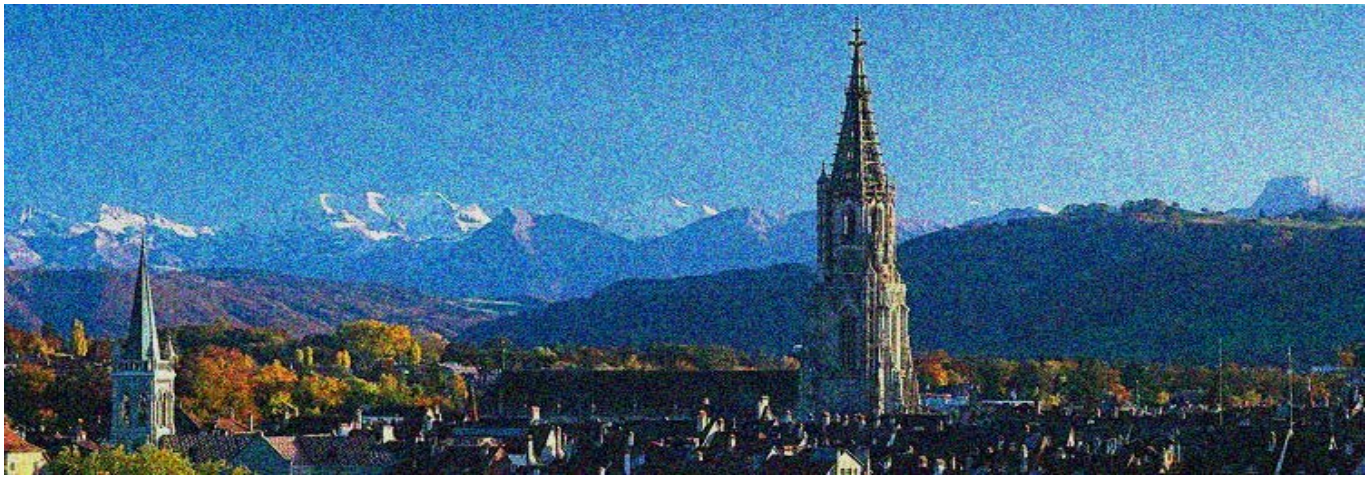

1st ENDOCANNABINOID PHARMACOLOGY MEETING IN SWITZERLAND

17th October 2014

University of Bern – Bern, Switzerland

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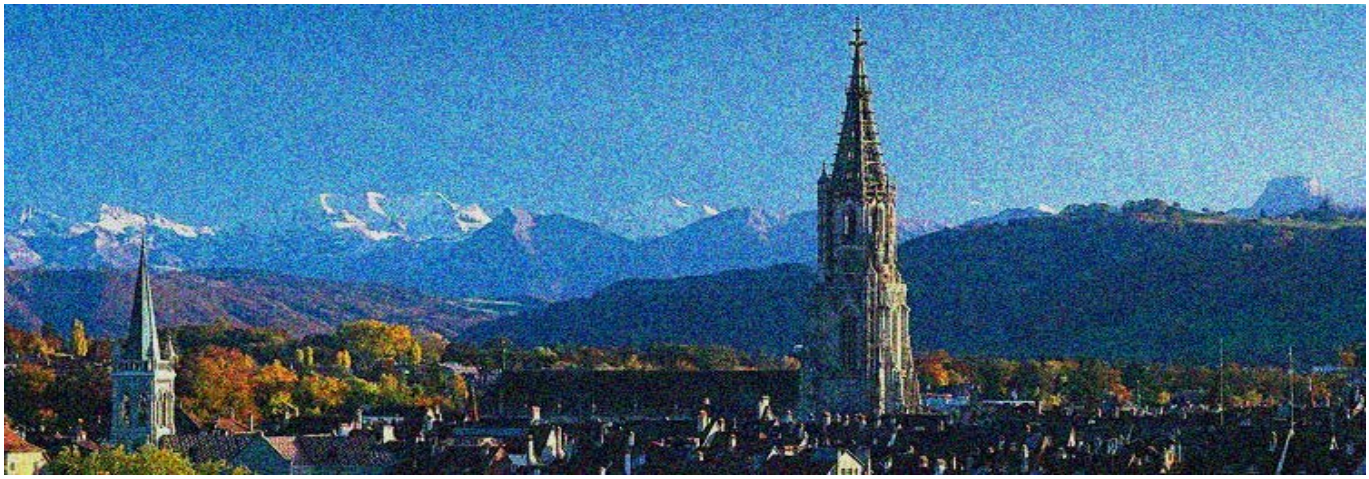

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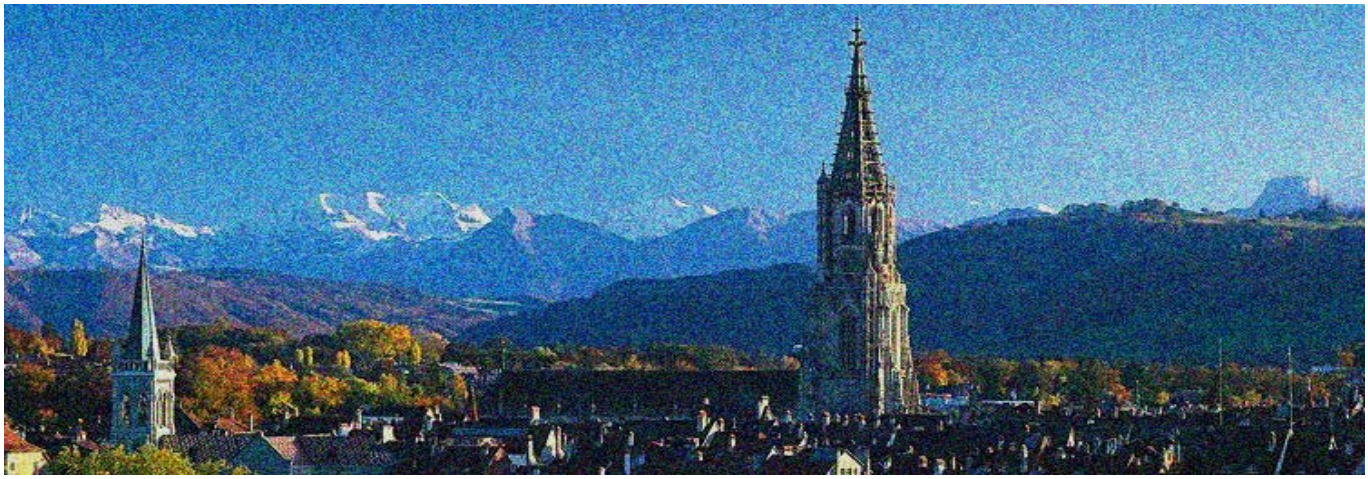


Duration: 1-day
FREE attendance
Fields of interest: Life and Medical Sciences with a special focus on the biology, biochemistry and pharmacology of the endocannabinoid system

Aim of the meeting: The main objective of the conference will be to provide a complete summary of the state-of-art of the pharmacology and therapeutic exploitation of the endocannabinoid system, focusing on the current and future directions undertaken by the scientific community and pharmaceutical companies.

