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Effects of polar κ receptor agonists designed for the periphery on ATP-induced Ca^{2+} release from keratinocytes

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Abstract

In order to obtain polar κ agonists, which cannot pass the blood brain barrier, the secondary amines **3a** and **3b** were reductively alkylated with pyridine-3-carbaldehyde to give the pyridylmethyl substituted perhydroquinoxalines **5a** and **5b**, respectively. Both **5a** and **5b** show very high κ-opioid receptor affinity with K_i values of 0.13 nM and 3.8 nM, respectively. Moreover they are very selective for the κ receptor. In the [^{35}S]GTPγS assay both quinoxalines reached full agonistic activity. With an EC_{50} value of 34 nM **5a** is only slightly less potent than the prototypical κ agonist U-69,593. For

the determination of the logD values a shake-flask method was developed making use of quantification by mass spectrometry which requires only very small amounts of compound (<0.8 mg). According to this method the $logD_{7.4}$ and $logD_{5.4}$ values of **5a** were 1.1 and -0.45 indicating very high polarity. The $logD_{5.4}$ value was recorded due the acidic milieu of the skin. The perhydroquinoxalines **3** - **5** reduced the release of Ca^{2+} ions into the cytoplasm after stimulation of HaCaT cells with ATP and thereby proved biological activity in human skin cells.

Keywords: atopic eczema; itching skin diseases; κ receptor agonists; perhydroquinoxalines; pyridylmethyl derivatives; receptor selectivity; agonistic activity; logD value; Ca^{2+} release

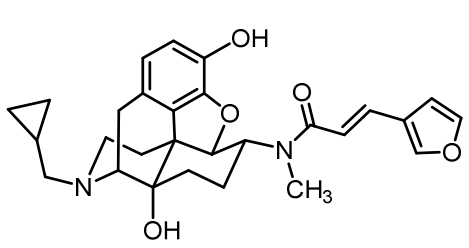
Introduction

Inflammatory and itching skin diseases, especially psoriasis and atopic eczema (syn. atopic dermatitis) are among the most frequent skin diseases. Atopic eczema is one of the most common skin diseases in Europe affecting 2-5 % of adults and up to 15 % of children.[1] Clinically it is characterized by symmetrically distributed, extensive eczema on the face, neck, trunk, and flexural surface of the extremities. The eczema are accompanied by a severe, excruciating pruritus which highly impacts the quality of life and induces sleep and word capability disturbance. Atopic eczema cannot be cured, but acute flares can be treated with topical or systemic immunosuppressants, which exhibit adverse effects limiting their chronic use.

The κ-opioid receptor plays a crucial role in the pathophysiology of atopic eczema and pruritus.[2] In healthy skin, κ receptors are expressed on keratinocytes, macrophages,

fibroblasts and mast cells.[3] In inflammatory skin diseases, diseased keratinocytes produce and release pro-inflammatory and pruritogenic mediators, i.e. ß-endorphin and interleukins, i.e. TSLP[4] acting on sensory nerves to induce itch.

In a mouse model of atopic eczema it has been shown that the systemic κ agonist nalfurafine (**1**, Figure 1) is able to significantly reduce severe pruritus and scratching behavior.[5] Furthermore, the concept of treating patients suffering from pruritus using systemic κ agonists but also μ-opioid receptor antagonists such as naltrexone,[6] is already clinically validated. The systemic κ agonist nalfurafine (**1**, Remitch®) is marketed in Japan for the treatment of patients suffering from chronic pruritus associated with chronic renal insufficiency and hemodialysis.[7] Moreover, nalfurafine has also been tested in a dose-finding study in the treatment of pruritus associated with atopic dermatitis in Europe.[8] However, nalfurafine is not restricted to the periphery causing typical, centrally mediated κ agonist side effects. More recently, a phase II study with the peripherally restricted κ agonist asimadoline for evaluating its safety, pharmacokinetics and preliminary efficacy in pruritus associated with atopic dermatitis was initiated.[9]



1 (nalfurafine)

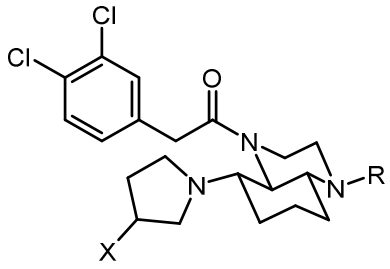


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Figure 1: The anti-pruritic compound nalfurafine (**1**) and κ agonistic perhydroquinoxalines **2**.

Very recently we have reported a novel class of κ agonists **2** based on the perhydroquinoxaline framework. (Figure 1) Some of the members of this class of compounds show low nanomolar κ affinity (K_i = 2-10 nM) and excellent selectivity over μ-opioid, δ-opioid, σ_1 and σ_2 receptors. In the GTPγS-assay full agonistic activity was observed.[10,11]

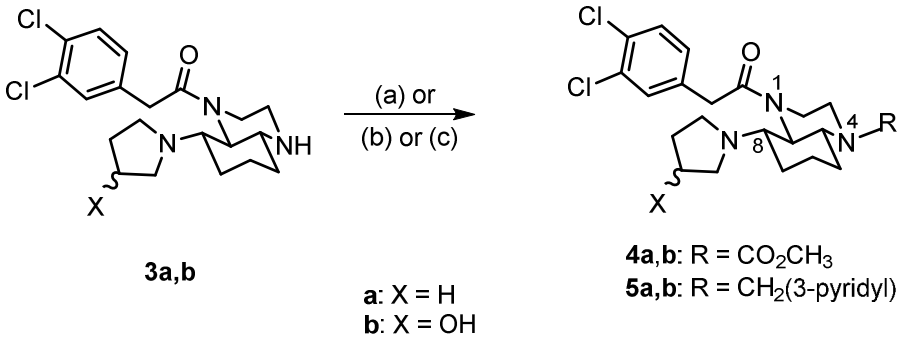
For the local treatment of pruritus associated with atopic eczema we are interested in very potent and very polar κ agonists, which penetrate into human skin but do not cross the blood brain barrier. For this purpose polar substituents should be introduced at the quinoxaline N-atom outside the κ pharmacophore.

