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Photoswitching the efficacy of a small-molecule ligand for a peptidergic GPCR: from antagonism to agonism

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Abstract: In order to obtain optical control of GPCR function, we set out to develop small-molecule ligands with photoswitchable efficacy in which both configurations bind the target protein but exert distinct pharmacological effects, i.e., stimulate or antagonize GPCR activation. Our design was based on a previously identified efficacy hotspot for the peptidergic chemokine receptor CXCR3 and resulted in the synthesis and characterization of five new azobenzene-containing CXCR3 ligands. G protein activation assay and real-time electrophysiology experiments demonstrated a photoswitching from antagonism to partial agonism and even to full agonism (VUF16216). SAR evaluation suggests that the size and electron-donating properties of the inner aromatic ring substituents are important for the efficacy photoswitching. These azo compounds are the first GPCR ligands with a nearly full efficacy photoswitch and may become valuable pharmacological tools for the optical control of peptidergic GPCR signaling.

Photopharmacology is an emerging discipline that involves the dynamic regulation of protein activity with light. Amongst others, freely diffusible photoswitchable ligands are used to regulate protein activity in a non-invasive and reversible manner with high spatiotemporal precision.[1] A commonly used strategy is to incorporate an azobenzene moiety into an existing bioactive ligand (azologization[1a]) to enable the isomerization from a planar *trans* configuration to a bent *cis* configuration in response to near-UV light. Longer wavelengths or thermal relaxation can revert this photoisomerization, offering a dynamic approach to modulate ligand geometry. These geometrical changes can alter the binding affinity and/or efficacy of the ligand for its target protein.

In view of their important role in numerous (patho)physiological processes and high therapeutic relevance, GPCRs represent an excellent protein class for the development of photoswitchable ligands.[2] Recently, several photoswitchable ligands have been reported for GPCRs[3] with some examples involving *in vivo* use.[4] Although it is not always straightforward to discern contributions by affinity or potency shift from the contribution by any efficacy shift, most reported photoswitchable GPCR ligands display light-induced alterations in affinity or potency. We present here a detailed photochemical and biochemical characterization of a class of photoswitchable ligands, of which the efficacy for a peptidergic G protein-coupled receptor can be controlled with light from antagonism to partial and, unprecedentedly, to full agonism.

To develop this proof of concept in GPCRs, we selected chemokine receptor CXCR3, which belongs to the class A GPCRs and is of interest since it plays a key role in T cell function and CXCR3 agonism can aid in tissue repair.[5] CXCR3 signals via $G\alpha_i$ proteins in response to the endogenous agonist peptide ligands CXCL9, CXCL10 and CXCL11.[6] However, small-molecule CXCR3 agonists are scarce and limited to peptidomimetic ligands[7] and non-peptidomimetic biaryl-type ligands.[8] Structure-activity relationship studies on the latter revealed that increasing the size of a halogen substituent on the *ortho* position of the outer aryl ring increases the agonist efficacy, while shifting it to the *meta* position yields antagonists (*figure 1A*).[8] Quantitative structure-activity relationships suggested that the electron density, a possible halogen bond and/or geometrical differences as a result of the biaryl dihedral angle contribute to the agonist efficacies.[8] Here, we aim to mimic this molecular efficacy hotspot by azologization of the biaryl moiety and as such enable optical control over efficacy of the CXCR3 ligands.

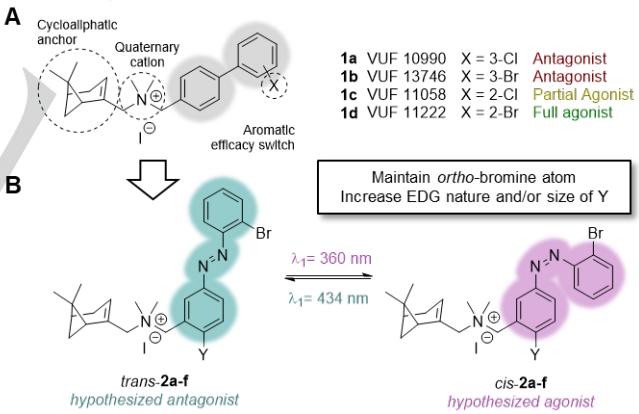

Figure 1: General design strategy. (A) Example of biaryl CXCR3 ligands that show differences in efficacy based on position and size of a halogen substituent. (B) Azologization of the scaffold of the biaryl CXCR3 ligands with maintained *ortho*-Br atom and tailored Y groups to give photoisomerizable analogs of **1a-d** (i.e. **2a-f**). Illumination with 360 nm enables a switch from *trans* to *cis* configuration and 434 nm illumination reverts the photoisomerization.