Venue: the meeting avenue is the main building of the University of Bern (Hauptgebäude H4, Hochschulstrasse 4 – 3012 – Bern). The morning session will take place in the « Kuppelraum (nummer 501)» and the afternoon session in «Raumnummer 201» (same building)

Kuppelraum (Raum 501 - 5th Floor):
http://www.hoerraeume.unibe.ch/detail.php?id=258577051004

Raumnummer 201 (2nd Floor):
http://www.hoerraeume.unibe.ch/detail.php?id=258577021030

Organizing committee: Andrea Chicca and Jürg Gertsch

MEETING PROGRAMME

Kuppelraum - Hauptgebäude H4 (Hochschulstrasse 4 – 3012 – Bern)

8.30 *Registration and coffee*

9.00 **Addresses Welcome**

Prof. Jürg Gertsch/Dr. Andrea Chicca

Prof. Christian J. Leumann vice-rector of the University of Bern

9.15-9.50 Prof. **Vincenzo Di Marzo** - National Council of Research (Italy) “Promising drugs from the endocannabinoid system "from simple to complex": DAGL inhibitors, FAAH/TRP modulators and phytocannabinoids”

9.50-10.10 Dr. **Ermelinda Lomazzo** - University of Mainz (Germany) “Inhibition of endocannabinoid degradation for the treatment of pain associated to chronic stress”

10.10-10.40 *Coffee break*

10.40-11.15 Prof. **Steve Alexander** - University of Nottingham (United Kingdom) “Breaking the conventions of cannabinoid receptor signalling”

11.15-11.50 Dr. **Jürgen Fingerle** - F. Hoffmann–La Roche (Switzerland) “Does CB_2 agonism protect from inflammation related organ damage and fibrosis?”

11.50-12.10 Dr. **Andrea Chicca** - University of Bern (Switzerland) “Beyond CB_2 receptor ligands, a multi-target approach to modulate the endocannabinoid system”

12.10-13.30 *Lunch break* (including poster session)

Raumnummer 201 - Hauptgebäude H4 (Hochschulstrasse 4 – 3012 – Bern)

13.30-14.05 Prof. **Daniele Piomelli** - University of Irvine (USA)/Italian Institute of Technology (Italy) “New tricks for an old dog: unexpected new functions for peripheral FAAH”

14.05-14.40 Prof. **Christoph Abels** - Dr. August Wolff GmbH & Co. KG Arzneimittel (Germany) “The endocannabinoid system of the skin as therapeutic target”

14.40-15.10 *Coffee break*

15.10-15.30 Prof. **Thomas Nevian** - University of Bern (Switzerland) “Retrograde signalling in spike-timing dependent plasticity”

15.30-16.05 Prof. **Jürg Gertsch** - University of Bern (Switzerland) “The emerging pharmacology of endocannabinoid uptake inhibitors”

16.05-16.40 Prof. **Raphael Mechoulam** - Hebrew University (Israel) “The endocannabinoid system: looking back and ahead”

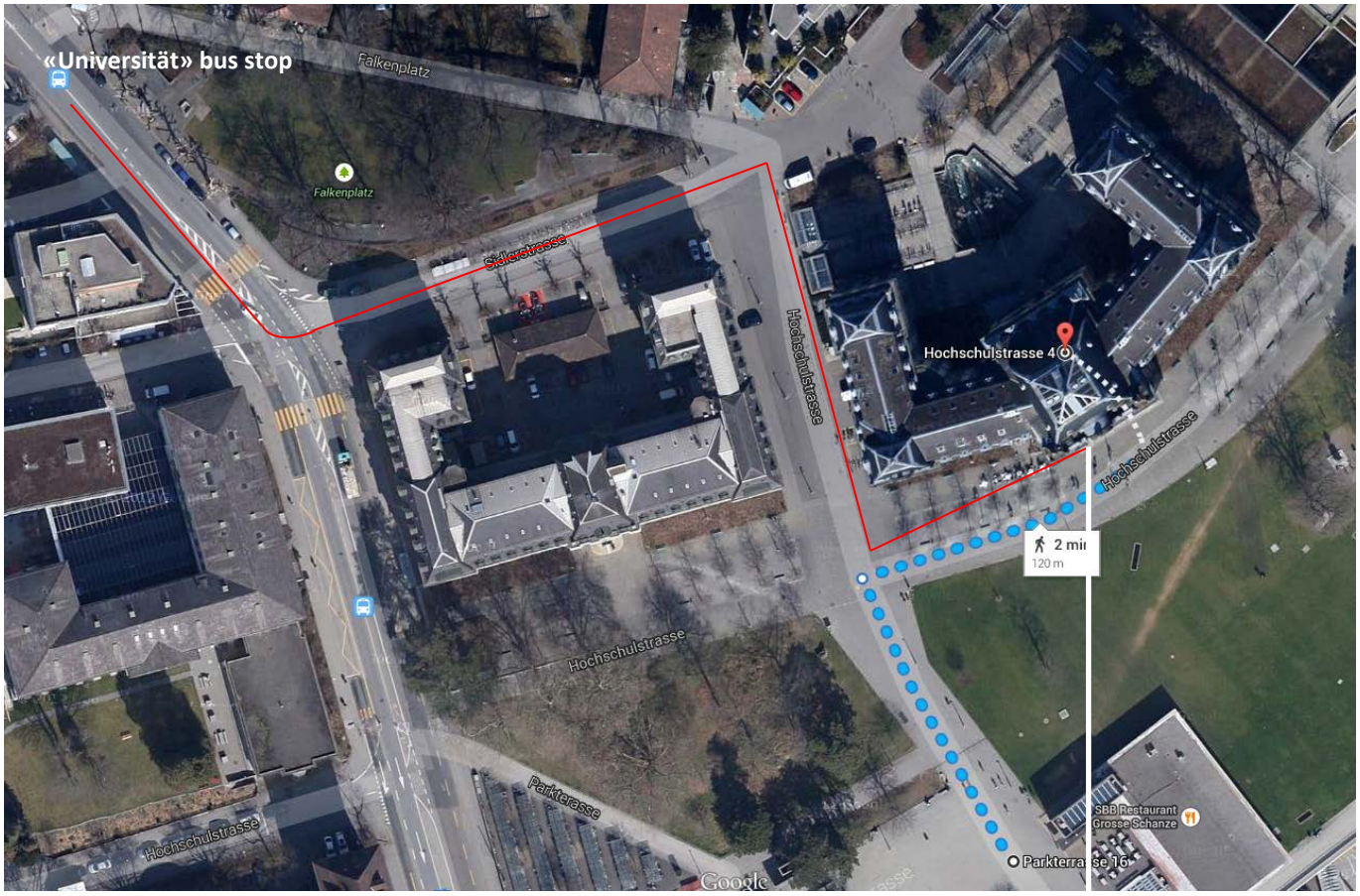
16.40-16.50 Concluding remarks and poster award