The release of TSLP and IL-31 induces production of the endogenous opioid ligand β-endorphin from keratinocytes, which is highly dependent on Ca^{2+} ions.[4,12] Both are reported to play a role in inflammatory processes in the skin, i. e. the production of pro-inflammatory cytokines by T-cells initiated by TSLP-activated Langerhans cells could be the cause in the development of atopic eczema.[13] The increase of cytosolic Ca^{2+} concentration by thapsigargin, a non-competitive inhibitor of the Ca^{2+} ATPase in the sarcoplasmic reticulum,[14] causes a significant increase in TSLP secretion from keratinocytes. The underlying mechanism which leads to increased cytosolic Ca^{2+} concentration is thapsigargin's ability of blocking the cell to pump Ca^{2+} ions into the sarcoplasmic and endoplasmic reticula. Therefore, thapsigargin is a useful tool for studying effects mediated by increased cytosolic Ca^{2+} concentration. To provide preliminary evidence that the newly synthesized κ agonists are functionally active, we selected as a first model the Ca^{2+}.release from intracellular storage into the cytoplasm of HaCaT, an immortalized human keratinocyte cell line.[15]

Results and discussion

Synthesis

The racemic secondary amines **3a** and **3b** activate κ receptors with K_i values of 2.1 and 8.7 nM, respectively. Acylation of the secondary amines **3a** and **3b** with methyl chloroformate led to the methyl carbamates **4a** and **4b** with slightly reduced κ affinity (K_i = 9.7 nM, K_i = 11 nM).[11] In order to obtain very polar κ agonists a 3-pyridylmethyl substituent should be introduced at 4-position. For this purpose the secondary amine **3a** was reductively alkylated with pyridine-3-carbaldehyde and $NaBH(OAc)_3$[16] in CH_2Cl_2 to give the pyridylmethyl derivative **5a** in 29 % yield. The yield of **5b** was slightly increased to 41 % by using $NaBH_3CN$[17] in methanol for the reductive alkylation of **3b** with pyridine-3-carbaldehyde. (Scheme 1) The hydroxypyrrolidine derviatives **3b**, **4b** and **5b** exist as 1:1 mixtures of diastereomers.



3a,b

a: X = H
b: X = OH

4a,**b**: R = CO_2CH_3
5a,b: R = CH_2(3-pyridyl)

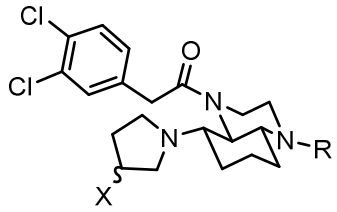
Scheme 1. Synthesis of κ agonists with perhydroquinoxaline framework.
Reagents and reaction conditions: (a) $ClCO_2CH_3$, CH_2Cl_2, rt, 2-3 h, **4a**: 59 %; **4b**: 62 %.[11] (b**)** **3a**, pyridine-3-carbaldehyde, $NaBH(OAc)_3$, CH_2Cl_2, HOAc, rt, 24 h, **5a**: 29 %. (c) **3b**, pyridine-3-carbaldehyde, $NaBH_3CN$, CH_3OH, HOAc, pH 5, rt, 2 h, **5b**: 41 %. Only one enantiomer of the racemic mixture is shown in the Scheme.

κ-Opioid receptor affinity

The κ-opioid receptor affinity of the pyridylmethyl substituted derivatives **5a** and **5b** was recorded in receptor binding studies using selective radioligands. In the first assay guinea pig brain preparations were used as receptor material, [^{3}H]U-69,593 as radioligand and the non-specific binding was recorded after addition of a large excess (10 μM) of the potent κ agonist U-69,593.[10,11,18,19] Additionally the κ affinity was recorded in a cell based assay using transfected HEK-293 (human embryonic kidney) cell lines expressing the human κ-opioid receptor. In this assay [^{3}H]CI-977 was employed as radioligand.[20,21] In Table 1 the κ affinities of **5a** and **5b** together with the analogs **3a**,**b** and **4a**,**b** as well the reference compounds U-69,593 and naloxone determined in both the guinea pig brain assay and the HEK-293 cell-based assay are summarized.

In the guinea pig brain assay the perhydroquinoxalines **3**-**5** show very high κ affinity. The K_i values of the secondary amines **3a**,**b** and the carbamates **4a**,**b** are between 2.1 and 11 nM, whereby the pyrrolidine derivatives **3a** and **4a** are slightly more potent than the hydroxypyrrolidine derivatives **3b** and **4b**.[11] However, the introduction of a 3-pyridylmethyl moiety at 4-position led to a dramatic increase of κ affinity. The pyrrolidine derivative **5a** reveals a K_i value of 0.13 nM and the corresponding hydroxypyrrolidine derivative **5b** a K_i value of 3.8 nM. Thus, the pyridylmethyl derivative **5a** represents the highest affinity κ agonist within the perhydroquinoxaline compound class reported so far.

Table 1: κ-opioid receptor affinities of perhydroquinoxalines and reference compounds correlated with agonistic activity in the [^{35}S]GTPγS-assay.



compd.	R	X	K_i ± SEM (nM) κ [^{3}H]U-69,593[a)]	κ [^{3}H]Cl-977[b)]	EC_{50} (nM) [^{35}S]GTPγS
3a[11]	H	H	2.1 ± 0.4	28	33
3b[11]	H	OH	8.7 ± 1.1	19	35
4a[11]	CO_2CH_3	H	9.7 ± 1.8	160	20
4b[11]	CO_2CH_3	OH	11 ± 2.8	104	79
5a	CH_2-(3-pyridyl)	H	0.13 ± 0.02	22	34
5b	CH_2-(3-pyridyl)	OH	3.8 ± 0.7	31	77
U-69,593			0.97 ± 0.40	-	12
naloxone			3.1 ± 0.40	15 ± 9.3	-

[a)] Guinea pig brain membrane preparation, [^{3}H]U-69,593, number of experiments 3 (n = 3).

[b)] Human κ-opioid receptors, HEK-293 cell line, [^{3}H]Cl-977, number of experiments 2 (n = 2).

The K_i values recorded with the human κ receptors expressed in HEK-293 cells are generally higher than the K_i values recorded with guinea pig brain preparations. With exception of the pair **5a**,**b** the pyrrolidines **3a** and **4a** are slightly less potent than the hydroxypyrrolidine derivatives **3b** and **4b**. The different behavior of the ligands in the two assays is explained by the different assay conditions, in particular different receptor material and different radioligands. However, the pyridylmethyl derivative **5a** has also a very high κ affinity (K_i = 22 nM) in the HEK-293 cell based assay.