A series of azobenzene-containing ligands was synthesized by replacing the biaryl moiety of **1a-d** with an azobenzene moiety to obtain **2a-f** (*Scheme S1*). The geometrical differences between *trans* and *cis* azobenzene isomers were hypothesized to be able to mimic the spatial features of the biaryl moiety in **1a-d**. In all cases, the bromine atom on the *ortho* position of the outer aromatic ring was maintained to accommodate a potential halogen bond that was suggested to be important for the agonism of the parent biaryl ligands.[8] To explore the

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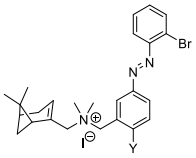
contribution of a possible conformational lock and electron density in the azobenzene-moiety to the targeted efficacy switch, substituents on the *para* position of the inner aromatic ring (*Y*, *figure 1B* and *table 1*) were varied. To that end, halogen atoms of different sizes and electronegativity (F, Cl and Br) and two different electron-donating groups (-OMe, -NMe_2) were used. Based on spatial considerations, agonism was hypothesized to reside in the *cis*-isomer of these designed compounds.

Table 1. Photoisomerization properties and binding affinities of **2a-f** for human CXCR3. Percentage of photoisomerization, pIC_{50} values and photo-induced affinity switches are shown as the mean ± standard error of the mean (SEM) of at least three independent experiments each performed in triplicate.



		Photoisomerization				[^{125}I]-CXCL10 binding[d]		
#	Y	*Trans* λ_{max}[a] (nm)	*Cis* λ_{max}[a] (nm)	PSS[b] (%)	$t_{1/2}$[c] (d)	pIC_{50} *trans*	pIC_{50} at PSS	PAS[e]
2a	H	325	420	88.9 ± 0.4	109	6.0 ± 0.1	6.0 ± 0.1	1.0 ± 0.1
2b	F	327	420	92.7 ± 0.1	92	6.0 ± 0.0	6.2 ± 0.0	1.6 ± 0.0
2c	Cl	331	419	92.6 ± 0.2	55	5.9 ± 0.1	6.2 ± 0.1	2.0 ± 0.4
2d	Br	334	424	92.6 ± 0.2	61	5.8 ± 0.1	6.4 ± 0.1	3.8 ± 0.4
2e	OMe	353	428	92.1 ± 0.1	29	5.9 ± 0.0	6.8 ± 0.0	8.3 ± 1.3
2f	NMe_2	387	dec.[f]	dec.[f]	dec.[f]			

[a] The absorbance maxima were extracted from UV-Vis spectra (10 µM in HEPES buffer with 1% DMSO at 25 °C, *figure 2A*, *figure S1*). [b] The photostationary state (PSS) was measured as the percentage of area of the *cis* isomer compared to combined areas of *cis* and *trans* isomers as detected by LC at 265 nm. [c] The thermal relaxation half-life was determined by allowing a PSS sample (10 µM in HEPES buffer with 1% DMSO) to thermally isomerize at 25 °C in the dark (*figure S2*). [d] The pIC_{50} value was measured by [^{125}I]-CXCL10 binding experiments using membranes prepared from HEK293T cells expressing CXCR3. [e] The photo-induced affinity switch (PAS) was calculated as the ratio of the IC_{50} values of *trans* isomers and those corresponding to the PSS. [f] dec. = decomposition under illumination at 360 nm

