Summary of the results

The aim of the research proposal is to develop new GnRH-II peptide based bioconjugates in order to enhance antitumor activity of GnRH-II. The main goals of the project are the development and synthesis of new GnRH-II bioconjugates, stability studies, *in vitro* cytotoxic and cytostatic effect of the bioconjugates, determination of cellular uptake profile of peptide conjugates, studying the apoptotic features and mechanism of GnRH-II conjugates.
In the first year of the project, daunomycin and/or proapoptotic peptide containing GnRH-II conjugates were synthesized and chemically characterized. The conjugates consist of [D-Lys^6]GnRH-II, as targeting moiety, and Bak-BH3 (H-^{71}MGQVGRQLAIIGDDINRR^{88}-NH_2) or PUMA-BH3 (H-^{137}IGAQLRRMADDLNAQ^{151}-NH_2) peptides, as proapoptotic part. In all cases, the drug molecule was daunomycin, a clinically used cytostatic drug. The corresponding GnRH-I conjugates, as controls were also prepared and characterized (Figure 1).

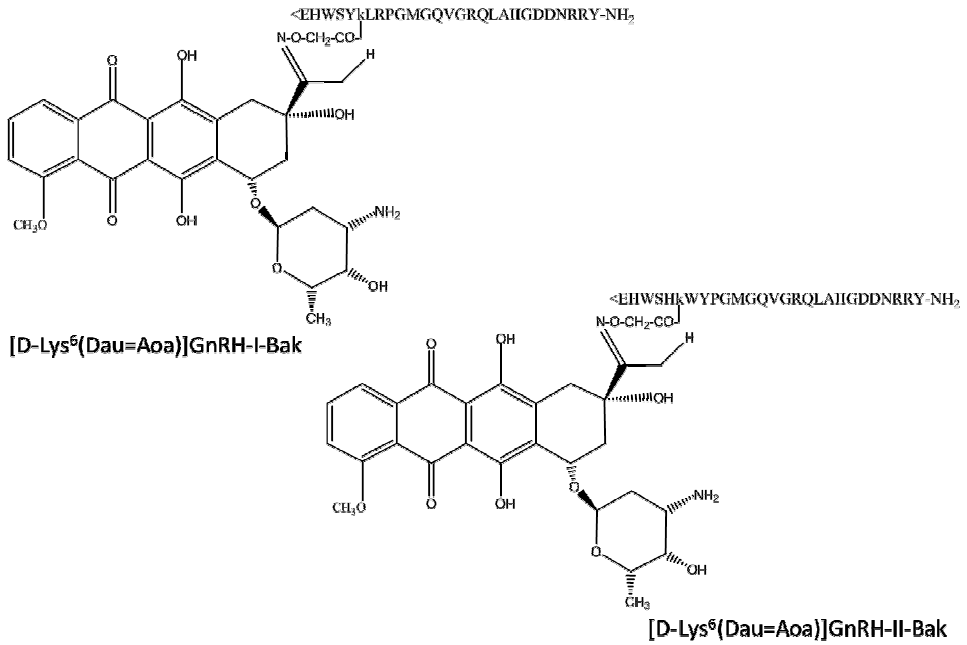

Figure 1. Chemical structure of daunomycin and proapoptotic (Bak BH3) peptide containing GnRH conjugates

The *in vitro* long-term cytotoxic effect of the conjugates was determined by MTT assay on human cancer cell cultures. It was found, that [D-Lys^6]GnRH-II and proapoptotic peptides themselves and their conjugates had similar *in vitro* long-term cytotoxic effect without cell type dependence activity. The attachment of the daunomycin was significantly increased the *in vitro* long-term cytotoxic activity of the GnRH-II-proapoptotic peptide conjugates. In all cases the activity of the conjugates („Dau-GnRH-II-Bak-BH3" and „Dau-GnRH-II-PUMA-BH3") was minimum one order of magnitude higher, than the control peptides. Furthermore, the daunomycin and the conjugates had almost similar *in vitro* long-term cytotoxic effect in all cell culture cases. To compare the *in vitro* efficacy of the GnRH-I and GnRH-II based conjugates, it was found, that there is no significant difference between the effectivity of the two kinds of conjugates, they had similar *in vitro* long-term cytotoxic activity. According to

the IC_{50} values, Bak-BH3 peptide containing GnRH conjugates („Dau-GnRH-Bak-BH3") were more effective, than the PUMA-BH3 containing ones; therefore, they were selected for the further *in vitro* studies (Table 1).

Table 1. In vitro long-term cytotoxic activity of GnRH-II and proapoptotic peptides (as controls) and their daunomycin conjugates on different type of cancer cells

	IC_{50} values (μM± SD)			
	MCF7	**SKBR3**	**HT29**	**T47D**
[D-Lys^6]GnRH-II	37.4±15.1	78.0±27.6	56.8±39.7	>100
Puma	34.0±14.3	60.9±40.6	67.2±36.4	>100
[D-Lys^6]GnRH-II-Puma	60.9±20.0	76.4±14.5	56.8±34.8	65.1±49.4
[D-Lys^6(Dau=Aoa)]GnRH-II-Puma	0.2±0.04	2.0±0.5	6.3±3.6	3.9±0.9
Bak-BH3	35.5±12.3	62.4±2.0	62.0±23.7	38.1±14.6
[D-Lys^6]GnRH-II-Bak-BH3	33.1±13.5	55.8±13.3	69.7±26.9	>100
[D-Lys^6(Dau=Aoa)]GnRH-II-Bak-BH3	0.01±0.0	0.3±0.08	0.6±0.5	0.5±0.3
Dau	0.4±0.05	0.2±0.03	0.4±0.08	0.3±0.07