Receptor selectivity

Table 2: Affinities of perhydroquinoxalines towards κ-opioid and related receptors.

compd.	R	X	K_i ± SEM (nM)[a]						
			κ[b]	κ[c]	μ	δ	σ_1	σ_2	NMDA (PCP)
			[^{3}H]U-69,593	[^{3}H]Cl-977	[^{3}H]naloxone	[^{3}H]deltorphine	[^{3}H]-(+)-pentazocine	[^{3}H]DTG	[^{3}H]MK-801
5a	CH_2-(3-pyridyl)	H	0.13 ± 0.02	22	2,460	40 %	20 %	1 %	n.c.
5b	CH_2-(3-pyridyl)	OH	3.8 ± 0.7	31	3,480	35 %	10 %	23 %	n.c.
U-69,593			0.97 ± 0.40	-	-	-	-	-	-
naloxone			3.1 ± 0.40	15 ± 9.3	2.3 ± 1.1	103	-	-	-
morphine			-	-	5.2 ± 1.6	-	-	-	-
SNC80			-	-	-	1.2 ± 0.5	-	-	-
(+)-pentazocine			-	-	-	-	5.4 ± 0.5	-	-
haloperidol			-	-	-	-	6.6 ± 0.9	78 ± 2.3	-
(+)-MK-801			-	-	-	-	-	-	2.9 ± 1.1
dexoxadrol			-	-	-	-	-	-	38 ± 9.9

[a] A value in % reflects the inhibition of the radioligand binding at a test compound concentration of 10 μM.

[b] Guinea pig brain membrane preparation, [^{3}H]U-69,593, number of experiments 3 (n = 3).

[c] Human κ-opioid receptors, HEK-293 cell line, [^{3}H]Cl-977, number of experiments 2 (n = 2).

n.c. = no correlation between concentration and radioligand binding.

In order to investigate the selectivity, the affinity of the very potent pyridylmethyl substituted κ agonists **5a** and **5b** towards the related μ- and δ-opioid receptors was determined in competitive radioligand receptor binding assays. (Table 2) Both compounds **5a** and **5b** show at least 100-fold selectivity for the κ-opioid receptor over both the μ- and δ-opioid receptors. Based on the affinity data recorded in the guinea

pig brain assay the selectivity over μ- and δ-opioid receptors is even higher than 10,000- and 1,000- fold, respectively.

In addition to the opioid receptor affinity, the binding of **5a** and **5b** at σ_1 and σ_2 receptors as well as at the phencyclidine binding site of the NMDA receptor was recorded. The σ receptor affinity was included here, since the σ receptor was originally considered to be an opioid receptor subtype. Later it was shown that the σ receptor (or the σ_1 and σ_2 receptor) represent independent receptors with a characteristic distribution in the central nervous system and the periphery and a characteristic ligand binding profile. The affinity towards the phencyclidine binding site of the NMDA receptor was recorded due to the similarity of κ agonists and NMDA antagonists. Thus the substitution pattern and the stereochemistry of benzomorphans determine whether a ligand reacts as a κ agonist (e.g. ketocyclazocine) or NMDA antagonist (e.g. normetazocine).

At the very high concentration of 10 μM the pyridylmethyl derivatives **5a** and **5b** did not compete with the radioligands [^{3}H](+)-pentazocine and [^{3H}]-di-o-tolylguanidine indicating high selectivity over these receptor types. In the PCP assay a clear correlation between the concentration of the test compounds **5** and the residual bound radioactivity could not be observed indicating also very low affinity.

Functional activity

The agonistic activity of the pyridylmethyl substituted perhydroquinoxalines **5a** and **5b** was determined in the [^{35}S]GTPγS ([^{35}S]-guanosine-5´-3-O-(thio)triphosphate) binding assay using human HEK-293 cells as source of human κ-opioid receptors.[11,22]

In Figure 2 the binding curves of the pyridylmethyl substituted perhydroquinoxalines **5a** and **5b** are displayed together with the binding curve of the prototypical κ agonist U-69,593, respectively. It can be clearly seen that **5a** and **5b** represent full κ agonists reaching 100 % intrinsic activity as the prototypical κ agonist U-69,593.

Compared to U-69,593, the EC_{50} values of **5a** and **5b** are slightly increased. (Table 1) Whereas U-69,593 activated the κ receptor with an EC_{50} value of 12 nM, the EC_{50} values of **5a** and **5b** are 34 and 77 nM, respectively. Although the κ receptor affinity of **5a** is more than 10-fold higher than the κ affinity of the secondary amines **3a** and **3b**, it shows the same agonistic activity as the secondary amines **3a** and **3b**.

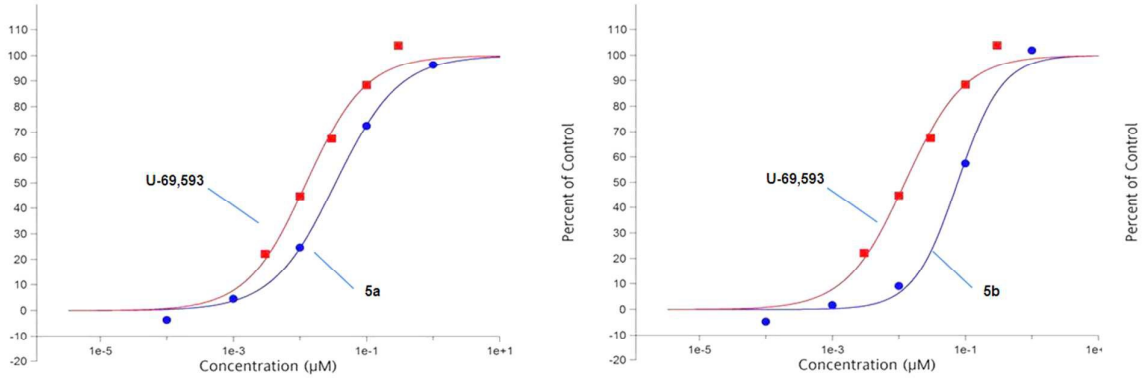


Figure 2: Binding curves of the pyridylmethyl substituted quinoxalines **5a** (left) and **5b** (right) in the [^{35}S]GTPγS assay. The binding curves of the test compounds are compared with the binding curves of the prototypical κ agonist U-69,593, respectively.