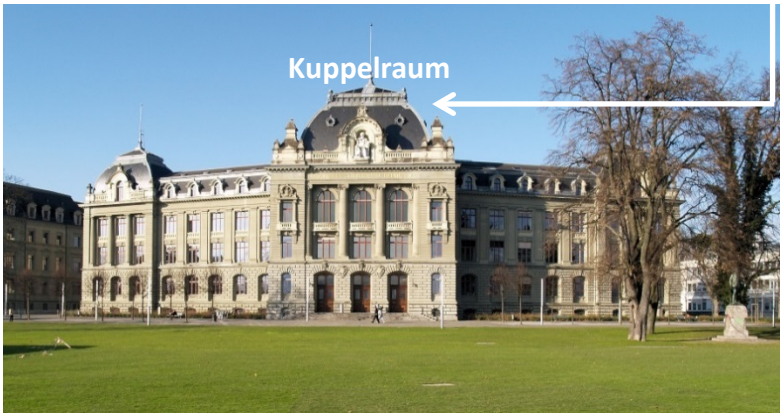
Directions:

By Train (www.sbb.ch): Arrive to «Bern Hauptbanhof» (Main Station). The train trip takes 1 hour from Basel, Zurich, Luzern and Lausanne; 1h40 from Geneve; 2h10 from Saint Gallen.

From the railway platform follow the indications to exit the train station at the «Universität». From the underground floor of the train station take the elevator until the floor number 4. From there turn around the corner and you will be in front of the main building of the University (Hauptgebäude).

By Bus (http://www.bernmobil.ch/): Take the Bus n.12 direction «Länggasse» and get off after two stops («Universität»). From the bus stop walk in Länggasse for approximately 30 mt, turn left in Sidlerstrasse and then turn right into Hochschulstrasse.





POSTER SESSION

12.10-13.30

P1

Development of Radiotracers for the PET Imaging of CB_2 Receptors in the Brain.

R. Slavik[1], D. Bieri[1], A. Herde[1], M. Weber[2], S. D. Krämer[1], R. Schibli[1], S. M. Ametamey[1], L. Mu[1,3]

[1] *Institute of Pharmaceutical Sciences, ETH Zurich, 8093 Zürich, Switzerland*
[2] *Kantonsspital St. Gallen, Neuromuscular Diseases Unit / ALS Clinic, 9007 St. Gallen, Switzerland*
[3] *Department of Nuclear Medicine, University Hospital Zürich, CH-8091 Zürich, Switzerland*

To develop a brain PET tracer towards CB_2 receptors, a series of novel CB_2 ligands were designed and synthesized based on the 4-oxo-quinoline structure of our previously published radiotracer [^{11}C]KD-2 [1] with focus on lowering its high lipophilicity while keeping its excellent binding properties. *In vitro* competitive binding assays were performed with membranes containing human CB_2 and CB_1, respectively, using [^{3}H]CP-55940. The most promising compound RS-016 exhibited a K_i value towards hCB_2 of 0.7 nM with a selectivity over hCB_1 >10'000 and was radiolabeled with carbon-11 isotope.

[^{11}C]RS-016 was obtained in ≥99% radiochemical purity with high specific radioactivity of 545 ± 154 GBq/µmol (n=39) at the end of synthesis. Log*D* value determined at physiological pH via the shake-flask method in octanol/water was 2.78 ± 0.04 (n=5). Autoradiography experiments on rodent spleen tissue demonstrated high specific binding to CB_2 receptors *in vitro*. Specific binding to rat spleen tissue *in vivo* was 78% determined using GW405833 (1.5 mg/kg), a CB2 receptor specific blocking agent. At 20 min p.i., 47% of intact parent compound was found in blood and 81% in spleen, respectively.

[^{11}C]RS016 is a very promising CB_2 PET tracer which might serve as novel tool for investigations on CB_2 receptor levels in healthy tissues and different disease stages *in vitro* and *in vivo*.

Reference

[1] Mu, L., et al., *Radiolabeling and in vitro /in vivo evaluation of N-(1-adamantyl)-8-methoxy-4-oxo-1-phenyl-1,4-dihydroquinoline-3-carboxamide as a PET probe for imaging cannabinoid type 2 receptor.* J Neurochem, 2013. **126**(5): p. 616-24.

P2

A Multi-fingerprint Polypharmacology browser for ChEMBL

M. Awale[1], G. Giuffredi[1], L. Ruddigkeit[1], J-L. Reymond[1*]

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awale@dcb.unibe.ch - jean-louis.reymond@dcb.unibe.ch

Drug discovery is increasingly influenced by the availability of very large databases of drug-like molecules, comprising of millions compounds from commercial or from theoretical sources awaiting biological evaluation, as well as large collections of bioactive compounds with annotated activities. Working with such large databases requires efficient tools to browse through very large lists of molecules, in particular to rapidly identify structurally similar molecules. Recently we showed that databases up to billions of molecules can be classified using simple descriptor sets such that similarity searches are completed within seconds, with optional browsing via interactive color-coded principal component maps if a query molecule is not available. The search principle was recently extended to design a multi-fingerprint browser for the ZINC database allowing to rapidly identify analogs of any screening hit and perform clustering to compose focused sets for hit confirmation. Here we report the extension of this multi-fingerprint approach to the problem of polypharmacology searches, i.e. how to find out if a newly identified bioactive molecule is closely related to molecules with documented bioactivity and therefore likely to interact with the corresponding biological target. Our polypharmacology browser searches within seconds through 717 groups of at least 20 bioactive molecules with documented activity against a biological target, as listed in ChEMBL, to identify analogs of any query molecule using nine different fingerprints or fingerprint combination, and displays results groups by targets as lists of bioactive compounds, which allows one to directly estimate whether the identified similarity is meaningful in the examined context. Compared to previous reports of related polypharmacology predictors, our browser application is much more versatile and gives more direct access to the chemical structures underlying the polypharmacology prediction. With the application of this browser we have deconvoluted the target for a nanomolar cytotoxic compound identified in cell-based phenotypic screen (data not shown).

