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REVIEW

# A cellular perspective of bias at G protein-coupled receptors

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**Abstract**

G protein-coupled receptors (GPCRs) modulate cell function over short- and long-term timescales. GPCR signaling depends on biochemical parameters that define the what, when, and where of receptor function: what proteins mediate and regulate receptor signaling, where within the cell these interactions occur, and how long these interactions persist. These parameters can vary significantly depending on the activating ligand. Collectivity, differential agonist activity at a GPCR is called bias or functional selectivity. Here we review agonist bias at GPCRs with a focus on ligands that show dramatically different cellular responses from their unbiased counterparts.

**KEYWORDS**

agonist bias, desensitization, GPCR, signaling

## 1 | INTRODUCTION

G protein-coupled receptors (GPCRs) are a superfamily of membrane-bound signaling proteins that upon activation change the cellular program. Agonist binding to a GPCR activates signaling and regulatory molecules to produce a stereotypical response.[1,2] Many of these proteins have been identified in the decades long work of molecular pharmacology, including: heterotrimeric G proteins, GPCR kinases (GRKs), β-arrestins, adenylyl cyclases, ion channels, kinases, and trafficking proteins. This abbreviated list only scratches the surface, and it is clear that new proteins await discovery.[3–6] In addition to "what" proteins function with GPCRs, it is increasingly clear that the cellular response to an agonist is shaped by "where" and "when" protein interactions occur (Figure 1).[7–10] Yet, these biochemical parameters are not inviolable. Structurally different agonists acting on the same receptor can alter these parameters and, as a consequence, change the downstream signaling and regulatory processes of the cell.[11,12] This phenomenon is called functional selectivity or biased agonism. Agonists exist on a continuum of bias and more strongly biased compounds are being developed.[13,14] Thus, it is important to determine the possible cellular responses to a biased agonist. For cases of extreme bias, how different are the cellular pathways of GPCR signaling and regulation?

## 2 | CLASSICAL PHARMACOLOGY AND AN INTRODUCTION TO BIAS

### 2.1 | Full, partial, and biased agonism

An important step from the early concept of a "receptive substance" to modern receptor theory was the recognition of partial agonism.[15–17] The differing capability of agonists to cause a response is called efficacy. Agonists can be classified based on relative efficacy with a full agonist causing a maximum response while a partial agonist, despite saturating concentrations, only stimulating a submaximal response. The above definition of efficacy is monodynamic. The cellular response to GPCR activation, however, is composed of the simultaneous, competitive, and sequential action of different proteins. The biased agonism hypothesis has two key tenets: (a) biased agonists stabilize receptor conformations distinct from full agonists and the result is differential coupling to receptor interacting proteins; (b) the response to GPCR activation arises from many cellular pathways and differential

  

