic proteins[89-91]. *In vivo* measurement of various isoforms may guide the design of drug product with improved ADME profiles; however the current methodology for such studies [mostly based on liquid chromatography-mass spectrometry (LC/MS) technologies] is limited and very labor intensive. While metabolic ID and metabolite quantitation studies for therapeutic proteins are not routinely conducted (and not required by the regulatory agencies), these studies are needed to understand mechanisms of unexpected ADME, PD, or toxicity profiles exhibited by some biotherapeutic drug candidates; however, the available methods for these investigations are in most cases not adequate.

Thus, a breakthrough in bioanalysis methodology will be necessary for advancement of the science of biotherapeutics' ADME to the next level. The next generation assay platforms, such as non-invasive imaging, LC/MS, immune-polymerase chain reaction (PCR) or aptamer-PCR, that are emerging to meet the demands of rapid growth in biologics discovery research are reviewed in references[87,92,93].

Unusual pharmacokinetic profiles of mAbs: With the advent of novel and advanced engineering tools, mAbs are being optimized to achieve higher affinity to targets, improve target specificity, reduce clearance and prolong half-life. However, an unwanted consequence of mAb optimization appears to be increased incidence of therapeutic candidates with unexpected disposition profiles. The causes of the unexpected pharmacokinetic profiles often remain unknown, including translatability from animals to humans.

Several recent publications illustrate the challenges of unexpected fast clearance and altered distribution behavior of protein therapeutics. Vugmeyster *et al*[45] and Bumbaca *et al*[94] case studies provided examples of species-dependent fast clearance, which was attributed to off-target binding in cynomolgus monkey (fibrinogen) and in mice (complement component 3), respectively.

Perhaps the more troublesome from the drug development standpoint are examples of species-independent fast clearance with unidentified causes. The examples include anti-IL-21R antibodies[95,96] and anti-RSV Ab[97] derived *via* a phage-display optimization, as well as a number of case studies with unrevealed therapeutic targets presented at scientific meetings. In these examples, common factors affecting mAbs/protein disposition (such as target binding, FcRn binding, whole blood stability, and ADA) were determined to be unlikely to account for the observed kinetic profiles. The disposition profiles of these mAbs with faster clearance are suggestive of low affinity and large capacity off-target binding: specifically, the observed early rapid declines in serum concentrations and linear pharmacokinetics over a large dose range. Of note is the possibility of multiple low affinity off-target binding epitopes for a given mAb, resulting in a net large capacity off-target sink. In addition to its impact on ADME profiles, the off-target binding may also be relevant for pharmacological and safety assessments, and may require changing the dosing regimens to improve

exposure.

In summary, there is still a significant gap in our understanding of disposition mechanisms of therapeutic proteins, even for the most common class, such as mAbs, which complicates commercial development.

The role of net charge (pI) and local charge clusters on disposition of therapeutic proteins: The role of charge (pI) on PK and biodistribution of therapeutic proteins remains to be systematically characterized. A recent review by Boswell *et al*[98] summarized the current knowledge. The mechanism behind the impact of charge on PK is believed to be the interaction of positively charged therapeutic proteins with negatively charged endogenous components within cell surface residues (sialic acids, glycosaminoglycans), i.e., a large capacity off-target sink, reminiscent and likely related to the phenomenon described above for mAbs with unexpected PK profiles. However, very few mechanistic studies are reported in the literature.

Charge heterogeneity or variability may be a consequence of deliberate changes, such as protein engineering or chemical modifications, or a result of spontaneous alterations occurring during manufacturing, such as post-translational modifications. In general, cationization is believed to shorten the half-life and decrease exposure, while anionization is believed to prolong the half-life and increase exposure[99-101]. However, the relationship between the net charge and disposition profile is not always straightforward[98]. As proposed by Boswell *et al*[98], net charge may alter tertiary or quaternary structure of the therapeutic molecule, resulting in indirect effects on disposition profiles, including altered FcRn interactions or altered charge localization. It is possible that the effective exposed local charge clusters, and not necessarily the total net charge, may ultimately account for the extent of interaction of therapeutic proteins with endogenous charged residues. Thus, deliberate engineering out of exposed positive charge clusters (not involved with target interactions) may be a rationale approach for improving PK profile of a therapeutic protein.