It can be concluded that the pyridylmethyl derivative **5a** binds with extremely high affinity towards the κ-opioid receptor (K_i = 0.13 nM). In addition to its high κ affinity, **5a**

shows very high selectivity over other opioid receptor subtypes and related receptors. In the [^{35}S]GTPγS assay **5a** behaved as full agonist with an EC_{50} value of 34 nM.

logD value

The novel κ agonists were designed for the periphery and should not penetrate the blood brain barrier. The logP value is an important physicochemical parameter indicating the ability of a compound to penetrate lipophilic membranes. The *n*-octanol/water partition coefficient (logP value) describes the polarity of a neutral compound without taking the ionization status under physiological conditions into account. Therefore, the logD value, i.e. the distribution coefficient of a compound between an *n*-octanol layer and a buffered water layer at a given pH value, describes in a more realistic manner the lipophilicity properties of a bioactive compound.[23]

Herein the logD value should be determined and calculated at physiological pH value 7.4 ($logD_{7.4}$). For a local, topical treatment with a κ agonist it is important that the compound penetrates into the skin. Thus, the $logD_{5.4}$ value at the more acidic pH value 5.4, which is closer to the acidic surface of the skin, should be determined experimentally and calculated theoretically.

In the literature several methods for the determination of logP and logD values have been reported including shake-flask method and subsequent quantification, correlation of the HPLC retention time or capacity factor with the logP/logD value or the use of artificial membranes.[24-26] Usually at the end of the synthesis only small amounts of the final test compounds are available. Therefore, a method consuming only very few amount of the test compounds and tolerating structural diverse scaffolds should be used for the logD value determination. At first a HPLC method with a RP-18 stationary

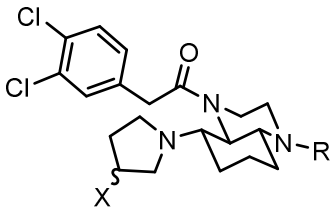
phase was employed. Although thoroughly modified and optimized, this method did not result in a clear correlation between the recorded and reported log $D_{7.4}$ values. This result can be explained by the heterogeneity of the analyzed compounds, which include neutral, basic and acidic compounds with diverse scaffolds (data not shown).

Therefore, a shake-flask method was established making use of quantification of the test compounds in the buffer layer by mass spectrometry. For the experiments the 10 mM stock solutions of the test compounds in DMSO were used. This standard solution, which is also used in the biological assays, was distributed between an *n*-octanol layer and a buffer layer. The buffer and the *n*-octanol layer were saturated mutually by stirring the two phase system for 24 h before use. The logD values were determined by experiments with different volume ratios of buffer and *n*-octanol keeping the amount of DMSO below 1 %. After vortexing for 2 min, the layers were separated by centrifugation and the amount of the sample in the aqueous layer was determined by quantitative mass spectrometry using a quadrupole MS detector. For quantification of the compounds external calibration curves were recorded with at least six concentrations covering the concentration range of the samples. MOPS buffers with pH 7.4 and PBS buffer pH 5.4 were used to determine $logD_{7.4}$ and $logD_{5.4}$, respectively. For the evaluation of the procedure the $logD_{7.4}$ values of six structural diverse approved drugs were determined and compared with $logD_{7.4}$ values reported in literature.[37] (see Table SI1 in Supporting Information) The $logD_{7.4}$ values recorded with the shake flask / LC-MS method and the reported $logD_{7.4}$ values differed by <0.3 units.

In addition to recording logD values, different programs were applied to calculate the $logD_{7.4}$ and $logD_{5.4}$ values of the perhydroquinoxalines **3**-**5**. It was found that the logD values calculated with MarvinSketch 15.5.18. ChemAxon, gave the best fit to the

experimentally determined logD values. Therefore in Table 3 the clogD values calculated using MarvinSketch 15.5.18. ChemAxon, are summarized and correlated with the experimentally determined logD values.

Table 3: LogD values of perhydroquinoxalines **3**-**5**



compd.	R	X	$logD_{7.4}$ [a] (experim. det.)	$logD_{5.4}$ [a] (experim. det.)	$clogD_{7.4}$ [b] (calcd.)	$clogD_{5.4}$ [b] calcd.	$clogP(H^+)$ [c] calcd.
3a	H	H	0.29 ± 0.04	-1.95 ± 0.11	1.05	-1.08	4.50
3b	H	OH	0.23 ± 0.05	-2.47 ± 0.04	0.36	-2.17	3.20
4a	CO_2CH_3	H	1.29 ± 0.01	0.39 ± 0.03	2.02	0.37	5.25
4b	CO_2CH_3	OH	1.31 ± 0.02	0.04 ± 0.08	1.33	-0.49	3.95
5a	CH_2-(3-pyridyl)	H	1.10 ± 0.08	-0.45 ± 0.19	2.04	0.46	6.04
5b	CH_2-(3-pyridyl)	OH	1.26 ± 0.01	-0.39 ± 0.19	1.36	-0.56	4.74

[a] $logD_{7.4}$ and $logD_{5.4}$ were determined by two-layer distribution and MS quantification (n = 9).

[b] $clogD_{7.4}$ and $clogD_{5.4}$ were calculated using MarvinSketch 15.5.18., ChemAxon (www.chemaxon.com).

[c] $clogP(H^+)$ was calculated using ChemBioDraw® 14.0.0.117.

The $logD_{7.4}$ values of the pyrrolidine and hydroxypyrrolidine pairs (**a**/**b**-pairs) are unexpectedly rather similar. The largest difference was found for the pyridylmethyl derivatives **5a** and **5b** displaying higher lipophilicity for the hydroxypyrrolidine derivative **5b**. The pyridylmethyl derivatives **5a**/**b** are less polar than the secondary amines **3a**/**b** but more polar than the methoxycarbonyl derivatives **4a**/**b**. The logD values at pH 5.4 are decreased by 1-1.5 units, indicating higher polarity due to higher amounts of protonated species. (Figure SI1) The high increase in polarity (negative

$logD_{5.4}$ values) found for the pyridylmethyl derivatives **5a** and 5**b** could be due to partial protonation of the pyridine ring. The high polarity of **5a** and **5b** indicates low passage of barriers, in particular the blood brain barrier.