References

[1] A multi-fingerprint browser for the ZINC database. M. Awale, J.-L.Reymond, Nucleic Acids Res. 2014, doi: 10.1093/nar/gku379.
[2] Discovery of Potent Positive Allosteric Modulators of the α3β2 Nicotinic Acetylcholine Receptor by a Chemical Space Walk in ChEMBL. J. Bürgi, M. Awale, S. Boss, T. Schaer, F. Marger, J. Viveros-Paredes, S. Bertrand, J. Gertsch, D. Bertrand, J.-L. Reymond, ACS Chem. Neurosci. 2014, DOI:10.1021/cn4002297.
[3] The SMIfp (SMILES fingerprint) Chemical Space for Virtual Screening and Visualization of Large Databases of Organic Molecules. J. Schwartz J, M. Awale and J.-L. Reymond, J. Chem. Inf. Model. 2013, 53, 1979-1989.
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P3

Guineensine as a Novel Inhibitor of Endocannabinoid Reuptake

R. Bartholomäus[1], S. Nicolussi[2], A. Simao[1], K-H. Altmann[2*], J. Gertsch[1*]

[1]*ETH Zürich, Institute of Pharmaceutical Sciences Vladimir-Prelog-Weg 1-5/10, CH-8093 Zürich*
[2]*University of Bern, Institute of Biochemistry and Molecular Medicine, Bühlstrasse 28, CH-3012 Bern*
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Guineensine was isolated from *Piper nigrum* and was recently shown to be a novel nanomolar inhibitor (EC_{50} = 290 nm) of cellular reuptake of the endocannabinoid anandamide.[1]

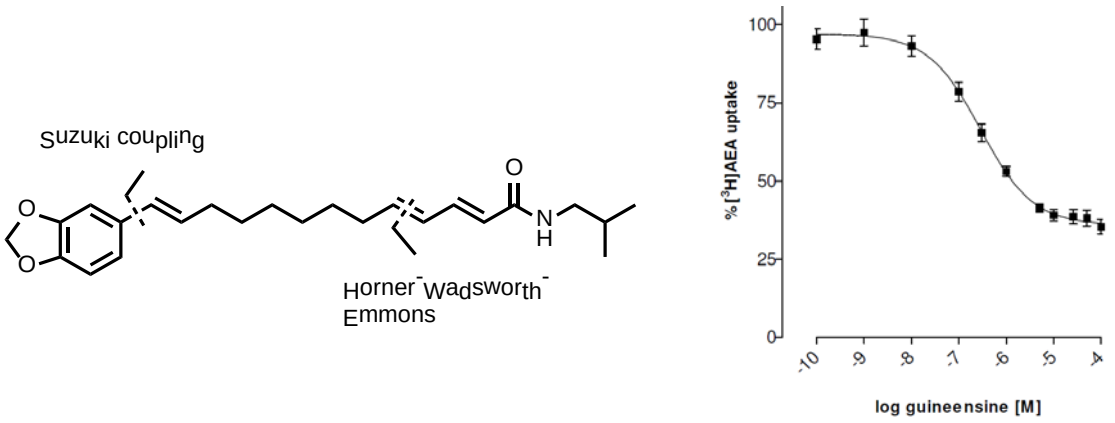


Figure 1: Structure of guineensine and retrosythetic key steps

One goal of the project is to gain knowledge about structure-activity relationships. Therefore we synthesized various head group analogs of guineensine (Figure 2, A). The most potent compound was found to be the 3,4-methylene-dioxyphenyl derivative **4**.

The saturated compound **7** reveals that the presence of the double bonds is essential in terms of inhibition of anandamide reuptake.

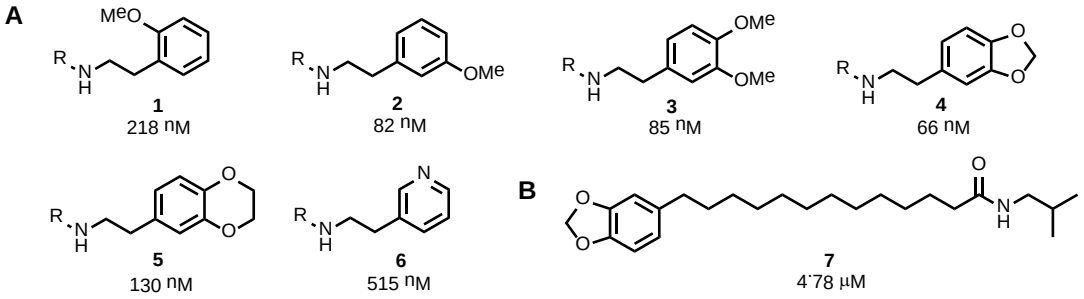


Figure 2: Synthesized headgroup derivatives (A) and saturated guineensine (B)

Reference

[1] S. Nicolussi et al., *Pharmacol. Res.* **2014**, 52-65.

P4

Pharmacological Evaluation of Guineensine, a potent CNS-active Inhibitor of Endocannabinoid Uptake showing Analgesic and Anti-inflammatory Effects

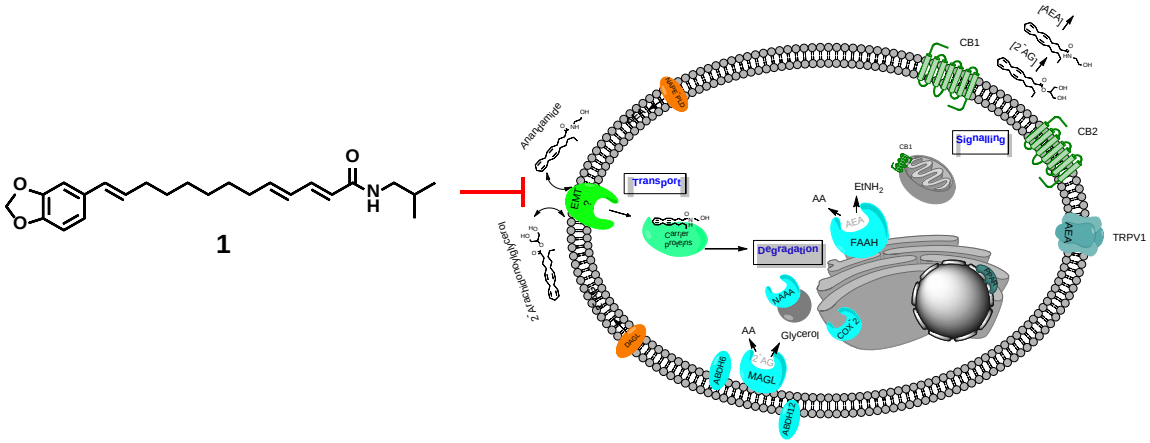
S. Nicolussi[1], J.M. Viveros-Paredes[2], M.S. Gachet[1], M. Rau[1], M.E. Flores-Soto[2], M. Blunder[3], J. Gertsch[1]

[1] *Institute of Biochemistry and Molecular Medicine, University of Bern, 3012 Bern, Switzerland*
[2] *Institute of Immunopharmacology, University of Guadalajara, Mexico*
[3] *Department of Neuroscience, University of Uppsala, Sweden*

Indirect agonistic modulation of the endocannabinoid system provides a promising therapeutic strategy during several pathological conditions [1,2]. Potentiation of anandamide and 2-arachidonoylglycerol signaling in neuropathic and inflammatory pain as well as in anxiety and mood related disorders showed beneficial effects in different rodent models [2]. Therefore we aimed to identify novel inhibitors of endocannabinoid reuptake using bioactivity-guided screenings, *in vitro* compound profiling and *in vivo* validations to identify novel drug scaffolds and potential tool compounds.