In summary, while many case studies that address the relationship between the protein charge and *in vivo* disposition have been described, a comprehensive assessment, including mechanistic and structural studies, remains to be conducted. The practical application of this emerging science is a potential for rational design of drug variants with desired PK and disposition properties. Thus, charge engineering may provide an alternative approach for modulation of ADME profiles of biotherapeutics and can be used instead of or in combination with other approaches, such as Fc-engineering or PEGylation.

FcRn: Role in absorption, distribution and *in vitro*/*in vivo* correlations: While significant scholarship has been acquired on the role of FcRn in disposition of antibodies and other Fc-containing biotherapeutic modalities (see "WHAT IS KNOWN" section), this remains to be a very active area of research with important applications in commercial development. Most published studies on the role of FcRn focus on serum pharmacokinetics and FcRn-mediated protection from lysosomal degradation; in contrast, the mechanistic studies on FcRn-mediated antibody absorption and distribution into tissue are sparse.

Current understanding of the role of FcRn in antibody absorption is limited. Deng *et al*[38] reported that the FcRn variant of an anti-TNF Ab with stronger affinity for FcRn at neutral pH appeared to have lower bioavailability after SC administration, possibly related to delayed release of the antibody leading to accelerated degradation at the injection site. Recent studies support the role of FcRn in SC absorption of mAbs in rodents[13,102] (and Balthasar lab, unpublished observations). Specifically, the bioavailability of an IgG1 antibody following SC administration was about 3-fold higher in WT mice compared to FcRn-deficient mice[102]. While the mechanism of FcRn-mediated effects on SC bioavailability is not known, it may include the protection during FcRn-mediated transport from interstitial fluid to the blood, as well as protection from catabolism at the site of injection and in the lymphatic system.

A mechanistic understanding of the role of FcRn in antibody distribution is also lacking. Studies with intestinal human cell lines suggested that FcRn transports IgG across cell monolayers, implicating FcRn in transport of mAbs from circulation to the interstitial fluid of tissues[103]. Several physiologically-based pharmacokinetic models that incorporate FcRn-mediated IgG transport have been developed and the model predicted an important role of FcRn-mediated mechanism on antibody distribution to various tissues in mice using a few test IgGs[104,105]. Comprehensive evaluations of the contribution of FcRn to transport of IgGs into various tissues in rodents and primates have not been performed, including quantitative assessment on the contribution of FcRn in tissue distribution of IgGs in animals and humans.

Following the discovery of the role of FcRn on antibody disposition, optimization of protein structures *via* improving FcRn binding has been pursued by pharmaceutical companies as an approach to produce a drug candidate with the desired half-life. Multiple studies have been performed with IgG variants engineered to have different binding affinities and/or kinetics and tested for the impact on PK profiles[33-43]. The cornerstone for these Fc-engineering efforts has been *in vitro* assays (such as surface plasmon resonance (SPR) or cell-based binding in FcRn-expressing cell lines) to test engineered variants for modulation of FcRn binding. However, the *in vitro* and *in vivo* correlations between the FcRn binding parameters determined from either SPR or cell-based methods remain controversial, with some studies reporting a good correlation of *in vitro* binding at pH 6.0 and the *in vivo* PK profiles[34,38-41] and other studies failing to demonstrate such a correlation[36,37,43]. The lack of the correlation between the binding at pH 6.0 and *in vitro* PK for some mAbs was linked to the hypothesis that efficient binding

at pH 6.0 needs to be complemented by the lack of binding and/or fast off-rate at neutral pH in order for IgGs or Fc-containing proteins to be salvaged by FcRn[37,106,107]. In general, it is likely that quantitative modeling that utilizes the combination of the kinetic parameters for FcRn/Fc-containing protein interaction (such as kon and koff rates at acidic and neutral pHs) and other "determinants" of clearance for a particular Fc-containing protein, will be needed to improve the *in vitro* and *in vivo* correlations, because the relative contribution of a given *in vitro* binding parameter to the overall *in vivo* clearance may differ across Fc-containing proteins (discussed by[38,107]). In addition, very recent studies suggested that mAbs with the same Fc sequence but different CDRs can exhibit differences in FcRn binding parameters and in PK profiles, possibly *via* CDR-mediated impact on tertiary structures of the Fc region, leading to altered FcRn binding[107,108]. Furthermore, the translation of FcRn effects from animal studies to humans is not straightforward, in part due to species differences in FcRn binding[31,32]. The recent report by Zheng *et al*[109] on pharmacokinetics of FcRn variants highlights the challenges in translating FcRn-mediated modulation of pharmacokinetics from animals to humans, including the cases when non-human primates are used for nonclinical investigations.