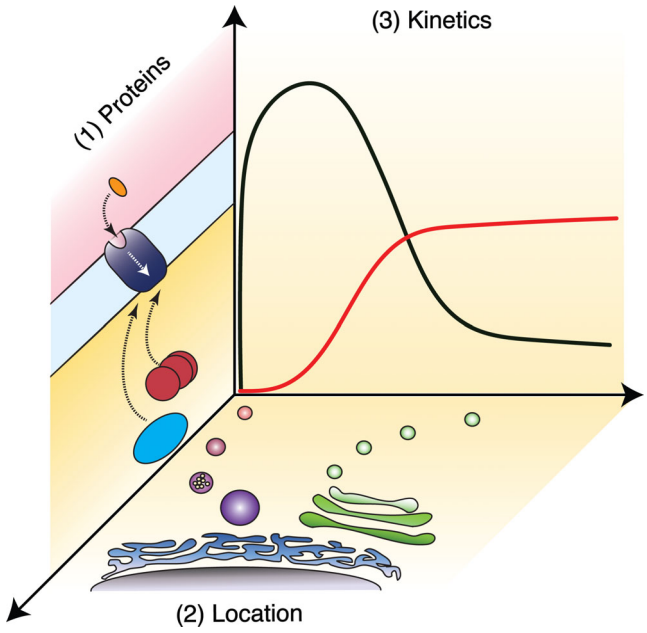


**FIGURE 1** Cellular dimensions of GPCR function. Different ligands acting at the same receptor can alter the "what," "where," and "when" parameters of GPCR function. Panel 1 shows an extracellular agonist (orange) activating a GPCR (purple) and subsequent coupling to intracellular proteins (red and blue). Panel 2 highlights intracellular locations including endosomes (magenta) and Golgi (green) where GPCRs may function. Panel 3 provides a hypothetical example comparing activity of two ligands (black and red traces). Observed efficacy (*y*-axis) can be dependent on when (*x*-axis) an assay is performed. Ligands which show agonist bias, location bias, or kinetic bias can change these parameters compared to their unbiased counterparts. Panel 2 was adapted from Lobingier and von Zastrow 2019.[10]

coupling to G proteins, GPCR kinases (GRKs), and β-arrestins can preferentially activate one pathway over another.[12,18,19] Recent biophysical, structural, and cell biological studies have provided important support to this model.[20–25] Thus, a G protein biased agonist will have greater relative efficacy in activation of the heterotrimeric G protein and subsequent signaling cascades.[26] In contrast, an arrestin biased agonist will have greater relative efficacy for the GRK/β-arrestin pathway, which includes: phosphorylation of the receptor by GRKs, formation of a GPCR/β-arrestin complex, and β-arrestin terminating G protein signaling, acting as an adaptor for endocytosis, and scaffolding additional signaling events.[1,11,27]

## 2.2 | Receptor reserve and system bias

Another important development in receptor theory was the recognition that full agonists need not occupy all receptors to drive a maximum response.[15,16] These unoccupied receptors are called spare receptors or receptor reserve. Receptor reserve represents a technical challenge when studying partial or biased agonists because of the inherent amplification in GPCR signaling combined with the fact that each tissue/cell type has a limited maximal response. With a large receptor reserve, lower efficacy agonists can appear as full agonists for G protein activity.[28,29] However, not all GPCR pathways are signal amplifying: binding of β-arrestin to the phosphorylated receptor and subsequent endocytosis requires a one-to-one interaction.[27] Thus, receptor reserve can differ between G protein and GRK/β-arrestin pathways and care must be taken in investigation of bias.[30] Receptor reserve is not the only variable between cell types. System bias describes the concept that the expression of any component in the GPCR pathway can vary between tissues.[19] Thus, an agonist/receptor pair can produce distinct activities in different cell types. In this review, we will note if agonists are considered full, partial, or biased with the recognition that these are system-dependent terms.

## 2.3 | Other forms of bias: Location bias and kinetic bias

Biased agonists can change the duration and location of receptor signaling by altering GPCR coupling to the G protein or GRK/β-arrestin pathways. However, these changes do not encapsulate the extent to which ligands can bias the location and timing of GPCR activity. Two recently described types of bias involve these where and when variables: location bias and kinetic bias. Location bias is a two part phenomenon: (a) GPCRs can activate signaling events from intracellular compartments as well as the plasma membrane; (b) certain ligands can preferentially drive GPCR activity from intracellular compartments or restrict activity to the plasma membrane and, as consequence, change signaling.[9,10] One type of intracellular signaling occurs at compartments such as the Golgi or nucleus. GPCRs at these compartments are activated by agonists which are actively transported into the cell or are inherently capable of passive diffusion through the cell membrane.[31–34] A second type of intracellular signaling occurs at endosomes following agonist-induced endocytosis.[9,10] Importantly, intracellular GPCR activation can drive distinct signaling events compared to agonists which restrict receptor activity to the cell surface.[34–40] Kinetic bias (also called temporal bias or kinetic context) highlights the interplay of ligand binding rates and the duration of GPCR signaling and regulatory pathways.[7,8,41] For example, when ligands with slow dissociation rates are examined for efficacy in transient signaling

events, the degree and even the direction of bias can change depending on when the assay takes place after agonist stimulation. Examples include serotonin and dopamine receptors while opioid receptors provide a counterexample.[42–44] Together, agonist bias, location bias, and kinetic bias can all shape the cellular response to GPCR activation.