First, the photochemical properties of **2a-f** were investigated by determining the UV-Vis absorption spectra after illumination (*table 1*). In the dark, the *trans* isomers were identified (*table 1, figure 2A, figure S1*). Depending on the electron donating properties of the Y substituent (*table 1*), a slight red-shifting in wavelengths of maximal absorbance of the π–π* band was observed. Upon illumination with 360 nm light, mainly the *cis* isomers of **2a-e** were observed (*figure 2A, figure S1A-D*), showing suitable photostationary states (PSS) containing 89-93% *cis* isomer (*table 1*). After subsequent illumination of the samples with 434 nm light, mostly *trans* isomers of **2a-e** were detected (*figure 2A, figure S1A-D*). Unfortunately, **2f** decomposes under 360 nm illumination (*figure S1E*) and was therefore omitted from further pharmacological characterization. Photoisomerization of **2e** was confirmed by ^{1}H NMR (*figure 2C*) and LC-MS (*figure 2D*) analyses. In both LC-MS and NMR analysis, only *trans*-**2e** was observed in the dark, while a PSS of 95% *cis*-**2e** after 360 nm illumination and a PSS of 75% *trans*-**2e** after 434 nm back-illumination were identified. Compound **2e** displays high resistance toward photochemical wear (*figure 2E*) and the long relaxation half-lives of *cis* **2a-e** indicate the bistable nature of **2a-e** (*figure 2B, table 1, figure S2*).

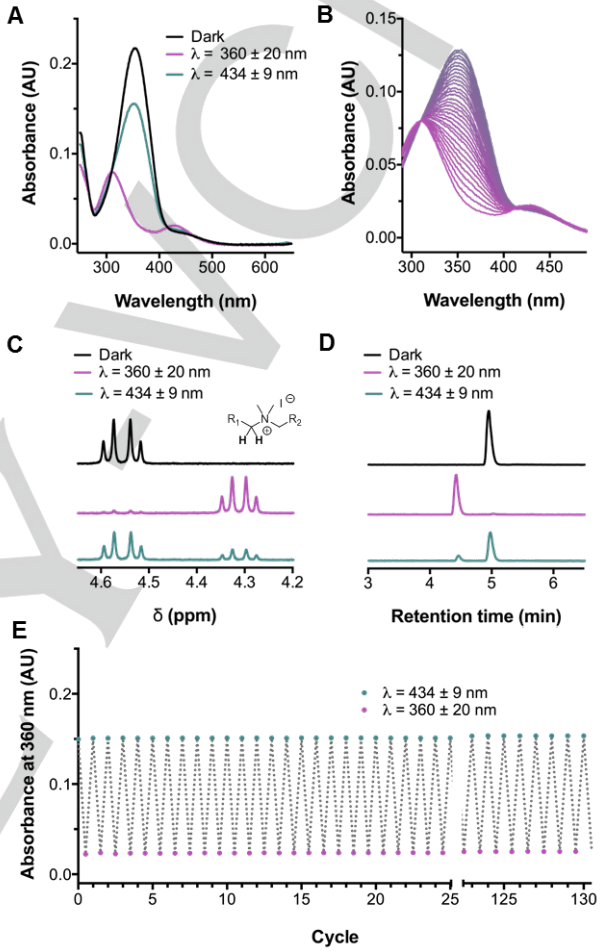


Figure 2. Photochemical properties of **2e**. (A) UV-Vis absorption spectra of 10 µM **2e** in HEPES buffer with 1% DMSO at 25 °C under dark conditions (black), after illumination with 360 nm light for 2 min (magenta) and after back-illumination for 2 min with 434 nm light (turquoise). (B) UV-Vis absorption spectra of 10 µM **2e** in HEPES buffer after obtaining the PPS, measured every 24 h during 30 d at 25 °C. (C, D) The isomerization of 1 mM **2e** in DMSO-*d6* analyzed by (C) ^{1}H NMR (α-ammonium methylene protons with signals at 4.56 ppm and 4.31 ppm for the *trans* and *cis* isomers, respectively) and (D) LC-MS analyses. (E) UV-Vis absorption measurements at 360 nm after repeated cycles of illumination with 360 nm (magenta) and 434 nm light (turquoise) of 10 µM **2e** in HEPES buffer.