In summary, the role of FcRn in antibody absorption after SC administration and in tissue distribution (after dosing *via* any route) remains to be characterized and quantified. Potential species differences in the role of FcRn in ADME of IgGs need to be systematically investigated to enable translation of the effects of FcRn modulation from animals to humans. The translation of *in vitro* FcRn binding kinetics to *in vivo* PK remains to be understood and is crucial for the success of rational Fc engineering.

Factors influencing SC absorption: Upon comparison of absorption parameters (such as bioavailability and Tmax) across various therapeutic proteins administered by SC injection, a wide range of mean/median values is apparent, especially for biologic modalities beyond mAbs (Table 1)[10,110]. The reason for this variability across different therapeutic proteins is not known, but many factors are likely involved (see "WHAT IS KNOWN" section for the list of possible factors), and the relative contributions of these factors are likely to vary with biologic modality or even a particular therapeutic protein within the same modality. Poor bioavailability translates into higher cost of goods and the need for higher doses and/or more frequent dosing. Therefore, a thorough understanding of key processes and factors that impact SC absorption and application of this knowledge for design of SC-administered biologic drug products with improved systemic exposure has significant commercial implications.

Surprisingly little is known about the mechanisms of absorption of therapeutic proteins following SC administration. In addition, it appears that there are significant species differences in physiology and mechanisms affecting kinetic profiles in disposition of therapeutic proteins upon SC dosing, such that the nonclinical models and methodologies for prediction of human SC profiles need to be further explored (Table 1)[10].

In general, SC bioavailability depends on pre-systemic metabolism/catabolism and systemic absorption[102]. Physiological processes that drive SC absorption of therapeutic proteins are believed to be convective transport across the lymphatic vessels ("lymphatic drainage"), passive diffusion across the blood vessels at the absorption site, and for molecules with an Fc, specific FcRn-mediated transport mechanisms. The relative contribution of these processes to SC absorption for a given biologic modality is controversial. Studies in sheep indicated that the main pathway of SC absorption is *via* lymphatic drainage for proteins larger than 20 kDa (using mostly non-Fc containing proteins)[111,112]. In contrast, in rodents and rabbits the relative contribution of lymphatic system in SC absorption is small[12,113,114]. There is no similar mechanistic data in monkeys or humans, and it is not known how to extrapolate the existing data to humans.

In summary, a comprehensive evaluation of factors and mechanisms influencing SC absorption in humans and animals needs to be conducted. Then models, including species-specific models that account for the complex interplay of the factors involved in SC absorption (for example, physiologically-based absorption and disposition models), need to be developed. Finally, these models need to be validated for predictability of human PK profiles across therapeutic modalities, species, and subjects.

Complex role of glycans: A variety of mechanisms are believed to account for the effect of glycans on the disposition and biological activity of therapeutic proteins, and the research in this field is rapidly expanding. In general, glycosylation can impact protein ADME properties by (1) masking of proteolytic or immunogenic site (decreasing degradation/clearance), or, conversely by introducing new immunogenic sites (increasing degradation/clearance); (2) changing total or local charge; (3) promoting or interfering with dimerization or multimerization; and (4) changing contribution of clearance and distribution pathways mediated by specific glycan receptors. If the clearance of the protein is increased by any of these mechanisms, then the PD effect is typically decreased.

For mAbs, glycosylation in the Fc region has been shown to modulate binding to Fc gamma receptors and complement components, which can either decrease or increase CDC and/or ADCC functions[115-117], depending on the type of the modification. However, the link between the Fc-related glycosylation and the PK profile of a mAb remains controversial, with some studies supporting such a link but not the others. It is possible that the impact of Fc-linked glycans on the disposition of mAbs is species- and molecule-dependent. Initial studies have indicated that in the Fc region, glycans are not thought to be accessible to receptors that can mediate glycan-dependent clearance and thus have minimal effect on PK[118]. Recent case studies have suggested this may not always be the

case, with some studies reporting increased clearance in humans and mice for high mannose enriched and afucosylated Fc-glycovariants of some mAbs[119-121], but not other variants[122,123]. It should be noted that the majority of antibodies (including therapeutic mAbs) have no glycans attached to their variable region, with a few possible exceptions[124,125].

