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Synthesis and in vitro biological effect of GnRH-protoporphyrin IX conjugates

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Photodynamic therapy (PDT) combines non-toxic components, a photosensitizer (PS), light and oxygen. If the photosensitizer is activated to its excited states by irradiation with visible light in the presence of molecular oxygen, electron and energy transfers can produce reactive oxygen species (ROS) in the tissue. These ROS react rapidly with the biomolecules leading to cell death *via* apoptosis or necrosis. In most cases a singlet oxygen (^{1}O) is generated that has a short intracellular life time (3 μs) and a very small intracellular diffusion distance that makes PDT highly selective. Unfortunately, photosensitizers accumulate in healthy tissues too, causing severe side effects like prolonged skin and eye photosensitivity. Therefore, the conjugation of the PS to a carrier peptide that itself has anti-tumor effect can increase the efficacy and selectivity of the treatment.

Protoporphyrin IX (PpIX) is a second-generation PS, it is non-toxic without irradiation, but can efficiently absorb light in the visible area. 635 nm irradiation is usually used to reach deeper (3mm) penetration. PpIX has two carboxyl groups that are suitable for conjugation to targeting moieties.[1]

Gonadotropin-releasing hormone (GnRH) receptors are overexpressed on several tumor cells, e.g. on tumors of the reproductive organs or on oral and laryngeal cancer cells, which makes this receptor a proper target for targeted tumor therapy.[2] GnRH-I is a decapeptide (<EHWSYGLRPG-NH_2, where <E is pyroglutamic acid) synthesized and released in the hypothalamus that plays a central role in the vertebrate reproduction by regulating gonadal activity.[3] Several different isoforms were isolated from different species, like chicken GnRH-II (<EHWSHGWYPG-NH_2) that is expressed also in human mainly in the kidneys, bone marrow and prostate, and is found to be a neuromodulator that stimulates sexual behavior[4] or GnRH-III (<EHWSHDWKPG-NH_2) that was originally isolated from sea lamprey.

GnRH-III binds to both type I and type II GnRH receptors and inhibits proliferation of different cancer cells while having insignificant hormonal activity.[5]

The sequences show that the N- and C- terminal parts are conserved, but the amino acids 5-8 can be changed without significant loss of efficacy.[4] In the sequence of GnRH-I and GnRH-II, the glycine in position 6 can be replaced by D-lysine, which serves as conjugation site, increases enzymatic stability and enhances the agonistic effect too.[6] Rahimipour *et al.* have already conjugated PpIX to GnRH-I[6DLys], and the selective receptor mediated phototoxicity could be demonstrated on T3-1 pituitary gonadotrope cell line.[7]

In most isoform the serine in position 4 can be replaced by a butyric acid modified lysine (Lys(Bu)) since this change increases the receptor binding affinity and the stability of the molecule against enzymes.[8]

In this work GnRH-protoporphyrin IX conjugates were synthesized using various GnRH analogues. All peptides were synthesized manually using solid phase peptide synthesis according to standard Fmoc/tBu strategy. GnRH-I and GnRH-II analogues modified with butyrylated lysin (Lys(Bu)) in position 4 were synthesized with using Fmoc-Lys(Dde)-OH. After finishing the protected decapeptides, Dde group of ^{4}Lys was removed on the resin and the acylation was performed by using butyric anhydride and DIPEA. The GnRH-III analogue was synthesized with aspartic acid methyl ester (Asp(OMe)) in position 6 since the free carboxylic acid could have interfered with the PpIX conjugation. The methyl ester group is labile under basic conditions, so in this case Fmoc-Lys(Mtt)-OH was incorporated into the peptide sequence instead of the Dde protected lysine and the butyrylation was performed after the Mtt removal.