In addition to the experimentally determined logD values the calculated clogD values using MarvinSketch 15.5.18. ChemAxon, are depicted in Table 3 showing a nice correlation between experimentally determined and calculated logD values. However, calculations with ChemBioDraw led to logD values differing from the experimentally determined logD values by 4-5 orders of magnitude.

Release of intracellular Ca^{2+} ions in HaCaT cells after stimulation with ATP

It has been shown that the κ agonist U-69,593 is able to reduce the Ca^{2+} release from intracellular Ca^{2+} storage into the cytoplasm of mouse thymoma cells.[27] An effect on the intracellular Ca^{2+} level after treatment with κ agonist is also described in astrocytes[28] and B-lymphocytes.[29] For a local therapy of atopic eczema the effect of the κ agonist on skin cells is of particular interest. Therefore, the effect of the κ agonists **3**-**5** on the release of Ca^{2+} ions in the human keratinocyte cell line HaCaT was investigated, which express the κ-opioid receptor.[30] For this purpose the cells were loaded with the Ca^{2+} binding fluorescence dye FURA 2AM. After careful washing, the cells were stimulated by ATP (100 µM) to release Ca^{2+} into the cytoplasm.[31] The reduced Ca^{2+} release after addition of the κ agonists **3**-**5** in a concentration of 1 µM is displayed in Figure.3. For purpose of comparison the prototypical κ agonist nalfurafine (**1**, 1 µM) was included into the assay.

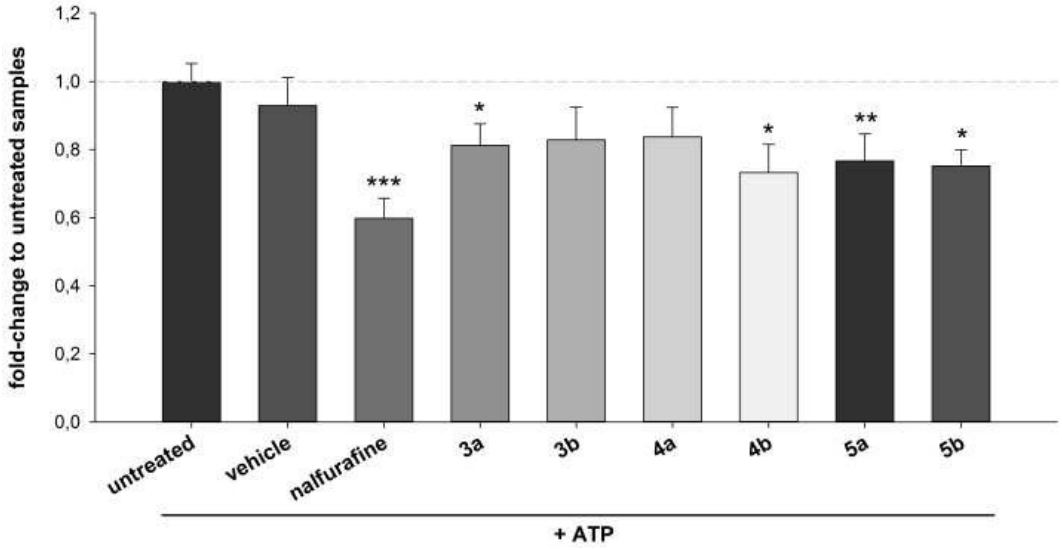


Figure 3: Decrease of Ca^{2+} release after stimulation of HaCaT cells with ATP and treatment with vehicle (DMSO), nalfurafine (1 µM) or the κ agonists **3**-**5** (each 1 µM). Shown are calculated fold-change values of the area under the curve of each experimental approach relative to untreated cells. (mean + SEM of data from 3 to 5 experiments; Significances calculated relative to the untreated sample *p<0.05, **p<0.01, ***p<0.001 by Student´s t-test)

Figure 3 shows a 40 % reduced release of Ca^{2+} ions induced by nalfurafine. Although the quinoxalines **3a**, **4b**, **5a** and **5b** also inhibited significantly the release of Ca^{2+} ions into the cytoplasm, their effects were considerably lower than the effect of nalfurafine. Nevertheless their Ca^{2+} ion release inhibition is statistically significant. A difference between the effects of the six quinoxalines can hardly be seen, but it could be assumed that the hydroxypyrrolidine derivatives **4b** and **5b** are slightly more potent than the pyrrolidine derivatives **4a**, and **5a**. In case of the secondary amines **3**, the hydroxypyrrolidine **3b** is slightly less potent than the pyrrolidine **3a**.

Conclusion

Very recently it was reported that *trans*,*trans*-configured perhydroquinoxalines of type **2** represent very potent κ-opioid receptor agonists. Introduction of a pyridylmethyl substituent at N-4 outside the κ pharmacophore resulted in the high affinity κ agonist **5a** with a K_i value of 0.13 nM. **5a** is very selective for the κ-opioid receptor over μ-opioid and δ-opioid receptors as well as over σ_1 and σ_2 receptors and the phencyclidine binding site of the NMDA receptor. In the [^{35}S]GTPγS-assay **5a** showed full agonistic activity and compared to the prototypical κ agonist U-69-593 slightly reduced activity (EC_{50} = 34 nM). The pyrrolidine **5a** and the hydroxypyrrolidine **5b** represent very polar compounds. The $logD_{7.4}$ and $logD_{5.4}$ values are 1.1 and 1.26 and -0.45 and -0.39, respectively. The perhydroquinoxalines **3a**, **4b**, **5a** and **5b** are able to significantly reduce the Ca^{2+} release of human keratinocytes, but to a lower extend than the prototypical κ agonist nalfurafine. However, the novel κ-opioid receptor agonists seem to represent promising candidates for treatment of itchy skin disease.

Experimental

Chemistry, general

Unless otherwise noted, moisture sensitive reactions were conducted under dry nitrogen. Thin layer chromatography (tlc): Silica gel 60 F_{254} plates (Merck). Flash chromatography (fc): Silica gel 60, 40–64 μm (Merck); parentheses include: diameter of the column, length of the column, eluent, fraction size, R_f value. MS: MAT GCQ (Thermo-Finnigan); EI = electron impact; Thermo Finnigan LCQ® ion trap mass spectrometer with an ESI = electrospray ionization interface. IR: IR spectrophotometer 480Plus FT-ATR-IR (Jasco). ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz): Mercury-400BB spectrometer (Varian); δ in ppm related to tetramethylsilane; coupling constants are given with 0.5 Hz resolution.