Although glycosylation has also been shown to play a prominent role in both disposition and PD for therapeutic proteins beyond mAbs[64], there are still numerous unanswered questions on mechanisms and predictability from biophysical profiling to *in vivo* PK and PD profiles. For example, the role of sialic acid in protein disposition and correlations between the extent/site of sialic acid content and effects on clearance and distribution has been an active research topic. Several reports indicate that increased sialic acid content in biotherapeutics is associated with reduced clearance and improved PK profiles[90,126-128]. Conjugation of polysialic acid (PSA) has been shown to increase half-life of several proteins, such as asparaginase, Fab fragment, and insulin[129]. The mechanism behind the beneficial effects of polysialylation on PK profile needs to be investigated and is likely related to be a combination of multiple factors, including masking of proteolytic and/or immunogenic sites and an increase in size beyond the renal filtration cut-off. In addition, relative contributions of these factors are likely to be different for each protein-polymer conjugate. This approach for half-life extension is similar to PEGylation[130], with a potential advantage of employing a natural, biodegradable polymer. Similar to PEGylation, polysialylation may lead to the decrease in biologic activity of a therapeutic protein; therefore for an optimal PD effect, a design that balances effects on PK and biological activity should be considered.

In summary, glycol-engineering and modulation of glycosylation during production of a biotherapeutic is a widely used approach for increasing exposure (by decreasing clearance), and for altering the biological activity of therapeutic proteins. The success of such modification in meeting the planned objective has been variable. Similar to considerations mentioned above for other protein engineering approaches, the key to commercial success of glycol-engineering is a mechanistic understanding of clearance pathway and species differences, as well as translation from the biophysical glycan profiles and *in vitro* activity to *in vivo* effects. An additional consideration for glycol-engineering is balancing the effects on PK *versus* PD to achieve optimal clinical efficacy.

Effect of ADA on clearance and distribution: While the impact of ADA on serum PK and PD profiles has been described qualitatively/semi-quantitatively in many case studies (see "WHAT IS KNOWN" section), the quantitative tools to link ADA characteristics [such as titer (which is related to both avidity and concentrations of ADA), persistence, isotypes, and neutralization potential] to PK or PD are lacking. For development of such a quantitative tool, significant advances in our knowledge of mechanisms behind ADA-driven clearance and distribution processes are needed. However, the mechanistic and quantitative studies on the impact of ADA on clearance and distribution of therapeutic proteins in both nonclinical and clinical settings are sparse[61,131].

The mechanisms responsible for the elimination of ICs in general and biotherapeutic/ADA complexes in particular remain to be fully delineated, although the important role of red blood cells and mononuclear and phagocyte system in the liver have been demonstrated (see "WHAT IS KNOWN" section). For example, both the Kupffer cells and sinusoidal endothelial cells in the liver are thought to be involved in the clearance of these ICs from the circulation *via* Fc-receptor dependent uptake[61]. However, because Fc receptors are expressed in many other organs, yet the largest relative uptake of ICs is reported in the liver, it is possible that there is an Fc receptor-independent uptake of ICs in this organ. In addition, some but not all studies implicate spleen in the clearance of ICs and suggest that size and type of ICs may influence the relative contribution of different elimination processes for ICs[61,132]. In the case of ADA, which are highly heterogenic across subjects or even in any given subject sample[55], ICs of different types and sizes are expected to form. Therefore, multiple ADA-mediated clearance and distribution pathways may be present for any given subjects or within the study population.

Johansson *et al*[61] provided a detailed case study on the *in vitro* IC formation and *in vivo* clearance and distribution of a model mAb ("Id") and its monoclonal anti-idiotype ("anti-Id") in mice. The *in vitro* results from this study suggested that the relative concentrations of the reactants (related to the dose of a mAb in the *in vivo* settings) played a role in the type and size of Id/anti-Id immune complexes generated, as examined by electron microscopic and other biochemical techniques. In the *in vivo* study, mice were given a single dose of the radiolabeled Id, followed by an injection of the unlabeled anti-Id antibody at different Id/anti-Id ratios; the total body clearance of Id, as well as uptake in various organs, were monitored. These studies indicated there is stoichiometric dependence on the impact of anti-Id antibody on the clearance of Id and that the metabolism of the ICs between Id and anti-Id occurred mainly in the liver.