Compounds **2a-e** were tested for their ability to bind to CXCR3. Control **1a** inhibits [^{125}I]-CXCL10 binding to membranes prepared from CXCR3-expressing cells with a pIC_{50} value of 6.4 ± 0.1, which is comparable to previous findings.[8] Compounds **2a-e** were tested in the dark (corresponding to >99% *trans* isomers) and at PSS after illumination during 15 minutes with 360 nm light. Due to the bistable nature of **2a-e**, the

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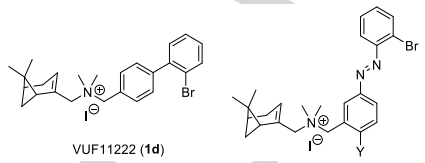
photostationary state was assumed to remain unaltered after illumination during the assay time. All *trans* isomers of **2a-e** inhibit [^{125}I]-CXCL10 binding in a concentration-dependent manner with similar submicromolar IC_{50} values (*table 1, figure 3A*). On the contrary, **2a-e** illuminated to PSS demonstrate increased pIC_{50} values with increasing size and electron donating properties of substituent Y (*table 1, figure 3A*). For example, **2a** shows no photo-induced affinity shifts (PAS) upon illumination, whereas **2e** displays the largest PAS with an 8-fold higher pIC_{50} value at PSS as compared to its *trans* form (*table 1*). A [^{35}S]-GTPγS functional binding assay was used to measure the intrinsic activity (α) as a means to quantify the efficacy of these photoswitchable ligands. As CXCL11 has the highest potency and efficacy of all three chemokines[9], it was deemed the best chemokine control for functional assays. Control **1d** (VUF11222) acts as a full agonist as compared to CXCL11 (*figure S3A-B)*, as was previously reported[8]. We did not observe any alteration of potency or efficacy of **1d** as a result of illumination (*table 2, figure S3B*). All *trans* compounds **2a-e** act as antagonists or very weak partial agonists with low efficacies (*figure 3C*, *table 2, figure S3C-F*). On the other hand, **2a-d** illuminated to PSS act as partial agonists with comparable submicromolar potencies (*table 2, figure S3C-F*). The efficacy of **2a-d** at PSS qualitatively correlates with the size and the electron donating properties of the *para* substituent (Y) of the inner aromatic ring (*figure 3B*, *table 2*). Confirming this trend, **2e** at PSS acts as a full agonist (α=0.99, *table 2, figure 3C*) and consequently shows the largest photo-induced difference of efficacy (PDE, *table 2*).

Table 2. Efficacy and potency of **2a-e** and control **1d** determined by [^{35}S]-GTPγS binding experiments using membranes prepared from HEK293T cells expressing human CXCR3. Potency, intrinsic activity and values for PDE are shown as the mean ± SEM of three independent experiments each performed in duplicate.



Cpd.	Y	α[a] *trans*	α[a] at PSS	PDE[b]	pEC_{50} *trans*	pEC_{50} at PSS
1d	-	1.04 ± 0.08	1.03 ± 0.07	0.00 ± 0.03	6.9 ± 0.0	6.9 ± 0.0
2a	H	0.05 ± 0.03	0.30 ± 0.01	0.25 ± 0.02	- [c]	6.5 ± 0.1
2b	F	0.12 ± 0.00	0.41 ± 0.03	0.30 ± 0.03	- [c]	6.2 ± 0.1
2c	Cl	0.11 ± 0.01	0.54 ± 0.01	0.42 ± 0.01	- [c]	6.3 ± 0.1
2d	Br	0.14 ± 0.02	0.58 ± 0.05	0.45 ± 0.04	- [c]	6.2 ± 0.1
2e	OMe	0.16 ± 0.01	0.99 ± 0.04	0.83 ± 0.06	- [c]	6.4 ± 0.1

[a] The ligand intrinsic activity (α) was determined relative to the maximum response of the endogenous full agonist CXCL11 (for which α is set to 1.0). [b] The photo-induced difference of efficacy (PDE) is obtained from subtracting the intrinsic activity under dark conditions from the intrinsic activity at PSS. [c] Could not be determined due to too low efficacy and/or potency.