Purity by HPLC, methods A and B

Merck Hitachi Equipment: UV detector: L-7400; interface: D-7000; autosampler: L-7200; pump: L-7100; degasser: L-7614.

Method A: column: LiChrospher® 60 RP-select B (5 µm), 250-4 mm; flow rate: 1.00 mL/min; injection volume: 5.0 µL; detection at λ = 210 nm; solvents: A: water with 0.05% (v/v) trifluoroacetic acid; B: acetonitrile with 0.05% (v/v) trifluoroacetic acid: gradient elution: (A %): 0-4 min: 90 % , 4-29 min: gradient from 90 % to 0 %, 29-31 min: 0 %, 31-31.5 min: gradient from 0 % to 90 %, 31.5-40 min: 90 %.

Method B: column: Phenomenex® Gemini C6-Phenyl 110A (5 µm), 250-4.6 mm; flow rate: 1.00 mL/min; injection volume: 5.0 µL; detection at λ = 220 nm; solvents: A: water with 0.05% (v/v) trifluoroacetic acid; B: acetonitrile with 0.05% (v/v) trifluoroacetic acid: gradient elution: (A%): 0-3 min: 90 % , 3-28 min: gradient from 90 % to 0 %, 28-31 min: 0 %, 31-31.5 min: gradient from 0 % to 90 %, 31.5-40 min: 90 %. According to HPLC methods A and B the purity of all test compounds was greater than 95 %.

Isolation of compounds by preparative HPLC, method C

Merck Hitachi Equipment: UV detector: L-7400; interface: D-7000, autosampler: L-7200; pump: L-7150; software: HSM; column: Phenomenex Gemini C18 (5 µm), 250-21.2 mm; flow rate: 9.00 mL/min; injection volume: 400 µL; detection at λ = 225 nm; solvent MeOH / H_2O / + 0.1 % diethylamine, the exact ratio is given in the individual experimental procedure; isocratic elution. The sample was dissolved in methanol (500 µL).

Synthetic procedures

2-(3,4-Dichlorophenyl)-1-{(*4aRS,8SR,8aRS*)-4-[(pyridin-3-yl)methyl]-8-(pyrrolidin-1-yl)perhydrochinoxalin-1-yl}ethan-1-one (5a, WMS-0631)

The secondary amine **3a** (120 mg, 0.30 mmol) was dissolved in CH_2Cl_2 (10 mL) and pyridine-3-carbaldehyde (65 mg, 0.61 mmol), $NaBH(OAc)_3$ (128 mg, 0.61 mmol) and glacial acetic acid (36 mg, 0.61 mmol) were added. The mixture was stirred at rt for 21 h. Then additional amounts of pyridine-3-carbaldehyde (65 mg, 0.61 mmol), $NaBH(OAc)_3$ (128 mg, 0.61 mmol) and glacial acetic acid (36 mg, 0.61 mmol) were added and the mixture was stirred for another 3.5 h. The solution was filtered and extracted with 1 M HCl (3 x). The aqueous layer was adjusted with 2 M NaOH to pH 8 and extracted with CH_2Cl_2 (3 x). The combined organic layers were dried (Na_2SO_4), filtered, the solvent was removed in vacuo and the residue was purified by preparative HPLC (method C, MeOH / H_2O / Et_2NH 80:20:0.1). MeOH was evaporated, the aqueous layer was extracted with CH_2Cl_2 (3 x). The combined organic layers were dried (Na_2SO_4), filtered and concentrated in vacuo. Pale yellow resin, yield 43 mg (29 %). $C_{26}H_{32}Cl_2N_4O$ (487.5). R_f = 0.05 (MeOH), 0.59 (CH_2Cl_2 / MeOH / NH_3 9:1:0.1). ^{1}H NMR (d_8-toluene, 100 °C): δ (ppm) = 0.85 (q broad, ^2J = ^3J = 10.7 Hz, 1 H, 7-H_a), 0.99 (q broad, ^2J = ^3J = 11.8 Hz, 1 H, 5-H_a), 1.04 – 1.13 (m, 1 H, 6-H_a), 1.49 – 1.59 (m, 5 H, 6-H_e, N(CH_2CH_2)$_2$ (4 H)), 1.63 – 1.69 (m, 1 H, 5-H_e), 1.70 –1.76 (m, 1 H, 7-H_e), 1.77 – 1.84 (m, 1 H, 3-H), 2.27 (t broad, ^3J = 9.7 Hz, 1 H, 8-H), 2.32 – 2.39 (m, 1 H, 3-H), 2.46 – 2.56 (m, 4 H, N(CH_2CH_2)$_2$), 2.78 (d, ^2J = 13.8 Hz, 1 H, Pyr-CH_2-N), 2.88 – 3.05 (m, 3 H, 2-H (2 H), 8a-H), 3.31 (s, 2 H, Ph-CH_2-C=O), 3.50 – 3.58 (m, 1 H, 4a-H), 3.55 (d, ^2J = 13.9 Hz, 1 H, Pyr-CH_2-N), 6.77 – 6.82 (m, 1 H, Pyr-5-H), 6.87 – 6.90 (m, 1 H, Ph-6-H), 7.04 – 7.07 (m, 1 H, Ph-5-H), 7.16 – 7.21 (m, 1 H, Pyr-4-H), 7.24 – 7.27 (m, 1 H, Ph-2-H), 8.16 – 8.39 (m, 1 H, Pyr-6-H), 8.45 – 8.48 (m, 1 H, Pyr-2-H). IR: $\tilde{\nu}$ (cm^{-1}) = 1643 (s, ν (C=O)), 875 (m, out-of-plane (Ar-H)), 821 (w, out-of-plane (Ar-H)). MS

(ESI): *m/z* (%) = 487 (MH^+, 2 · ^{35}Cl, 100), 489 (MH^+, $^{35}Cl/^{37}Cl$, 69), 491 (MH^+, 2 · ^{37}Cl, 11). Purity (HPLC, method A): 98.7 %, t_R = 15.5 min.