## 3 | A "CELLULAR PERSPECTIVE" OF GPCR SIGNALING: PHOSPHOPROTEOMICS AND TRANSCRIPTOMICS

### 3.1 | Phosphoproteomics

GPCR activation stimulates cellular kinases. Activity of these kinases can be probed with phospho-specific antibodies or genetically encoded reporters.[45] What these approaches miss is the breadth of cellular remodeling evoked by GPCR activation. Phosphoproteomics is an alternative approach to understand how GPCR activation changes kinase and phosphatase activity in the cell. One advantage to phosphoproteomics is that it can be used to detect and identify thousands of phosphosites; thus, examining GPCR activity from the "cellular perspective." For example, GPCR activation is estimated to change 1–10% of all phosphorylation sites in the cell.[46–49] As a comparison, an estimated 16–20% of phosphosites can be regulated upon receptor tyrosine kinase (RTK) stimulation.[50,51]

Before discussing examples in which biased agonists change the targets of GPCR signaling when compared to their unbiased counterparts, it is important to highlight an example demonstrating that this is not always the case. Tsvetanova et al. compared the cellular phosphoproteome of HEK293 cells endogenously expressing β2-adrenergic receptor (β2AR) when stimulated by the full agonist isoproterenol compared to a partial agonist with G protein bias, salmeterol.[48,52,53] The authors found that salmeterol induced changes in the same phosphorylation sites as isoproterenol and the only difference was that the changes were smaller in magnitude with salmeterol.[48] In contrast, a pair of highly complementary studies examined the cellular phosphoproteome downstream of the angiotensin II type 1 receptor (AT1R) when activated by the endogenous agonist angiotensin II (AngII) or a biased agonist, SII ($Sar^1$, $Ile^4$, $Ile^8$-angiotensin).[46,47] AngII is a full agonist that activates Gαq and GRK/β-arrestin pathways while SII is a partial agonist for GRK/β-arrestin and shows minimal activation of Gαq.[54–57] Both studies employed HEK293 cells stably overexpressing AT1R. Together, these papers demonstrated that one-third of phosphosites responding to AT1R activation were linked to GRK/β-arrestin activity (observed with both SII and AngII), while two-thirds were linked to Gαq (observed with only AngII).[46,47] Thus, the majority of the cellular phosphorylation sites remodeled by AngII were untouched by SII-mediated AT1R signaling. Additionally, the authors noted it was unlikely that SII caused AT1R to gain to new signaling targets.[47]

A more recent study examined how the cellular phosphoproteome was remodeled by activation of the protease-activated receptor-1 (PAR1) with thrombin or anticoagulant protease (APC).[49] Thrombin cleavage of PAR1 causes coupling to Gα12/13, Gαq, and β-arrestin while APC causes PAR1 to couple to β-arrestin.[58–61] Phosphoproteomic comparison of PAR1 signaling in endothelial cell derived EA.hy926 cells revealed broadly different targets of thrombin and APC-induced activity.[49] Targets of thrombin activated PAR1 signaling were enriched in proteins linked to endothelial barrier function and adherens junctions while APC-induced signaling modulated phosphorylation of proteins linked to gene transcription.[49] Additionally, APC modulated a much smaller number of phosphosites than the full agonist thrombin.[47,49] Thus, certain biased agonist/GPCR pairs can greatly change the cellular phosphoproteome—and consequently cellular function—compared to their unbiased counterparts.

### 3.2 | Transcriptomics

GPCR signaling can alter gene transcription and cause persistent changes to the proteome. Multiple methods, including RT-qPCR and transcriptional reporters, allow study of transcription factor activity or levels of specific mRNAs. Yet analogous to what was discussed above, these transcriptional assays cannot capture the breadth of changes induced by GPCR activation. Microarrays and RNAseq provide an alternative approach to more holistically capture how GPCR activation changes the cell. Such approaches show that GPCR signaling can activate the transcription of hundreds of genes.[39,62–64] As a point of comparison, activation of RTKs can induce changes to the transcription of ~1,000 genes.[65–67] Christensen et al. compared how the full agonist AngII and the biased agonist SII changed transcription downstream of AT1R.[62] They observed that SII regulated only 12% of the 212 transcripts that were regulated by AngII, and had no additional transcriptional targets. They also observed kinetic bias: AngII-induced transcription returned to basal after 48 hr of stimulation while SII induced transcripts remained upregulated at 48 hr.[62] In an investigation of

location bias, endogenous β2AR-induced transcription in HEK293 cells was compared under normal conditions or when signaling was restricted to the cell surface by inhibition of endocytosis.[39] Fifty-five genes were found to be induced by activation of β2AR, and transcription of over half of these targets was suppressed by inhibition of endocytosis.[39] In these two studies, agonist bias or location bias caused a strong reduction in the number of transcribed genes.