Guineensine (1), isolated from *Piper nigrum* L., was identified as a novel nanomolar inhibitor of endocannabinoid uptake (IC_{50} = 288 nM (95% CI = 190 - 437 nM) in U937 cells) [3]. It did not inhibit EC degrading enzymes (FAAH, MAGL/ABHD) nor interact directly with CB1 or CB2 receptors. Further, no binding to FABP5, a cytosolic AEA carrier protein, could be demonstrated. These properties characterize guineensine as an inhibitor of high selectivity for the putative endocannabinoid membrane transporter. Further, weak but selective inhibition of the cyclooxygenase COX-2 could be detected (IC_{50} = 33 µM).

In vivo guineensine triggers a full tetrad in BALB/c mice whereas catalepsy and analgesia could be abolished with rimonabant (SR141716A), a CB1 selective inverse agonist. The hypothermic effects of seem to be mediated by serotonergic mechanisms. In a mouse model of endotoxemia, guineensine potently inhibited the acute expression of TNF-α and IL-10.



Guineensine was the first plant-derived product shown to selectively inhibit endocannabinoid uptake. In mice it indirectly activates CB1 receptors and is a surprisingly potent anti-inflammatory, analgesic and CNS active polypharmacophoric agent. Therefore, guineensine might have the potential as a lead substance for drug development and contributes novel insights to the modulation of the ECS *in vivo*.

References

[1] Di Marzo V. Nature Rev Drug Discov 2008; 7: 438-455.
[2] Batista et al. Beh. Pharmacol.2014; 25: 425-433
[3] Nicolussi S et al. Pharmacol Res 2014; 80: 52-65

P5

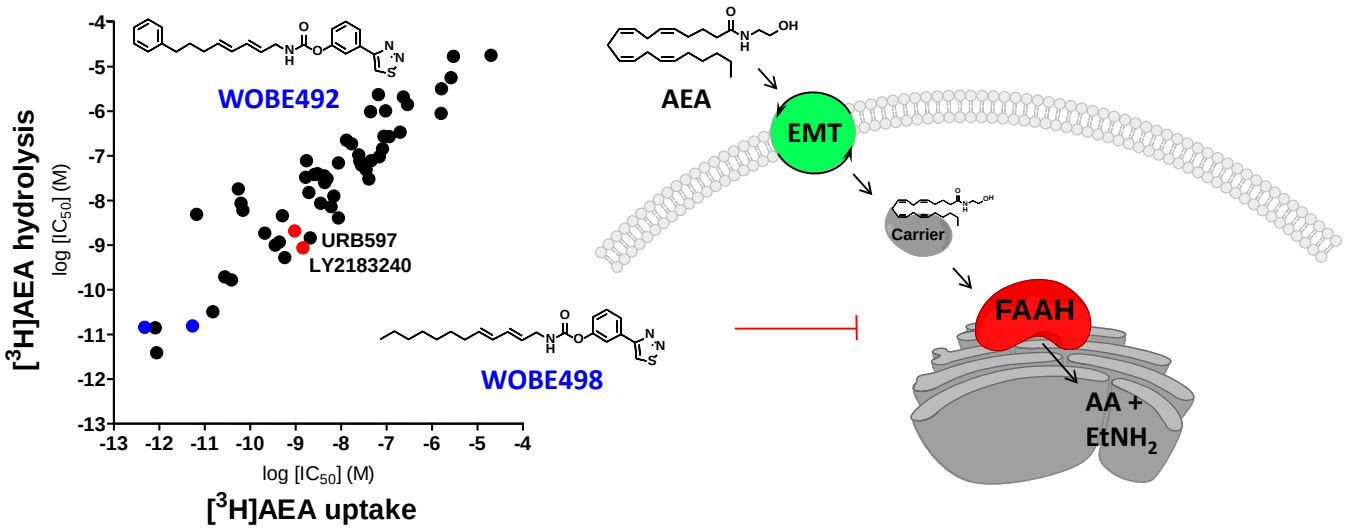
Correlating FAAH and anandamide cellular uptake inhibition using N-alkylcarbamate inhibitors: From ultrapotent to hyperpotent

S. Nicolussi[1], A. Chicca[1], M. Rau[1], S. Rihs[1], M. Soeberdt[2], C. Abels[2], J. Gertsch[1]

[1] *Institute of Biochemistry and Molecular Medicine, University of Bern, 3012 Bern, Switzerland*
[2] *Dr. August Wolff GmbH & Co. KG Arzneimittel, D-33611 Bielefeld, Germany*

The cellular uptake of the endocannabinoid anandamide (AEA) and its degradation by fatty acid amide hydrolase (FAAH) are intrinsically coupled mechanisms. However, differential blockage of each mechanism is possible using specific small-molecule inhibitors. Starting from the natural product-derived *2E,4E*-dodecadiene scaffold previously shown to interact with the endocannabinoid system (ECS), a series of diverse *N*-alkylcarbamates were prepared with the aim of generating novel ECS modulators.

While being inactive at cannabinoid receptors and monoacylglycerol lipase, these N-alkylcarbamates showed potent to ultrapotent picomolar FAAH inhibition in U937 cells. Overall, a highly significant correlation (Spearman's rho = 0.91) was found between the inhibition of FAAH and AEA cellular uptake among 54 compounds. Accordingly, in HMC-1 cells lacking FAAH expression the effect on AEA cellular uptake was dramatically reduced. Unexpectedly, 3-(4,5-dihydrothiazol-2-yl)phenyl carbamates and the 3-(1,2,3-thiadiazol-4-yl)phenyl carbamates WOBE490, WOBE491 and WOBE492 showed a potentiation of cellular AEA uptake inhibition in U937 cells, resulting in unprecedented femtomolar (hyperpotent) IC50 values. Potential methodological issues and the role of cellular accumulation of selected probes were investigated. It is shown that albumin impacts the potency of specific N-alkylcarbamates and, more importantly, that accumulation of FAAH inhibitors can significantly increase their effect on cellular AEA uptake. Taken together, this series of N-alkylcarbamates shows a FAAH-dependent inhibition of cellular AEA uptake, which can be strongly potentiated using specific head group modifications. These findings provide a rational basis for the development of hyperpotent AEA uptake inhibitors mediated by ultrapotent FAAH inhibition.