In a study by Rojas *et al*[131] cynomolgus monkeys were given a single IV dose of a therapeutic antibody infliximab (IFX), followed by injection of either ^{125}I-labeled, purified monkey anti-IFX IgG (test group) or ^{125}I-labeled monkey non-binding control IgG (control group). This study was designed to model the formation of ADA/biotherapeutic ICs in the presence of excess antigenic protein, such that IFX was given in excess of ^{125}I anti-IFX IgG or ^{125}I-control IgG. *In vivo* formation of IFX/^{125}I anti-IFX ICs of variable size was confirmed by high-performance liquid chromatography analysis. The serum PK profile of IFX, although somewhat lower in concentration over time for the test group (i.e., IFX given in combination with anti-

IFX Ab), was not statistically different relative to the control group. In contrast, the terminal half-life and clearance of the ^{125}I- anti-IFX IgG (circulating largely in complex with IFX) was significantly shorter because of more rapid elimination compared with the ^{125}I- control IgG. The authors noted that these data illustrated that detection of ADA-containing ICs may be challenging because of the rapid clearance of the ADA/biotherapeutic ICs, especially in cases when a transient anti-drug immune response is triggered. Interestingly, the authors also provided evidence that red blood cells appeared to play only a limited role in the elimination of ICs.

It should be noted that in some cases the relative impact of ADA on serum drug concentrations cannot be directly extrapolated to tissue profiles[19], which may have significant implications for projections of efficacious dosing regimens for a biotherapeutic which has its site of action in tissues. For an anti-IL-21R Ab administered to wild-type or lupus-prone mice, a differential impact of ADA was shown for tissues serum. Specifically, after a single dose of ^{125}I-labeled anti-IL-21R Ab to mice, there was a rapid decline of serum drug concentrations at 10-21 d post dose, associated with development of ADA. However, tissue drug concentrations declined at a slower rate. Interestingly, the difference between serum and tissue drug concentrations was more pronounced in the disease model *vs* healthy animals, related primarily to faster clearance in the serum in the disease model. In general, it appears that in single-dose studies in which formation of ADA leads to a reduction in serum drug concentrations, an increase in tissue-to serum concentration ratios is observed and tissue concentrations often approach or even exceed those in serum as the ADA removes the test article from the serum more rapidly than tissues[19]. In these cases, it is likely that the relatively high tissue concentration reflected the high serum concentrations before the onset of ADA.

In summary, comprehensive mechanistic studies on ADA-mediated distribution and elimination of therapeutic proteins in nonclinical and clinical settings will be instrumental in building PK-PD relationship that take into account the ADA-mediated effects, including dose-dependency and inter-subject variability of these effects. However, because of highly heterogenic nature of ADA response, the quantitation of these ADA-mediated effects is expected to be challenging and will require unique modeling approaches.

Toxicology

Species selection: As noted above (in the "WHAT IS KNOWN" section), there are standard paradigms for selecting species for toxicity studies. However, how closely the pharmacologic effects of a biotherapeutic in a given species truly mimics the effects in humans are often not really known. In some cases, *in vivo* assays, including efficacy models, may be available. This is particularly true when the biotherapeutic has activity in rodents, but is often less common if the molecule only has activity in primates. If a biotherapeutic demonstrates activity *in vivo*, it is usually based on a limited set of parameters, and it is always possible that other parameters that were not evaluated might also be affected. Furthermore, the translatability of *in vitro* effects to *in vivo* effects is often not clear, given the complex, overlapping, and/or redundant pathways that can exist. For example, if a biotherapeutic in an *in vitro* assay causes a 20% effect, it is possible that a full 100% effect might still occur *in vivo*. The TGN1412 clinical trial was one case where it is believed that unrecognized species differences led to significant adverse events in human subjects, because the cynomolgus monkey used in the testing were not as sensitive to the biological effects of the test article[133,134].

In cases where there is no pharmacologically-relevant species from standard toxicology species, toxicologists may turn to the use of animal models of disease[135]. While this may be the only option, it should be recognized that little is often known about the model from a toxicology standpoint, historical data may be limited or completely lacking to assist in interpretation of findings, the disease may confound interpretation of toxicological effects, and the models may not be suitable for long-term dosing.