### 3.3 | Bias in GPCR signaling

As more holistic methods, transcriptomics and proteomics provide a cellular perspective of agonist bias at GPCRs. [39,47,49,62] These studies provide insight about whether biased agonists cause GPCRs to gain new signaling targets or simply change the number/magnitude of full agonist targets. In several cases, biased agonists strongly reduced the number of targets while in one case bias caused GPCR signaling to target new phosphosites.[47–49,62] Why this occurs is not currently understood and these phenomena deserves further study. Together, these data raise the question: if the cellular state induced by GPCR signaling can differ significantly between full agonists and biased agonists, how then can GPCR regulation be effected by bias?

## 4 | REGULATION OF GPCR SIGNALING

### 4.1 | GPCR kinases: Partial agonism and changes in GRK/GPCR pairing

Acute termination of GPCR signaling—called desensitization—requires GRKs to phosphorylate the agonist-activated receptor. Genetic or chemical inhibition of GRK function, or mutation of GPCR Ser/Thr phosphorylation sites, inhibits desensitization.[68,69] Nonvisual GRKs are grouped into two subfamilies by sequence homology: GRK2/3 and GRK4/5/6.[68] Two important questions remain incompletely resolved: (a) why GRKs pair with certain GPCRs; (b) what are the consequences of these pairings? A critical difference between GRKs is the mechanism by which they are recruited to activated GPCRs. GRK2/3 proteins contain a pleckstrin homology (PH) domain which directly binds free Gβ/Gγ while GRK4/5/6 proteins lack this PH domain and are instead membrane associated through palmitoylation or direct lipid binding.[27] Thus, a component of GRK2/3 recruitment requires GPCR signaling to liberate free Gβ/Gγ.[25] Additionally, it has been shown that GRKs can phosphorylate non-receptor proteins and the targets of this "extramural" activity appear to be GRK-specific.[68,70] Here we review studies in cultured cells showing how partial and biased agonists can change the GPCR/GRK pairing.

Studies of agonist-driven phosphorylation of β2AR in model human cells demonstrated that both GRK2 and GRK6 are required to obtain full phosphorylation of β2AR.[71–73] RNAi-mediated knockdown of GRK6 reduced agonist-driven phosphorylation of two sites in β2AR (Ser355 and Ser356) while GRK2 knockdown reduced phosphorylation on the other six (Thr360, Ser364, Ser396, Ser401, Ser407, and Ser411).[73] Intriguing, the ligand carvedilol—an inverse agonist for the G protein pathway and partial agonist for the GRK/β-arrestin pathway—was shown to only stimulate phosphorylation of the GRK6-sensitive sites, Ser355/Ser356.[73,74] One possible consequence of ligand-specific GPCR phosphorylation was defined in the "bar-code" hypothesis: differential patterns of GPCR phosphorylation cause distinct activities of β-arrestin and/or preferential coupling to different signaling pathways.[73,75–78] Thus, in cultured cells, a full agonist at β2AR can activate both GRK2 and GRK6 while carvedilol drives a partial version of the same response and only activates GRK6.[73]

The mu opioid receptor (MOR) provides a contrasting example of the cellular response to partial/biased agonism. In cultured cell models, the full agonist DAMGO was shown to cause GRK2/3-mediated phosphorylation of MOR phosphorylation sites: S356, T357, T370, S375, T376, and T379.[79–84] This result was compared with the lower efficacy agonist morphine, which is typically considered a partial agonist in vitro although with systems-dependent efficacy.[44,85–88] Morphine caused phosphorylation of S375 in MOR, produced minimal phosphorylation of T370, T376, and T379, and thus drove 5- to 15-fold-less multiphosphorylation of MOR.[79–84] Unexpectedly, and in contrast to β2AR, morphine caused a switch to GRK5 (part of the GRK4/5/6 subfamily) as the kinase primarily required for phosphorylation of MOR in cultured cells.[80,82] Importantly, agonist-dependent and GRK-specific phosphorylation of MOR is largely conserved in animal models. GRK3 knockout mice, but not GRK5 knockout mice, showed reduction in MOR phosphorylation stimulated by higher efficacy agonists.[80,89] In comparison, MOR phosphorylation driven by morphine was reduced in both GRK5 and GRK3 knockout mice.[80,89] These data suggest that the lower efficacy agonist morphine can cause a fundamental re-wiring of GRK/GPCR pairing compared to higher efficacy opioids.