2-(3,4-Dichlorophenyl)-1-{(*4aRS,8SR,8aSR*)-4-[(pyridin-3-yl)methyl]-8-[(*3SR*)- and (*3RS*)-3-hydroxypyrrolidin-1-yl]perhydrochinoxalin-1-yl}ethan-1-one (5b, WMS-0638)

The secondary amine **3b** (103 mg, 0.25 mmol) was dissolved in MeOH (15 mL), pyridine-3-carbaldehyde (53 mg, 0.49 mmol) and $NaBH_3CN$ (157 mg, 2.5 mmol) were dissolved in MeOH (5 mL) and the solution was added dropwise. The mixture was adjusted with conc. acetic acid to pH 5 and stirred at rt for 2 h. Saturated Na_2CO_3 solution (15 mL) was added and the mixture was stirred at rt for 15 min. The precipitate was filtered off and the solvent was removed in vacuo. The residue was purified by fc (2 cm, CH_2Cl_2 / MeOH / NH_3 9.5:0.5:1, 16 cm, 3 mL). The solvent was removed in vacuo. The residue was purified by preparative HPLC (method C, MeOH / H_2O / Et_2NH 70:30:0.1). MeOH was evaporated, the aqueous layer was extracted with CH_2Cl_2 (3 x). The combined organic layers were dried (Na_2SO_4), filtered and the solvent was removed in vacuo. Pale yellow resin, yield 52 mg (41 %). $C_{26}H_{32}Cl_2N_4O_2$ (503.5). R_f = 0.06 (MeOH), 0.35 and 0.43 (CH_2Cl_2 / MeOH / NH_3 9:1:0.1), 0.25 and 0.33 (CH_2Cl_2 / MeOH / NH_3 9.5:0.5:0.1). ^1H NMR (d_8- toluene, 100 °C): δ (ppm) = 0.81 – 0.91 (m, 1 H, 7-H_a), 0.93 – 1.12 (m, 2 H, 5-H_a, 6-H_a), 1.49 – 1.89 (m, 6 H, 3-H, 5-H_e, 6-H_e, 7-H_e, N(CH_2CH_2) (2 H)), 2.20 – 2.29 (m, 1 H, 8-H), 2.30 – 2.37 (m, 1 H, 3-H), 2.38 – 2.45 (m, 1 H, N(CH_2CHOH)), 2.50 – 2.56 (m, 1 H, N(CH_2CHOH)), 2.62 – 2.69 (m, 1 H, N(CH_2CH_2)), 2.69 – 2.81 (m, 1 H, N(CH_2CH_2)), 2.79 / 2.86 (d, ^2J = 13.9 Hz, 1 H, Pyr-CH_2-N), (m, 3 H, 8a-H, 2-H (2 H)), 3.14 – 3.22 (m, 1 H, 4a-H), 3.23 – 3.36 (m, 2 H, Ph-CH_2-C=O), 3.49 / 3.56 (d, ^2J = 13.9 Hz, 1 H, Pyr-CH_2-N), 4.00 – 4.08 (m, 1 H, N(CH_2CHOH), 6.79 – 6.86 (m, 1 H, Pyr-5-H), 6.87 – 6.92 (m, 1 H, Ph-6-H), 7.01 – 7.06

(m, 1 H, Ph-5-H), 7.17 – 7.22 (m, 1 H, Pyr-4-H), 7.23 – 7.27 (m, 1 H, Ph-2-H), 8.16 – 8.39 (m, 1 H, Pyr-6-H), 8.45 – 8.48 (m, 1 H, Pyr-2-H), a signal for the OH proton is not seen in the spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3600 – 3100 (m, ν (O-H)), 1634 (s, ν (C=O)), 876 (w, out-of-plane (Ar-H)), 822 (w, out-of-plane (Ar-H)). MS (ESI): *m/z* (%) = 503 (MH^+, 2 · ^{35}Cl, 100), 505 (MH^+, $^{35}Cl/^{37}Cl$, 60), 507 (MH^+, 2 · ^{37}Cl, 11). Purity (HPLC, method A): 96.7 %, t_R = 14.5 min. Purity (HPLC, method B): 95.7 %, t_R = 11.8 min.

Determination of $logD_{7.4}$ and $logD_{5.4}$ values

Instruments and Materials

UPLC-UV/MS (Agilent Technologies): Degasser: 1260 HiP (G4225A), Pump: 1260 Bin Pump (G1212B), Autosampler: 1260 HiP ALS (G1367E), Column Oven: 1290 TCC (G1316C), MS-Detector: 6120 Quadrulpol LC/MS (G1978B). Precolumn: Zorbax Eclipse Plus-C_{18} (2.1 x 12.5 mm, 5 µm particle size). Main column: Zorbax SB-C_{18} (2.1 x 50 mm, 1.8 µm particle size). MS Source: Multimode source; ESI mode; SIM mode. 1.5 mL safe lock tubes, Eppendorf. 3-Morpholinopropanesulfonic acid (Fisher BioReagents), MOPS sodium salt (Sigma Aldrich), disodium hydrogen phosphate salt and sodium dihydrogen phosphate salt (Merck, Darmstadt), *n*-octanol (Chromasolv® for HPLC; Sigma Aldrich), DMSO (Fisher Scientific).

LC-MS Method

The alignments of the capillaries of the column oven of the instrument were rebuilt that the six-port-valve which normally switches between two columns was used as a divert valve to protect the mass spectrometer from salts of the buffer solution. After 1 minute the valve was switched from “waste” to “MS-source”. At the end of a single run the valve was switched to “waste”.

Eluents: solvent A: bidest. water : CH_3CN 95 : 5 with 0.1% formic acid; solvent B: bidest. water : CH_3CN 5 : 95 with 0.1% formic acid; gradient elution (A %): 0 – 0.5 min: 100 %; 0.5 – 2 min: gradient from 100 % to 0 %; 2 – 5 min: 0 %; 5 – 6 min: gradient from 0 % to 100 %; 6 – 10 min: 100 %. Flow rate: 0.3 mL/min. MS parameter: ESI mode. Gas temperature: see supporting information, vaporizer temperature: 200 °C, drying gas: 12 L/min, nebulizer pressure: 35 psi, VCap: -4000 V.

Optimization of fragmentor voltage and gas temperature

Flow: 0.3 mL/min isocratic elution solvents A : B (20 : 80).