Another option when there is no pharmacologically-relevant toxicity species is to use a surrogate molecule[135]. This surrogate would ideally have activity in a standard species used in toxicity testing, and in particular in the mouse or rat because they represent a more controlled population, smaller size and associated lesser amount of drug needed, lower animal cost, and in keeping with trying to minimize monkey use. However, the use of surrogates requires careful manufacturing and characterization of the surrogate to a degree that may come close to mimicking the efforts required for the actual drug candidate. Because of this extensive effort, most consider the use of a surrogate to be a last resort. If a surrogate is used, questions still remain. Does the surrogate interact with the target in the exact same way and lead to the exact same biological effect? If not, what are the differences? Are the downstream effects the same as those that occur in humans (which is usually not known at the time the studies are conducted because *in vivo* testing has not occurred in people)? Again, if not, what are the differences? In reality, it is unlikely that the interaction with the target and downstream effects will exactly mimic those in humans, and it is also unlikely that these differences will be completely understood.

Still another option is the use of genetically-modified animals, typically mice[135]. These animals may have reduced or loss of function for a particular target (knock-out), or may have gain-of-function to mimic agonists (knock-in). In addition, animals can be constructed to express the human target. In all these cases of using genetically-modified animals, much is unknown. As with disease models, historical data may be limited or completely lacking to assist in interpretation of findings. In the case of loss or gain of function mutations, this alteration is often complete and present from conception.

This differs substantially from the therapeutic case, which usually involves variable drug concentrations and corresponding variable PD effects over time, only partial loss or gain of function, or complete loss or gain of function only intermittently. In addition, the therapeutic effect of an exogenously administered drug is typically not present from conception. How these differences affect the toxicity of a biotherapeutic is often not known, but one usually considers such knockout or knock-in animals to represent a worst case scenario for loss or gain of function. Regarding animals which have been modified to express human molecules, whether the cellular distribution, signaling, and function in the animal biologically matches the human is usually not completely clear.

Immunogenicity: The administration of biotherapeutics to animals often results in immune responses to the drug. The immune responses to the drug can take many forms, including production of ADA and cellular immune responses[55,136]. Impact of ADA on PK/PD of therapeutic proteins is discussed above. From the standpoint of clinical signs in a toxicity study, the effects related to immunogenicity can be quite diverse, ranging from no effect to hypersensitivity reactions (up to and including fatal anaphylaxis) to loss of function of the endogenous molecule. For example, in the case of replacement proteins, the ADA can result in neutralization of the endogenous protein as well as the administered protein, as discussed above. From a clinical and anatomic pathology standpoint, effects may also be diverse. One may see subtle evidence of inflammation, with alteration in white cell counts and microscopic evidence of lymphoid hyperplasia from immune stimulation, immune-mediated vasculitis or glomerulonephritis, or effects secondary to loss of function of the endogenous protein (for example, aplastic anemia in the case of neutralization of erythropoietin). It is generally accepted that immune reactions in animals are not predictive of what will occur in humans, and therefore, the effects related to immune reactions in animals are generally not considered to be relevant to humans[136]. However, differentiating these immunogenicity-related findings from direct test article-related effects can be challenging, in particular when the test article is an immunomodulator.

Tools that can assist in determining whether an effect is related to an immune reaction include the presence of ADA, effects on PK, loss of PD effect, activation of complement, the presence of circulation immune complexes, and evidence of histamine release. Clinical signs consistent with anaphylaxis (including having the effects shortly after dosing) can also be useful in determining whether effects are related to an immune response to the biotherapeutic. Microscopically, anti-drug immune responses may result in findings such as vasculitis or glomerulonephritis[137-140]. It may be possible to detect animal IgG, IgM, and/or complement in the lesions. In some cases, it may also be possible to detect the test article in the lesions, for example using specific anti-human antibodies or anti-CDR antibodies that can detect the human biotherapeutic without binding to animal molecules. If the study has a range of doses, as most toxicity studies have, findings overall may exhibit an inverse or bell-shaped dose-response relationship. This pattern may be related to development of tolerance, or may be related to the pharmacologic activity of the test article in the case of immunomodulators that may down regulate the immune response to a greater magnitude at higher doses. When all analyses are done, there are some cases in which there is a strong correlation between animals with clinical or pathologic effects and evidence of ADA. However, in many cases the correlation is not as strong, and findings are somewhat variable between individual animals. In these cases, one must use a weight of evidence approach to reach a final conclusion that the study or program may be impacted by anti-drug immune responses.