### 4.2 | Atypical regulation of opioid receptors via PKC and JNK

The canonical mechanism for homologous desensitization of GPCRs involves GRKs and β-arrestin. However, not all agonists efficiently recruit these proteins. How do GPCRs desensitize under these conditions? A series of observations demonstrated that lower efficacy opioid ligands can fundamentally re-write the mechanisms of cellular desensitization and physiological tolerance.[90] Here we refer to this process as atypical regulation to differentiate it from canonical homologous regulation involving GRK/β-arrestin.

In cultured cells, desensitization of MOR caused by the full agonist DAMGO was sensitive to dominant negative mutants of GRK2 but not PKC inhibitors.[83,91] In contrast, MOR desensitization driven by the partial agonist morphine was sensitive to PKC but not GRK inhibition.[83,91] These findings were extended to electrophysiological recordings in the locus coeruleus (LC) from acute brain slice.[92,93] Cell-type differences play a role in atypical desensitization of MOR: the PKC dependence of morphine induced desensitization could be seen in naive HEK293 or AtT20 cells while neurons assayed in brain slice required co-activation of PKC or pretreatment of the animal with morphine.[83,91–96] Thus, morphine opens the door to an atypical mode of GPCR regulation but cell-type specific parameters determine if it occurs. One model suggests that the mechanism of MOR desensitization exist on a continuum informed by ligand efficacy and shaped by cell-type specific parameters.[90] In this model, morphine and DAMGO occupy ends of the continuum, and primarily use either PKC or GRK, while other opioids may be able to use both mechanisms.[90,92,97,98]

In vitro studies have provided insight into how PKC may be mediating atypical regulation. Efficient β-arrestin binding to a GPCR is thought to require three or more correctly spaced phosphorylations in the receptor.[12] In cultured cell models, PKC activity can drive phosphorylation of two residues in MOR, S363 and T370.[81,99,100] Thus, it is possible that the desensitizing activity of PKC may not occur exclusively at the receptor but at other points in the pathway.[90] In support of this model, a mutant MOR lacking all phosphorylation sites in its carboxy-terminal tail was shown to still be able to desensitize in response to morphine.[83] Additionally, it has been demonstrated that Gαi is directly phosphorylated by PKC downstream of morphine-activated MOR and this phosphorylation contributed to atypical regulation.[101]

Development of opioid tolerance in vivo can also occur through opioid-specific mechanisms involving canonical pathways (GRK) or atypical pathways (PKC or JNK).[102,103] Here we will discuss behavioral assays comparing opioid analgesic activity when combined with genetic or chemical inhibition of GRKs, PKC, or JNK. Such experimental paradigms are powerful but have potential caveats: (a) opioids have different pharmacokinetic/pharmacodynamic properties such that dosing can vary significantly between compounds; (b) tissue-specific expression of proteins in the opioid receptor pathway means opioids can behave differently between physiological assays; (c) kinases phosphorylate many proteins and thus the kinase inhibitors will be pleiotropic in their effects as well as having potential off-target activities. With these caveats in mind, data from multiple groups suggest the mechanisms for development of opioid tolerance differ between the lower efficacy agonist morphine and higher efficacy opioids.