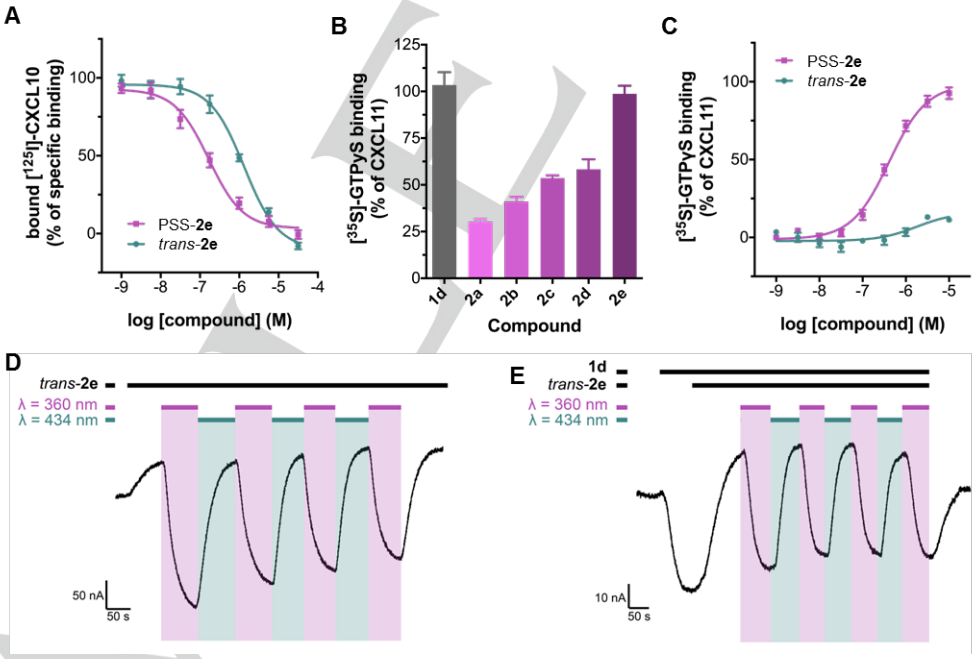


Figure 3. Pharmacological characterization of **2a-e**. (A) Binding experiments with [^{125}I]-CXCL10 were performed in the presence of increasing concentrations of **2e** in the dark (turquoise) or at PSS after 360 nm illumination (magenta) using membranes prepared from HEK293T cells expressing human CXCR3. (B, C) Maximum responses (efficacy) of **1d** and **2a-e** after 360 nm illumination (B) or concentration-response curves of *trans*-**2e** and **2e** at PSS (C), as determined in a [^{35}S]-GTPγS binding experiment using membranes prepared from HEK293T cells expressing human CXCR3. Data are presented as percentage of specific [^{125}I]-CXCL10 binding (A) or as percentage of the maximum response induced by the endogenous agonist CXCL11 (B, C) and represent the mean ± SEM from at least three independent experiments each performed in triplicate (A) or duplicate (B, C). (D, E) Representative traces of two-electrode voltage clamp experiments in *Xenopus laevis* oocytes expressing human CXCR3 and GIRK channel in the presence of **2e** (10 μM) (D) or **2e** (10 μM) in competition with **1d** (1 μM) (E) and using 360 nm (magenta) and 434 nm light (turquoise) illumination cycles (concentrations given are in high potassium solution with 0.1% DMSO).

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To address the possibility of nonspecific effects on the receptor activity due to compound microprecipitation,[10] nephelometry experiments were performed (*figure S5*)[11] and revealed no microprecipitation of *trans*-**2a-e** or *cis*-**2a-e** at pharmacologically relevant concentrations.

The full agonism of **2e** at PSS can possibly be explained by the size of the -OMe group in Y position and/or the more important electron-donating properties as compared to the halogen substituents. Unfortunately, this explanation could not be further explored with a -NMe_2 moiety due to chemical instability of **2f** under illumination. We observed a correlation between PDE and PAS values (r = 0.986, P = 0.0024, *figure S4*). This is probably a result of more efficient inhibition of [^{125}I]-CXCL10 binding by photoisomers with higher efficacy through preferential binding to a subset of CXCR3 conformations that overlap with those preferentially binding the endogenous agonist [^{125}I]-CXCL10[12].

