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Characterization of the novel opioid and nociceptin peptides

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Opioid system is consists of μ, δ, κ and nociceptin (NOP) receptors and their respective endogenous neuropeptide ligands. In this study we have characterized novel non-mammalian opioid peptides Ile-enkephalin and Phe-enkephalin, and artificial NOP receptor partial agonist hexapeptide Ac-RYYRIR-ol.

Leu- and Met-enkephalin were the first endogenous opioid peptides identified in different mammalian species including the human. Comparative biochemical and bioinformatic evidence indicates that enkephalins are not limited to mammals. Lower vertebrate enkephalins were investigated with in vitro biochemical experiments using rat brain membrane preparations and turned out to be moderate affinity opioids with a definite preference for the δ-opioid receptor sites. Phe-enkephalin from the lungfish displayed low affinities toward the μ- and δ-opioid receptor, while exhibited moderate affinity toward the κ-opioid receptor. In [^{35}S]GTPγS binding studies, Met-enkephalin produced the highest stimulation followed by Leu-enkephalin, Ile-enkephalin from the clawed frog and Phe-enkephalin, was the least efficacious among these endogenous peptides (Bojnik et al. 2009a).

Some N/OFQ sequence unrelated hexapeptides can effectively bind to the NOP receptor and they were used as template for structure activity studies that lead to discovery of the new NOP selective ligands. The pharmacological profile of the novel hexapeptide Ac-RYYRIR-ol was investigated using various in vitro assays including receptor binding and G protein activation in rat brain membranes, mouse vas deferens, rat vas deferens, guinea pig ileum, mouse colon and calcium mobilization. In rat brain membranes Ac-RYYRIR-ol displaced [^{3}H]Ac-RYYRIK-ol (Bojnik et al. 2009b) with high affinity and stimulated [^{35}S]GTPγS binding with high potency. The stimulatory effect of Ac-RYYRIR-ol was antagonized by the selective NOP receptor antagonist UFP-101. In antagonist type experiments Ac-RYYRIR-ol inhibited the stimulatory effects induced by N/OFQ. In the electrically stimulated mouse vas deferens Ac-RYYRIR-ol displayed negligible agonist activity while antagonizing in a competitive manner the inhibitory effects of N/OFQ. In the mouse colon Ac-RYYRIR-ol produced concentration dependent contractile effects with similar potency and maximal effects as N/OFQ. Finally, in the Ca^{2+} mobilization assays Ac-RYYRIR-ol displayed lower potency and maximal effects compared with N/OFQ assays.

In conclusion, two novel, non-mammalian enkephalins were described and compared with those of the well-known Leu- and Met-enkephalin. Among the four structures tested, the 'mammalian type' Met-enkephalin exhibited the highest affinities in receptor binding assays and produced the most efficacious G-protein stimulation in brain membranes, while the newly identified 'lower vertebrate type' Ile- and Phe-enkephalins were found to be less effective. On the other hand, novel NOP receptor selective hexapeptide Ac-RYYRIR-ol has been shown to have fine selectivity, high potency, furthermore agonist and antagonist effects toward the NOP receptors were measured in various assays. This is likely due to its partial agonist pharmacological activity.

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Genetic modification of carotene producing Zygomycetes

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Carotenoids are terpenoid-type chemical compounds. These yellow to orange-red natural pigments are used in the food, pharmaceutical and cosmetic industry and as feed colour additives. Recently, they are attracting an increasing attention, due to their beneficial effects on health. In Zygomycetes fungi, β-carotene is the predominant carotenoid. Traditionally three species: *Blakeslea trispora*, *Phycomyces blakesleeanus* and *Mucor circinelloides* have been involved in the study of the carotene biosynthesis.

Mucor circinelloides has several characteristics advantageous for molecular genetic studies. For example, well functioning methods are available for the genetic transformation of this fungus based on autonomously replicating plasmids (Papp et al. 2008). However, integrative transformation methods are not well established and the fate of the transforming DNA has not yet been analyzed.