Reference

[1] Nicolussi et al. Biochem. Pharmacol 2014, *in press* (doi : 10.1016/j.bcp.2014.09.020)

P6

A quantitative LC-MS/MS method for the measurement of arachidonic acid, prostanoids, endocannabinoids, N-acylethanolamines and steroids in human plasma

M.S. Gachet[1], P. Rhyn[1], O. G. Bosch[2], B. B. Quednow[2], J. Gertsch[1*]

[1]*Institute of Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, 3012 Bern, Switzerland*
[2]*Experimental and Clinical Pharmacopsychology, Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital of the University of Zurich, Lenggstr. 31, CH-8032 Zurich, Switzerland*

Free arachidonic acid is functionally interlinked with different lipid signaling networks including the prostanoid pathways, endocannabinoid system, N-acylethanolamines, but also steroids. A sensitive and specific LC-MS/MS method for the quantification of arachidonic acid, prostaglandin E2, thromboxane B2, anandamide, 2-arachidonoylglycerol, noladin ether, lineoyl ethanolamide, oleoyl ethanolamide, palmitoyl ethanolamide, steroyl ethanolamide, aldosterone, cortisol, dehydroepiandrosterone, progesterone, and testosterone in human plasma was developed and validated. Analytes were extracted upon acetonitrile precipitation followed by solid phase extraction. Separations were performed by UFLC using a C18 column and analyzed using a triple quadrupole MS after electron spray ionization. Analytes were run in negative mode and subsequently in positive mode in two independent LC/MS/MS runs. For each analyte, two MRM transitions were collected in order to confirm identity. All analytes showed good linearity over the investigated concentration range ($r > 0.98$). Validated LLOQs ranged from 0.1 to 192 ng/mL and LODs ranged from 0.04 to 12.3 ng/mL. Our data show that the LC-MS/MS method is suitable for the quantification of these bioactive lipids in plasma from human donors (n=32). The determined plasma levels are in agreement with literature, thus providing a versatile method to explore pathophysiological processes in which changes of these lipids are implicated.

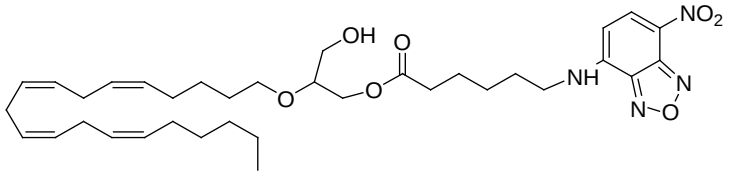
P7

Fluorescent noladin ether as new hydrolysis-resistant endocannabinoid analogue to investigate endocannabinoid cellular uptake and trafficking

A. Chicca[1], S. Hofer[1], V. Petrucci[1], S. Gachet[1], S. Nicolussi[1], S. Ortega Gutierrez[2], J. Gertsch[1]

[1]Institute of Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, 3012 Bern, Switzerland
[2]Department of Organic Chemistry, Universidad Complutense de Madrid, Spain

Endocannabinoids (ECs) are endogenous molecules which activate primarily cannabinoid CB1 and/or CB2 receptors. Anandamide (AEA), *N*-arachidonoyl dopamine (NADA) and 2-arachidonoyl glyceryl ether (noladin ether; 2-AGE) are functionally more selective for CB1; virodhamine appears to prefer CB2 while 2-arachidonoyl glycerol (2-AG) is equipotent at both receptor subtypes [1]. ECs effects are regulated by cellular biosynthesis, release, re-uptake, trafficking and enzymatic metabolism. Unlike the clear knowledge about ECs biosynthetic and metabolic pathways, their cellular re-uptake mechanism is not fully elucidated yet. The most supported theory relies on a transporter-mediated mechanism. One of the main issues in elucidating the uptake process is the tight inter-play between ECs plasma membrane movement and their rapid and almost complete cellular cleavage mainly dependent on FAAH and MAGL activity. Recently we have shown that all ECs compete for the same putative membrane transporter (EMT) independently of their intracellular fate (trafficking and enzymatic inactivation) [2].



NBD-2AGE

Noladin ether was identified in pig brain and rodent brain and peripheral tissues [3,4], but afterwards several groups could not repeat this quantification *in vivo* raising some doubts about the real endogenous presence of this molecule. In our work we developed a sensitive LC-MS/MS based method that allowed the quantification of noladin ether in human, pig and rodent plasma. Interestingly, noladin ether could not be detected neither in pig nor in rodent brain suggesting a peripheral distribution of this endocannabinoid. Based on this, we synthesized a fluorescent-analogue of noladin ether (NBD-2-AGE), the only hydrolysis-resistant endocannabinoid molecule. We have characterized the properties of NBD-2-AGE by performing the classical radioactivity-based method and by fluorescence HPLC analytical quantification. The results show that the fluorescently-tagged noladin ether possesses the same biochemical features as noladin ether in terms of CB receptor binding, cellular uptake and trafficking, and hydrolytic cleavage resistance. We made use of this new tool compound to study cellular EC uptake and release kinetics in different cell types (immune and neuronal cells) by FACS measurement. Both processes could be selectively inhibited by the classic EMT inhibitors. When NBD-2-AGE was co-incubated with AEA, 2-AG or noladin ether a selective competition in cellular uptake was detected. Other *N*-acetylethanolamines did not show any significant effect on the uptake as previously shown for the main endocannabinoids AEA and 2-AG [2]. NBD-2AGE was also incubated with mouse brain slices and after extensive washing, the distribution of the probe was analysed by using confocal microscopy and high-resolution laser scanner (Typhoon FLA 9500). The results showed that NBD-2AGE accumulates in certain brain regions and that neurons are target cells for the probe. Altogether, our data suggest that NBD-2-AGE is a very useful probe to investigate ECs cellular uptake and trafficking kinetics with a sensitive and radioactivity-free based method. Unlike the other ECs, NBD-2-AGE is resistant to the fast and very efficient FAAH- and MAGL-mediated hydrolysis, which is a well known confounding factor for studying cellular uptake and trafficking of AEA and 2-AG. Finally, fluorescently tagged noladin ether would allow monitoring the ECs distribution in different cell types when applied to complex matrices such as whole blood or brain slices.

References

[1] Bisogno T, et al. Pharmacol Biochem Behav 2005
[2] Chicca A, et al. J Biol Chem 2012
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[4] Fezza F, et al, FEBS Lett 2002

P8

Functional role of presynaptic NMDA receptors during the induction of long-term depression at neocortical L4-L2/3 synapses in juvenile rats

F. Neubauer[1], R. Min[1], T Nevian[1]

[1]Department of Physiology, University of Bern

Spike-timing dependent depression (t-LTD) at layer 4-to-layer 2/3 synapses in the developing primary somatosensory cortex is presynaptically expressed but depends on a chain of retrograde signaling events initiated at the postsynapse. We previously have shown that endocannabinoids which are postsynaptically released during the induction of t-LTD do not directly signal to the presynaptic membrane but rather act on cannabinoid receptors located on the third cellular synaptic element, the astrocyte. Activation of astrocytic cannabinoid receptors triggers an increase in astrocytic calcium activity leading to the release of glutamate from the astrocyte which, in turn, activates presynaptic NMDA receptors (pre-NMDAR). Hence, pre-NMDAR are at the interface between the postsynaptic/astrocytic events of LTD induction and the presynaptic signal transduction pathway which eventually leads to the presynaptic expression of LTD. Here we investigate whether calcium influx through pre-NMDAR is a necessary part of this presynaptic pathway. Making use of our finding that astrocytes are mediating the effect of endocannabinoids, we have established an experimental paradigm which allows us to image the putative calcium influx through presynaptic NMDA receptors. Bath application of 2-AG resulted in astrocyte-dependent induction of LTD, bypassing the requirement of timing-dependent activation of neurons. Using high-resolution two-photon calcium imaging and appropriate pharmacology we then monitored whether axonal boutons show pre-NMDAR-dependent calcium increases upon astrocyte activation.