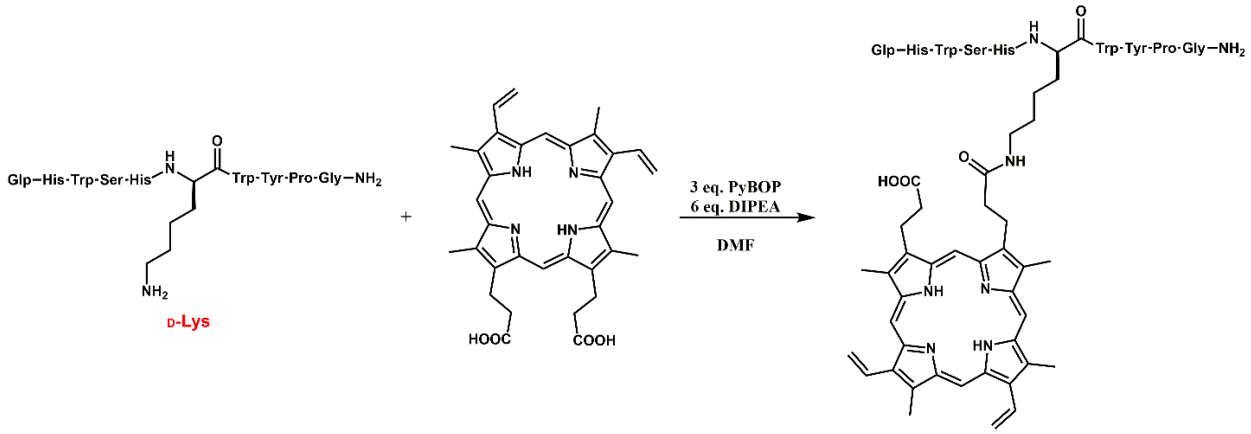

Figure 1: Synthesis of the GnRH-I - PpIX conjugate via amide bond formation

The purified peptides were conjugated to PpIX *via* an amide bond in solution phase using PyBOP (Figure 1).

The *in vitro* biological assays were performed on Detroit-562 human pharynx carcinoma cells that highly express GnRH receptors.[9] The cells were co-incubated with the conjugates, then after wash-out the cells were irradiated with 635 nm. MTT assay was performed after 72h.

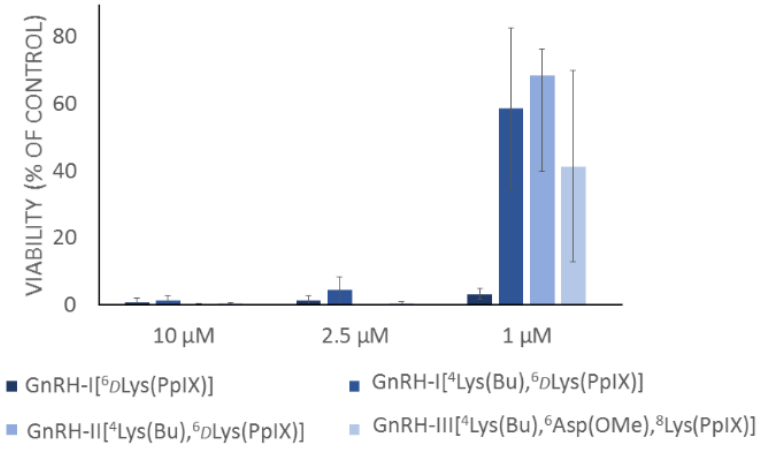

Figure 2: In vitro cytotoxicity of the GnRH-PpIX conjugates

We have investigated the compounds with and without irradiation and we found that the compounds alone, without irradiation, were not toxic in the assays (>10 μM). We could also show that a short irradiation time (10 min) is enough for the tests, since the compounds showed the same *in vitro* effect with 30 min than with 10 min irradiation. The shorter time is more beneficial for the further *in vivo* treatments. Furthermore, we found that all GnRH-Pp conjugates showed excellent anti-tumor effect at low concentrations (~1 μM), the best conjugate was GnRH-I[^{6}D-Lys(PpIX)] (Figure 2).

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