When immunogenicity does develop, it is sometimes recommended to dose through the immunogenicity, and perhaps to increase the dose (dose level, dosing frequency, or both). The latter strategy may have several potential effects. It may simply overwhelm the immune response and allow free drug to have whatever biological effects it will have, it may induce tolerance, and/or it may increase immunosuppression in the case of immunosuppressants. On the other hand, the relevance of this situation to humans is often unclear. While some concerns have been raised related to protein overload in situations where doses are high, from a practical standpoint the large amounts of administered protein alone do not appear to cause any significant effects in most cases.

Another concern related to immunogenicity is the impact of previous exposure to other biotherapeutics. As biotherapeutics become more commonly used, there is an increased chance that the patient may have been dosed with other biotherapeutics in the past. The impact of this cannot be modeled well nonclinically, as immune responses in animals are not predictive of what happens in humans. However, several scenarios are possible in humans. There may be no immune response and no impact; there may be an immune response to the previous biotherapeutic that reacts with the new biotherapeutic and potentially results in clearance, neutralization, or hypersensitivity reactions; or the previous biotherapeutic many have modified the immune response to the new biotherapeutic, either increasing or decreasing the response. Because of these variable impacts, clinicians should carefully assess patients who have previously received biotherapeutics.

Latent infections: While rodents, rabbits, and dogs used in toxicity studies are usually purpose-bred and do not contain background infectious agents or parasites, the same is not true for monkeys[141-143]. While the monkeys used in toxicity studies are usually tested for a variety of infectious agents and parasites, and should not have overt infections or parasitic infestations at the time of study initiation (or they should be excluded from the study), there are limits to the number of agents that are tested and the

assays are not full proof. For example, recrudescence of malaria, polyomavirus, and lymphocryptovirus can occur in toxicity studies evaluating immunosuppressants. It is possible that unknown agents may also play a role. In a recent study, pretest blood cultures revealed an unspeciated organism in greater than 80% of animals (Leach MW, unpublished data). Differentiating the pharmacologically-mediated effects of immunnosuppressants from secondary stress-induced immunosuppression can be challenging.

Juvenile toxicity assessments: When the clinical population involves children, testing of juvenile animals may be necessary[144]. Paradigms for juvenile toxicity testing have been well established through many years of experience for rodents[145]. However, because many biotherapeutics only have activity in primates, juvenile toxicity evaluations (when they need to be conducted to support the clinical program) may need to be conducted in monkeys. In contrast to rodents, protocols to assess juvenile toxicity in monkeys have only recently been developed and there is very limited experience with these studies[146]. Furthermore, while the time from birth to sexual maturity is only several 2 months in rodents, it is years in monkeys, and covering this entire period in a toxicity study is not practical. How to adequately and rationally address juvenile toxicity when monkeys are the only pharmacologically active toxicology species remains an area of discussion.

Carcinogenicity assessment: The assessment of carcinogenic risk for biotherapeutics can be challenging. Biotherapeutics typically do not have direct effects on DNA, and thus are secondary carcinogens when they cause tumors. Therefore, many of the standard assays for detecting potential carcinogens, such as the Ames test, are not relevant and should not be conducted[74]. For small molecules, many compounds are assessed in lifetime rodent studies. However, because of the lack of pharmacologic activity of many biotherapeutics in rodents, such studies cannot be conducted. Furthermore, immunogenicity can be an issue, resulting in neutralization of the test article and/or in long term immune stimulation. In either case, interpretation of the data and assessment of the relevance to humans can be challenging. Lifetime dosing of monkeys is not considered practical. At the present time, it is recommended that the need for a product-specific assessment of the carcinogenic potential for biopharmaceutical be determined with regard to the intended clinical population and treatment duration[75]. The presence or absence of cell proliferation in general toxicity studies may be useful. If a carcinogenicity assessment is warranted, for example for chronic dosing with a potential mechanistic concern for an increased risk of tumors, then a strategy should be developed to address the potential hazard[75]. It should be noted that standard rodent carcinogenicity studies with the test article, or carcinogenicity studies with homologous products, are not usually considered useful in most situations[75]. As noted above, latent infections can occur, and some of these are known to result in lymphoproliferative disease[141]. In some cases, additional *in vitro* or *in vivo* cancer models may be conducted in an attempt to shed some light on the potential risk for carcinogenicity.