The aims of our work were (1) to investigate and compare the effect of overexpression of different isoprene biosynthesis genes for the carotene production; (2) to produce oxygenated β-carotene derivatives by heterologous expression of the *crtW* gene (encoding β-carotene ketolase) of the marine *Agrobacterium aurantiacum*; (3) to integrate the *crtW* gene into the *Mucor* genome by different methods; (4) and to reveal the carotenoid spectra and to characterize the carotenoid production of some Zygomycetes in order to determine new producer strains potentially applicable in further analysis.

Transformation of fungal protoplasts was carried out by the polyethylene glycol-mediated method. Three different isoprenoid genes were involved in the study. Expression vectors that contained one of these genes driven either by their own promoter or by the regulator

sequences of the *Mucor* glyceraldehyde-3-phosphate dehydrogenase 1 gene (*gpd1*), were constructed. The *Mucor leuA* or *pyrG* genes were used as selection markers; they complement the leucine or uracil auxotrophy of the recipient *M. circinelloides* strain, respectively. Vectors were introduced alone ore in co-transformations to combine the isoprenoid genes. All transformants proved to be stable under selective conditions and some of them under non-selective conditions as well. Transformants were analyzed with hybridization and PCR techniques. Real-time PCR analysis revealed a relatively high copy number of the plasmids in the transformants and an unequal proportion of them in the co-transformants. Higher expression of the genes was also verified. The carotene production was analyzed by spectrofotometric, TLC and HPLC methods.

It has been found that *M. circinelloides* has β-carotene hydroxylase activity, therefore introducing the *crtW* gene may result in the production of several types of oxygenated β-carotene derivatives. Transformation with vector, containing the *crtW* gene under the control of *gpd1* promoter, was carried out (Papp et al. 2006) and co-transformations with the isoprene genes were also done. Changes in the carotenoid production due to expression of the *crtW* gene have been proven.

Integration the *crtW* gene into the *Mucor* genome was achieved by three different methods: homologous recombination with double crossing over, *Agrobacterium tumefaciens*-mediated transformation and restriction enzyme-mediated integration. The integration had been proven and analysed in several transformants by PCR, inverse-PCR, real-time PCR and hybridization techniques.

Carotene content of twenty one Zygomycetes strains was also analyzed. Some of them produced the same or higher amount of carotenoids than the wild type *M. circinelloides* or *B. trispora* strains. These strains were analyzed under different conditions, e.g. temperature, light and carbon source. For some of these strains, we started the development of new transformation systems that allows the direct selection of the transformants.

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Increased genetic stability of a rationally designed reduced-genome *Escherichia coli*

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In an attempt to engineer a simplified, core-genome *Escherichia coli*, we have reduced the wild-type K-12 MG1655 genome by making surgically precise scarless deletions (Pósfai et al. 2006). Genome reduction was achieved without compromising the basic metabolic circuits of the cells. The new strains, with genomes up to 22% smaller, were designed by bioinformatic comparative genomics of four *E. coli* strains to identify non-essential genes and recombinogenic, mobile or cryptic virulence sequences, as well as genes with unknown functions for elimination.

These so-called multi-deletion strains (MDS) have several attractive properties which can make them useful in a wide variety of biotechnological applications. One of the most important of these properties is the increased genetic stability of these strains which includes an increase in both genomic and plasmid stability. This work focuses on the quantification of these different aspects of genetic stability. This was done using novel methods we developed for calculating mutation rates (Fehér et al. 2006) as well as rates of recombination within the cells.

Removal of all mobile genetic elements from the *E. coli* genome resulted in a lower mutation rate because of the lack of insertion events. In addition, the genes of three so-called error-prone DNA polymerases (*polB*, *dinB* and *umuDC*) were deleted resulting in a lower point-mutation rate. The resulting strain has a mutation rate that is close to one order of magnitude lower than the wild-type.

In addition to the increased fidelity of replication, lentiviral expression vectors harbored within different MDS strains proved to be more stable than in other commonly used cloning strains (Chakiath and Esposito 2007). By developing a plasmid-based system to measure recombination rates, we were able to quantify this improved stability. The most stable of our strains has a recombination rate that is over five times lower than the wild-type.

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