In a GRK3 KO mouse, tolerance to the high efficacy agonist fentanyl but not the lower efficacy agonist morphine was attenuated in the hot plate assay for analgesia.[97,104] In a separate study, GRK/β-arrestin function was disrupted by mutation of S375 in MOR.[105] S375 is the initiating site for MOR multiphosphorylation and mutation of this site to alanine reduced overall MOR phosphorylation, β-arrestin recruitment, and agonist-dependent MOR endocytosis.[79,82,84] In a S375A-MOR knock-in mouse, development of tolerance to high efficacy opioids was reduced while development of tolerance to morphine was retained in the electrical tail root assay for analgesia.[105] In conjunction with these genetic studies, kinase inhibitors have provided insight into the pathway(s) by which tolerance to morphine can develop in the animal. Inhibitors to PKC were shown to inhibit the development of tolerance to morphine in both the tail-flick and hot plate assays for analgesia.[98,106–108] Importantly, PKC inhibitors did not inhibit the development of tolerance to the high efficacy agonist DAMGO in assays for opioid-induced analgesia.[98] In addition to PKC, inhibition of JNK has been broadly reported to inhibit the development of morphine tolerance but not affect tolerance to higher efficacy opioids.[104,109,110] Consistent with the model that JNK activity is involved in the development of morphine tolerance, a JNK2 knockout mouse was shown to have reduced tolerance to morphine in tail-flick and hotplate assays of analgesia.[104,111] However, it is important to underscore pathway/tissue specific differences in opioid efficacy and mechanisms of desensitization. For example, in opioid-induced respiratory depression, PKC inhibitors—but not JNK inhibitors—reduced the development of morphine tolerance.[112] Together these in vitro data examining MOR desensitization and in vivo data studying development of tolerance suggest that the cell contains the ability to muster different homeostatic responses to opioid receptor activation: a "canonical" response utilizing GRK/β-arrestin and an

"atypical" response which utilizes kinases such as PKC or JNK.

Is atypical regulation a broader phenomenon observed with other GPCRs? While not conclusively resolved, evidence suggests that D2 dopamine receptor, CB1 cannabinoid receptor, and 7A serotonin receptor may undergo analogous types of regulation.[113–116] Another intriguing example involves kappa opioid receptor (KOR). Norbinaltorphimine (nor-BNI) was originally identified as a KOR antagonist.[117] It was subsequently recognized that nor-BNI, as well as the KOR antagonists JDTic and 5′-GNTI, had properties of biased agonists in that they inhibited G protein signaling but activated JNK signaling through KOR.[118] Additionally, several atypical properties were noted about these KOR ligands: (a) they evoked long-lasting (~3 weeks) antagonism of the G protein pathway in vivo without any sign of covalently modifying the receptor; (b) inhibition of JNK blocked long-term inactivation of KOR; (c) inhibition of PKC blocked phosphorylation of JNK, implying that PKC was part of atypical KOR regulation.[104,115,118,119] A recent study identified an increased association between KOR and Gαi after nor-BNI treatment, and proposed a mechanism for nor-BNI mediated inactivation of KOR proceeding through loss of G protein palmitoylation via a JNK-mediated increase in local reactive oxygen species production.[115]

## 5 | THE BOUNDARIES OF LIGAND BIAS AT GPCRS

Much is still unknown about how agonist bias, location bias, and kinetic bias shape GPCR activity. In this review, we have highlighted examples in which biased agonists can substantially change the targets of GPCR signaling or the mechanisms by which GPCRs are regulated. While it is clear that not all biased ligands evoke such distinct cellular responses compared to their unbiased counterparts, the examples reviewed here help to draw boundaries around what is possible in a cellular response to GPCR activation. It is noteworthy that many of the biased agonists discussed in this review are, in fact, partial agonists for the pathway(s) they activate. This raises the intriguing question of how to disentangle partial agonism from differential pathway efficacy (e.g., bias)? Toward that point, a recent paper investigating bias at opioid receptors developed an approach to minimize the effects of system bias and receptor reserve and found that many opioids previously described as biased agonists are in fact low efficacy partial agonists without significant bias.[88] In this same paper, they show that many of these low efficacy partial agonists have an improved therapeutic window for analgesia compared to higher efficacy opioids.[88] Thus, further study of partial agonism and bias in all its forms will be necessary to define the boundaries of what is possible and harness those understandings for improved GPCR-targeting therapeutics.

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## AUTHOR CONTRIBUTIONS

**Thomas Fernandez:** Conceptualization; writing-original draft; writing-review and editing. **Monica De Maria:** Conceptualization; writing-original draft; writing-review and editing. **Braden Lobingier:** Conceptualization; funding acquisition; supervision; writing-original draft; writing-review and editing.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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