P9

Towards the development and characterization of ABHD6 & ABHD12 selective inhibitors

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Endocannabinoid (EC) levels are tightly regulated by the activity of their biosynthetic and degrading enzymes, which consequently control the extent of ECs effect on CB1 and CB2 receptors. While AEA is degraded by FAAH, the hydrolysis of 2-AG can be mediated by multiple enzymes: MAGL, ABHD6 and ABHD12 thus constituting a more complex system. Among them, MAGL is considered the main contributor to 2-AG hydrolysis in the brain and the most extensive studied enzyme for which several potent and selective inhibitors have been developed (i.e. JZL184, [1]). Much less is known regarding the role of ABHDs, which is mainly due to the lack of structural information and specific inhibitors. Although ABHDs and MAGL have been suggested to share a similar catalytic triad, there are several differences which point towards a differential role of these enzymes in controlling 2-AG levels. In particular, ABHDs and MAGL have been proposed to show different cellular localizations in the brain - e.g. MAGL co-localizes with CB1 receptors in presynaptic neurons where would be responsible for while ABHD6 resides post-synaptically where is believed to control the accumulation and efficacy of 2-AG at the receptors. ABHD12 is highly expressed in microglia and accounts for approximately 9% of total brain 2-AG hydrolysis (reviewed in [2]) - and also different compartmentalization at the cellular level: while MAGL is a soluble enzyme found both associated to the inner leaflet of the plasma membrane and in the cytosol, ABHDs have been suggested to be integral membrane proteins, with the catalytic site either facing the cytoplasmic lumen - ABHD6, or the extracellular milieu -ABHD12 [3].

Our focus is to develop potent and selective inhibitors for ABHD6 and mainly ABHD12 to further explore their involvement in the endocannabinoid system, with particular attention on the bidirectional movement of 2-AG across the plasma membrane. With this aim, we generated HEK293 cell lines stably transfected with hABHD6 and hABHD12. The initial plasmids (gently donated by Prof Laitinen) were designed only for transient transfection and we optimized the constructs for the stable transfection. The transfected cell lines were validated for the ability to hydrolyse [3H]2-OG. Transfected cell lines showed significant increase (5-6 times for ABHD6 and ABHD12, respectively at 10 µM 2OG) of [3H]2-OG hydrolysis compared to non-transfected cells. HEK293 cells showed negligible FAAH activity while no MAGL expression and activity. These features make HEK293 cells an optimal system to study the physiological role of ABHD6 and ABHD12 on 2-AG trafficking across plasma membranes. The use stably transfected cell lines has a double advantage: (i) they provide a permanent and reproducible source of sample (cell homogenate/membrane preparation) enriched with the hydrolase of interest (i.e. ABHD6 or ABHD12) allowing a reliable screening format for different libraries of compound and (ii) they allow to investigate the effects of a specific inhibitors in living cells. This enables an experimental set-up for the developing of specific inhibitors by modifying their cell permeability based on the prediction that the catalytic site of the hydrolases face opposite directions in the plasma membrane.

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P10

Pepcan localization and production in the rodent

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Introduction: The activation of cannabinoid receptors CB1 and CB2 has shown to positively affect pathological conditions in the CNS and in the periphery. Interestingly, in the CNS, CB1 activation may lead to detrimental effects, such as memory and learning deficits and hyperalgesia under certain conditions. 1 In addition to the classical endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG), which have been studied extensively, we have recently identified a new class of endogenous peptide ligands of CB1 receptors (Pepcans), which includes twelve peptides of varying length (Pepcan 12-23) that derive from the alpha chain of hemoglobin and show negative allosteric modulation at CB1 receptors.

Aims: We sought to determine the localization of Pepcans in the CNS and pinpoint the site of their production. Free-floating 40 µm cryosections were stained with immunofluorescence or immunoperoxidase (3,3'-Diaminobenzidine (DAB)) using an antibody that detects all Pepcans.2 Furthermore, we wanted to quantify the total amount of Pepcans (Pepcan 12-23; numbers indicating the total number of amino acids) as well as the most bioactive Pepcan12 (RVD-hemopressin) in the CNS. To do so, we are using a newly established quantitative LC-MS/MS method (for Pepcan12) as well as ELISA (for Pepcan 12-23) on whole brain and spinal cord tissue.

Results: Our results show comparable specific Pepcan staining patterns in both sexes of mouse and rat (infant and adult) with peptidergic projections throughout the brain and spinal cord. Both species stain for Pepcans to a high extent in the forebrain and brainstem. Colocalizations of Pepcans with tyrosine hydroxylase lead us to identify the locus coeruleus as the main brain nucleus harboring noradrenergic neurons, to produce Pepcans. Pepcans also colocalized - in projection areas as well as the locus coeruleus neurons - with galanin but not neuropeptide Y, somatostatin or vasopressin, which are all putative peptide transmitters found in the locus coeruleus neurons3. Consistent with their innervation pattern, our results suggest, that Pepcans might originate in a subset of neurons in locus coeruleus nucleus.

Conclusions: Gaining insight into Pepcan production and localization has opened new doors, allowing to investigate their physiological function, which - given their high abundance in the CNS - might be of high importance.

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P11

Peptide endocannabinoids (Pepcans) are the first endogenous PAMs for CB2 receptors

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Peptide endocannabinoids (pepcans) is a family of hemoglobin-derived peptides which has been recently identified [1,2]. The length of endogenous pepcans ranges from 12 to 23 aminoacids and are N-terminal extended versions of the smallest representative of the family, pepcan-12 (RVD-hemopressin). Pepcan-12 was reported to interact with CB1 receptors as agonist and inverse agonist [1,2]. We recently identified that the entire family of pepcans acts as negative allosteric modulator (NAM) of CB1 receptors and pepcan-12, which is the most abundant pepcan, showed also the most potent and efficacious allosteric modulation of CB1 receptors [3].
Here we show that pepcans behave as positive allosteric modulators (PAMs) of CB2 receptors. In binding assays pepcan-12 increased the affinity of orthosteric ligands ([3H]-CP55,940 and [3H]-WIN55,212-2) towards hCB2 receptors. When pepcan-12 was co-incubated with synthetic (CP55,940) and endogenous (2-AG) ligands it induced an increased efficacy and potency of the orthosteric ligand in functional assays ([35S]GTPγS and cAMP). Pepcan-12 per se did not elicit any effect either in the binding or functional assays for CB2 receptors, thus behaving as pure PAM. We quantified the levels of pepcans in different animal tissues by competitive ELISA exploiting our in-house generated specific antibody (1A12) [3]. The results showed that pepcans are present in different tissues with amounts ranging from 0.07 ng/mg tissue in the brain to 0.6 ng/mg tissue in the liver, 0.5 ng/mg tissue in the kidney and 2.1 ng/mg tissue in the spleen. In LPS-challenged mice, the level of pepcans significantly raised suggesting a modulation of pepcan formation and/or degradation upon inflammation. In a mouse model of liver ischemia reperfusion, pepcans levels showed an increase after 2h and 6h of reperfusion returning to basal levels after 24h, following the kinetics of endocannabinoids as previously shown [4].
In conclusion, the different allosteric modulation properties of pepcans (negative at CB1 and positive at CB2) make them a unique and versatile class of endogenous modulators of cannabinoid receptor activity.