The photo-induced efficacy switch of **2e** was dynamically evaluated in real-time electrophysiology measurements using two-electrode voltage clamp (TEVC) in *Xenopus laevis* oocytes transiently co-expressing CXCR3 and G protein-coupled inwardly-rectifying potassium (GIRK) channel. Control agonist **1d** induced GIRK channel activation, which was not affected by several illumination cycles of 360 nm and 434 nm light, and the initial current was restored upon washing-out with buffer (not shown). *Trans*-**2e** did not activate GIRK channels (*figure 3D*), as expected for an antagonist/weak partial agonist, but a slight outward current was observed. Indeed, it is well known that quaternary ammonium ions may interact with GIRK channels[13] or other potassium channels[14]. Illumination of perfused oocytes, expressing CXCR3 and GIRK with 360 nm light prompted the photoisomerization of *trans*-**2e** to the *cis* configuration, and consequently induced the activation of CXCR3 in turn leading to GIRK channel activation (*figure 3D*). This confirms CXCR3 agonism of **2e** at PSS in the oocyte expression system**.** This effect could be reverted by 434 nm illumination and repeated cycles of illumination with 360 and 434 nm light clearly show the reversible activation of CXCR3 by photoswitching *trans*-**2e** to its *cis* isomer and *vice versa* (*figure 3D*). The antagonist behaviour of *trans*-**2e** was confirmed by competition with agonist **1d** in the same CXCR3-expressing oocyte system (*figure 3E*). In the dark, *trans*-**2e** antagonized the CXCR3 activation induced by **1d** and this was reverted upon illumination with 360 nm light. Subsequent illumination with 434 nm recovered the antagonism of *trans*-**2e.**

Taken together, we have demonstrated optical control of CXCR3 activity by photoswitching small-molecule antagonists/weak partial agonists to agonists with different efficacies up to full agonism. The isomerization from the *trans* to *cis* configuration was designed to probe a very subtle efficacy hotspot that was identified in earlier studies. Real-time electrophysiology measurements confirmed the reversibility of an efficacy switch from antagonism to agonism upon several illumination cycles. These compounds, particularly **2e**, will be excellent tool compounds to elucidate CXCR3 signaling due to the distinctive spatio-temporal control of functional activity provided with light. Such photocontrol may also be of interest for topical treatments, such as wound healing where CXCR3 agonism plays a key role[5]. Further efforts will be undertaken to understand the mechanism of action of these compounds and improve their biocompatible properties, (e.g. red-shifting photoisomerization wavelengths).[15] To our knowledge, **2a-e** are the first small-molecule GPCR ligands that harbor a rationally designed efficacy photoswitch. Moreover, **2e** (VUF16216) is the highest photo-induced efficacy switch for a GPCR ligand reported to date and provides a real-time efficacy switch from antagonism to agonism. Our results together with other reported GPCR efficacy photoswitchers may contribute to the development of a second generation of photoswitchable ligands with light-dependent functional activity switches.

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Keywords: Azo compound • Efficacy Photoswitching • G Protein-Coupled Receptor • Photochromism • Photopharmacology

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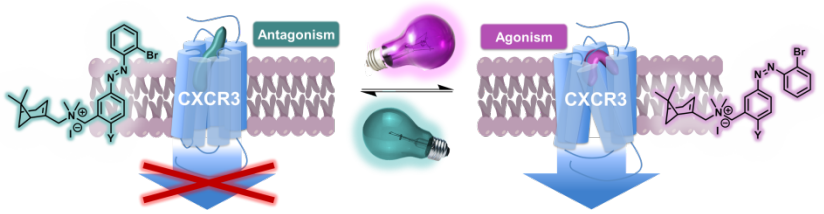

In order to obtain unprecedented spatiotemporal optical control of GPCRs, we set out to develop efficacy photoswitchable ligands. G protein activation assays and real-time electrophysiology experiments validated a photoswitching from antagonism to partial agonism and even to full agonism. VUF16216 is the first GPCR azo-ligand with almost full efficacy photoswitch and it may become a valuable tool for the optical control of GPCR activity.

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Photoswitching the efficacy of a small-molecule ligand for a peptidergic GPCR: from antagonism to agonism

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