Flow injection analysis was used in order to find the optimum value of the fragmentor voltage for each analyte. For this purpose solutions consisting of 990 µL of bidest. water : CH_3CN 50 : 50 and 10 µL of the 10 mM DMSO stock solutions were prepared. 1.0 µL of these solutions was injected with varying values for the fragmentor voltage or dry gas temperature but keeping all other MS parameters constant. The maximum area under the curve (SIM mode) for each analyte indicates the optimum value for the fragmentor voltage.

Chemicals, solvents and stock solutions

8.9 mM MOPS (3-morpholinoppropanesufonic acid) and 11.1 mM MOPS Na salt (sodium 3-morpholinoppropanesufonate) were dissolved in dist. water to prepare 20 mM MOPS buffer pH 7.4. 0.4 mM Na_2HPO_4 and 19.4 mM NaH_2PO_4 were dissolved in dist. water to prepare 20 mM PBS buffer pH 5.4. MOPS as well as PBS buffer and *n*-octanol were mutually saturated by stirring a two phase system for 24 h before use. Then the aqueous and organic layers were separated.

10 mM stock solutions of the test compounds in DMSO were prepared by dissolving an exactly weighted amount of the test compound and adding the calculated amount of DMSO (Fisher Scientific).

$logD_{7.4}$ and $logD_{5.4}$ determination

Calibration curves were generated with at least six concentrations covering the concentration range of the samples. The final concentration of DMSO was kept below 1 % in all samples. The $LogD_{7.4}$ and $logD_{5.4}$ values were determined using different volume ratios of buffer and *n*-octanol (1:1, 2:1, 1:2).The different solutions where pipetted into safe lock tubes. Afterwards the cups where vortexed for 2 min and then centrifuged at rt with 16,000 rpm for 5 min. An aliquot of the aqueous layer was transferred to a vial and 1 µL was analyzed with the above mentioned LC-MS method. Each sample and calibration point was measured in triplicate. Each experiment was repeated three times. For very polar compounds (logD < 0) the *n*-octanol layer was analyzed accordingly.

Calculation of logD values

The $clogD_{7.4}$ and $clogD_{5.4}$ values (logD values at a pH value of 7.4 or 5.4) were calculated using the Evaluation Mode of Marvin 15.5.18, 2015 (www.chemaxon.com). The clogP(H^+) values were calculated using ChemBioDraw® 14.0.0.117.

Receptor affinity

Affinity towards the κ-opioid receptor using guinea pig brain preparations

The affinity towards the κ-opioid receptor was recorded as described in references 11, 12, 18, and 19.

Affinity towards human κ-, μ- and δ-opioid receptors using preparations from different cell lines

The affinity towards κ-, μ- and δ-opioid receptor using different cell lines was recorded as described in references 20 and 21.

Affinity towards the σ_1 and σ_2 receptors

The affinity towards σ_1 and σ_2 receptors was recorded as described in references 32-34.

Affinity towards the PCP binding site of the NMDA receptor

The affinity towards the PCP binding site of the NMDA receptor was recorded as described in references 35 and 36.

[^{35}S]GTPγS binding assay, agonistic activity at the κ-opioid receptor

The [^{35}S]-guanosine-5´-3-O-(thio)triphosphate (GTPγS) assay was carried out as described in reference 21.

Measurement of intracellular calcium level

HaCaT keratinocytes were cultured in DMEM culture media (Dulbecco's Modified Eagle's Medium, Sigma Aldrich, Seelze, Germany) supplemented with 10% fetal calf serum, 2 mM glutamine and penicillin/streptomycin (GE Healthcare, Solingen, Germany). Cells were seeded on glass slides coated with collagen and grown to a confluence of 90 – 100 % under normal cell culture conditions (37°C, 5 % CO_2). Than cells were washed twice with HEPES buffer containing 0.1 % BSA (pH 7.4) and then

incubated for 45 min in HEPES buffer (+ 0.1 % BSA) with 2.5 µM FURA 2AM (Life Technologies, Darmstadt, Germany) at 37 °C in the dark. After that, cells were carefully washed eight times with HEPES buffer. Intracellular calcium levels were measured on the LS55 luminescence spectrometer (Perkin Elmer, Hamburg, Germany). For the measurement, the glass slides with the cells were fixed in a holder, then placed in a cuvette and inserted into the spectrometer. The cuvette was connected to a water bath which held the temperature constant at 37 °C. The calcium release was induced by ATP (100 µM). EGTA (10 mM) served as a negative control for a minimum calcium level in the cells. Different newly developed κ opioid receptor agonists were tested at concentrations of 1 µM. The agonist nalfurafine (1 µM) was used as a control for κ receptor mediated calcium release.

Ca^{2+}-induced change was measured in fluorescence at 340 and 380 nm and was determined over a time interval of 180 s. The data obtained were analyzed with the software FL WinLab (Perkin Elmer, Hamburg, Germany) and transferred to Microsoft Excel for further analysis. Areas under the curves (AutC) were determined between the EGTA curve and the curves of the test compounds. Values of the AutC were related to the mean value of the untreated sample and fold-change of each experimental approach was calculated.

The differences between the ATP control (untreated sample) and treated samples were tested using Student´s t-test, significance was set *$p<0.05$, **$p<0.01$ or ***$p>0.001$. Experiments were repeated 3 to 5 times.

Supporting Information Available

Supporting Information contains the $logD_{7.4}$ values of reference compounds and the ESI source parameters used for the ionization of the compounds.

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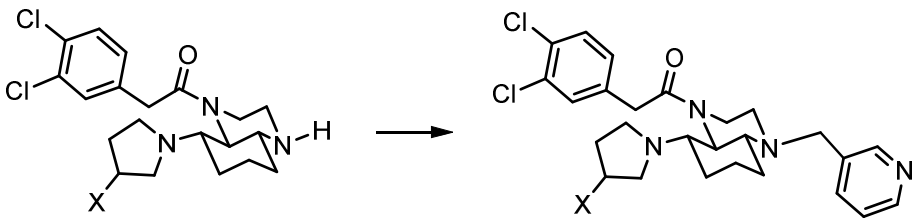
Graphical Abstract



X = H
K_i (κ) = 0.13 nM
high selectivity
full agonism
$logD_{7.4}$ = 1.1
$logD_{5.4}$) -0.45
reduction of Ca^{2+} release
in human ketatinocytes