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P12

Rational design and synthesis of new 2-oxo-1,2-dihydropyridine-3-carboxamide derivatives as highly selective CB_2R ligands

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Cannabinoid receptors (CB1 and CB2) are key components of a ubiquitous complex lipid signaling system known as endocannabinoid system (ECS), which also consists of the endocannabinoids anandamide (AEA) and 2-arachidonoilglycerol (2-AG), the enzymes implicated in their biosynthesis, and inactivation (MAGL and FAAH) and several other components (membrane transporter, intracellular carrier proteins, and other catabolic enzymes) [1]. As CB1R probably mediates most of the psychotropic effects of cannabinoids [2]. CB2R selective ligands are attractive as therapeutics for treating inflammation, pain, neurodegenerative disease and cancer [3] because they would presumably lack this psychoactivity.

In a research program aimed at obtaining CB2R selective ligands, we have individuated a series of 2-oxo-1,2-dihydropyridine-3-carboxamide derivatives (**A,** Figure 1) that exhibited good CB2R affinity. Furthermore, we discovered that the substituent in position 5 is responsible for the functional activity of this class of compounds [4] (**B,** Figure 1). In particular, replacing the hydrogen atom in position 5 with a phenyl group (**B1**) and with a 4-methoxyphenyl group (**B2**), the CB2R activity shifts from agonism to inverse agonism and neutral antagonism, respectively [5]. Conversely, substituting the hydrogen with a bromine atom (**B3**) the CB2R activity does not vary. To further deepen this discovery, in this work we designed and synthesized novel potential CBR ligands (**C,** Figure 1) modifying the 5-substituted 2-oxo-1,2-dihydropyridine-3-carbaxamide nucleus (**A**, **B,** Figure 1) through the insertion of a methyl group at the position C-6 of the 2-oxopyridine. These new compounds were tested in order to evaluate the influence of a small substituent at the 6-position of pyridine ring on CBR affinity. The results obtained lay solid foundations for future studies of the structure-activity relationships within this class of compounds

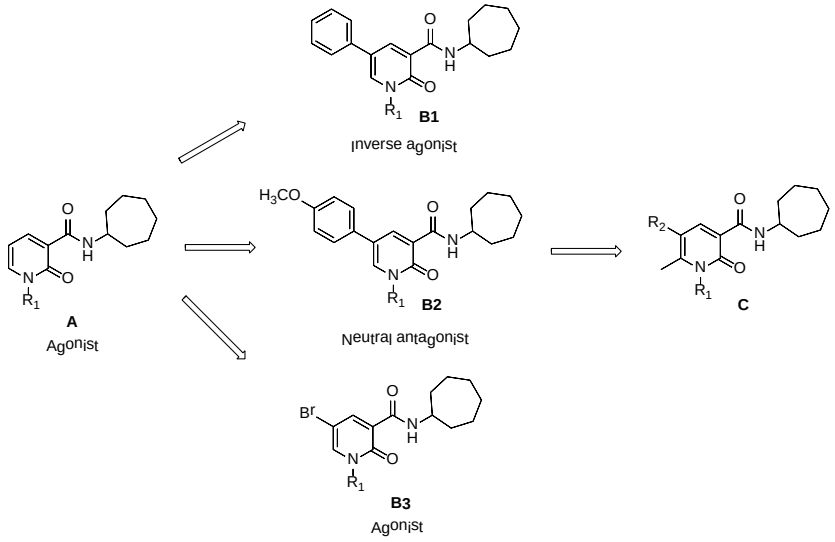

Figure 1. Derivatives with general structures **A**, **B** and **C**.

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P13

6-Methyl-2-oxo-1,2-dihydropyridine-3-carboxamide derivatives as a source of CB2 receptor modulators with polypharmacology features

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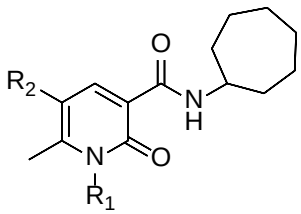
6-Methyl-2-oxo-1,2-dihydropyridine-3-carboxamide derivatives showed potent and selective binding to CB2 receptors. It was shown that the nature of the substituent in position C5 of the heterocyclic nucleus controls the switch among the different types of pharmacological modulation (agonism, inverse agonism and antagonism) of the receptor (Lucchesi et al, 2014). To further study the structure-activity relationship of this class of CB2 ligands, the insertion of a methyl group in position C6 of the 1,2-dihydro-2-oxopyridine ring was investigated.



1,2-Dihydro-6-methyl-2-oxopyridine-3-carboxamide

The binding properties of 1,2-dihydro-2-oxopyridine-3-carboxamide derivatives were not affected by the presence of the methyl group in position C6 similarly to the influence of the substituent in position C5 on the functional modulation of CB2 receptor activity. The C5 bromide derivative (FM6b) showed agonist property, the C5 phenyl derivative (AD15b) showed inverse agonist property while the C5 p-methoxyphenyl derivative (FM5b) behaved as neutral antagonist up to the concentration of 1 microM. Interestingly, FM5b competitively antagonized the effect of FM6b in the [35S]GTPγS assay, while it behaved as non-competitive antagonist in presence of the inverse agonist (AD15b). Since the role of the CB2 receptors in certain physio-pathological conditions still remains unclear, our library of compounds which contains the full repertoire of receptor modulators, will help to elucidate this issue. We also tested the effect of the 1,2-dihydro-6-methyl-2-oxopyridine-3-carboxamide derivatives on all the main targets of the ECS. Interestingly, some of the compounds, which showed also the best binding properties at CB2 receptors, showed potent inhibition of AEA and 2-AG uptake with IC50 values in the nanomolar range. Some of these inhibitors might exert part of their action by inhibiting FAAH, while others not. Therefore, the presence of compounds which modulate different targets of the ECS (i.e. putative endocannabinoid membrane transporter and FAAH) beyond the CB2 receptors, will allow investigating the pharmacological relevance of these multi-target approaches in different cellular and animal models.

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