Biosimilars: The topic of biosimilars is receiving extensive discussion, as many biotherapeutics are losing patent protection in the near term[147,148]. From a nonclinical perspective, global regulatory agencies are determining what they feel is necessary for development and approval[149-152]. The US Food and Drug Administration (FDA) and European Medicines Agency (EMA) appear to be favoring a scientific approach with relatively limited *in vivo* toxicity studies, and a greater reliance on *in vitro* characterization of the product. This is related to relative lack of sensitivity in detecting small differences in innovator *vs* biosimilar products *in vivo* toxicity studies under most circumstances. However, it is unclear exactly how similar a biosimilar must be to the innovator, and in what assays, to demonstrate equivalent biologic/therapeutic effect. It is likely that what characteristics matter may differ between molecules, or especially between classes of molecules. As the global scientific community gains additional experience with biosimilars, some of these questions may be answered. Another unresolved issue with biosimilars is related to global harmonization. At the present time it is not clear whether the scientific approach being adopted by some countries and regions that limits animal studies will be accepted globally. Thus, it is possible that different regions of the world will ask for a variety of toxicity studies, increasing the total number of studies required for global registration, and increasing animal use. It is hoped that all regions of the world will utilize strong scientific principles and only require the studies that are truly needed.

CONCLUSIONS AND FUTURE PERSPECTIVE

Despite the rapid increase in knowledge of mechanisms involved in ADME of therapeutic proteins, many fundamental questions remain answered. Some of the emerging questions and active research topics include the role of charge and glycosylation, factors influencing SC absorption, role of FcRn beyond serum half-life extension, as well as anti-drug antibody-mediated clearance and distribution mechanisms. A comprehensive evaluation of factors influencing ADME of biotherapeutics and mechanistic studies in nonclinical and clinical settings is needed to build *in vitro* tools that can be used to predict disposition and biological activity profiles and to establish structure activity relations (SARs). The acquisition of mechanistic knowledge is currently hindered by the limited bioanalytical methods to assess the concentration of biotherapeutics in tissues, as well as tools to study metabolism/catabolism in both blood and tissues and to assess potential differences in ADME profiles of drug product isoforms. A breakthrough in bioanalysis, includ-

ing MS-based techniques and imaging tools, will be instrumental for the success of rational protein engineering aimed at optimizing ADME profiles. In addition, because of complex interplay of factors influencing ADME and biological activity of protein therapeutics and potentially multiple sites of actions, modeling tools ranging from "fit for purpose" and "site of action" to full physiologically-based pharmacokinetic models may be needed to build *in vitro*/*in vivo* correlations and enable translation from animals to humans.

In toxicology, much has been learned since the advent of biotherapeutics regarding what studies are needed to safely develop these drug, and what studies do not provide relevant information. The concept of appropriate species selection has become relatively well accepted. However, there is room in some cases to develop better models that more closely mimic the pharmacologic activity in humans. In cases where there is no pharmacologic activity in standard toxicology species, the appropriate design of toxicology programs, including the use of animal models of disease, surrogate molecules, and genetically-modified animals, is still an area of need. The design of reproductive toxicity studies in monkeys has made substantial progress in the past decade. However, these studies lack sufficient power to identify uncommon findings. How to adequately assess juvenile toxicity, when monkeys are the only pharmacologically-relevant toxicology species, remains an area of need. Immunogenicity can cause significant issues in nonclinical development, and strategies to minimize immunogenicity in animals while still testing the molecule in a relevant manner are needed. Despite the issues, a large number of biotherapeutics have been successfully brought to market with acceptable benefit: risk ratios, providing better treatments to innumerable patients.

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Dubai, United Arab Emirates

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Applied Pharmaceutical Analysis-India
Ahmedabad, India

February 20, 2012
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February 20, 2012
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New Delhi, India

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Metabolomics2012
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February 24, 2012
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May 16, 2012
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INSTRUCTIONS TO AUTHORS

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1949-8454/g_info_20100309232449.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

Examples for paper writing

Editorial: http://www.wjgnet.com/1949-8454/g_info_20100316155524.htm

Frontier: http://www.wjgnet.com/1949-8454/g_info_20100312091506.htm

Topic highlight: http://www.wjgnet.com/1949-8454/g_info_20100316155725.htm

Observation: http://www.wjgnet.com/1949-8454/g_info_20100316155928.htm

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Original articles: http://www.wjgnet.com/1949-8454/g_info_20100316160646.htm

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