In the second year of the project, further comparative *in vitro* studies were performed to define and compare the *in vitro* activity of the GnRH-I and GnRH-II conjugates. In the first step, the receptor binding affinity of the daunomycin containing GnRH conjugates was determined using human pituitary and human prostate cancer. It was found, that the GnRH-I and GnRH-II conjugates displayed slightly higher binding affinity to the prostate cancer cells (0.89±0.09 nM and 2.11±0.27 nM, respectively), than to the human pituitary cells (1.62±0.32 nM and 4.24±0.81 nM, respectively). The GnRH-II conjugate compared with GnRH-I conjugate showed twice as lower binding affinity. Based on the literature, the GnRH-I receptor has two binding sites, a high- and a low-affinity one. In the case of high-affinity binding sites, the ligand binding affinity of the receptor is in the nanomolar concentration range. On tumor cells both of these binding sites were detected, while in the pituitary cells mainly high-affinity binding sites are preferred. In our case both GnRH-I and GnRH-II conjugates were bound to the receptor in a nanomolar concentration suggesting, that these conjugates might activate the high affinity binding sites of the GnRH-I receptor.

According to the binding affinity evaluation, the *in vitro* cellular uptake profile of the Dau-GnRH-Bak-BH3 conjugates was determined and compared by flow cytometry and fluorescence microscopy on MCF-7 human breast, and HT-29 human colon cancer cells. It was found, that the conjugates can be taken up in a concentration dependent manner without any significant differences. The *C*-terminal elongation of the GnRHs with Bak-BH3 peptide significantly increased the internalization ability of the conjugates (Figure 2).

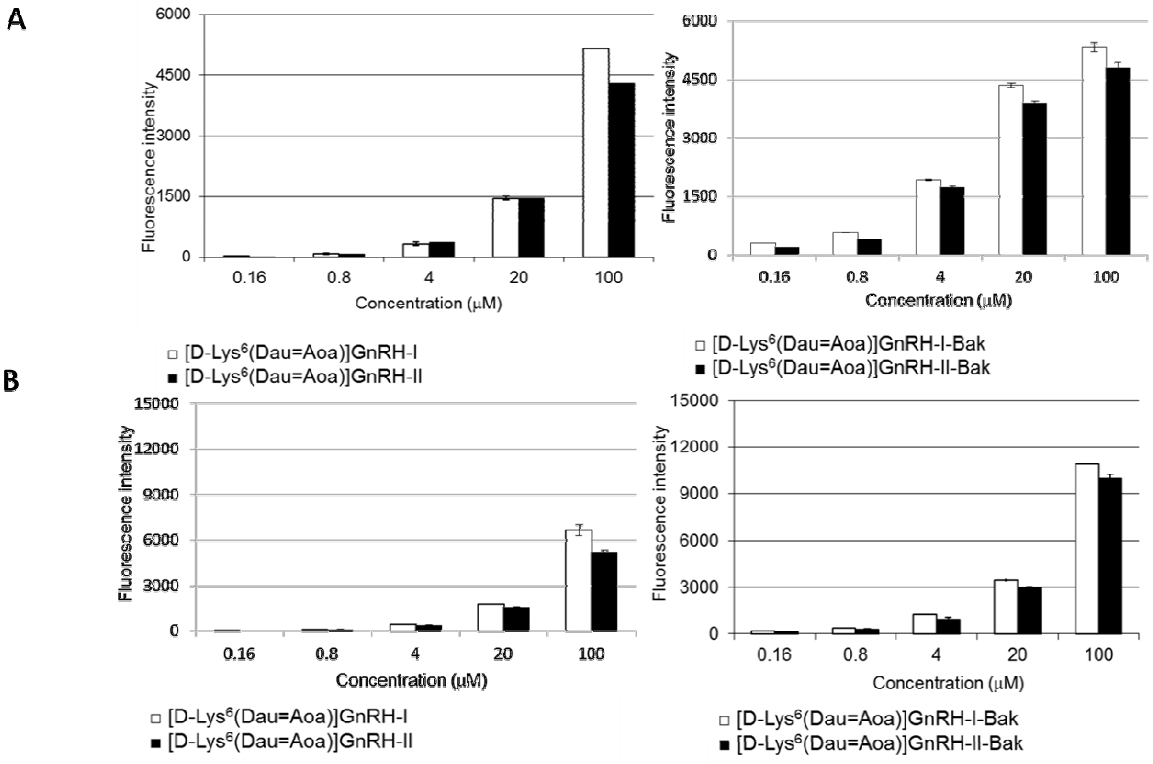

Figure 2. Determination of cellular uptake profile of daunomycin and proapoptotic peptide containing GnRH conjugates compared to daunomycin-GnRH conjugates on MCF-7 (A) and HT-29 (B) cells

The apoptotic and/or necrotic effect of Dau-GnRH-II and Dau-GnRH-II-Bak-BH3 conjugates and their corresponding GnRH-I conjugates, as controls was determined by flow cytometry analysis using fluorescence labeled annexin-V and propidium iodide. However, the GnRH-I and GnRH-II conjugates have significant *in vitro* long-term cytotoxic efficacy (IC_{50} values are around: 4 and 1 μM, respectively), unfortunately it coupled with low apoptotic activity (2 % and 3 %, respectively) on MCF-7 and HT-29 cells. They mainly caused necrotic cell death. The *C*-terminal elongation of the GnRH-I carrier moiety with the Bak peptide resulted in significantly enhanced *in vitro* long-term cytotoxic effect (10-fold) and increased apoptotic activity (2-fold) on MCF-7 cells. In the case of GnRH-II type targeting moiety, the *C*-terminal elongation with Bak BH3 peptide could not influence the *in vitro* long-term cytotoxic activity, but the apoptotic effect of these conjugates was significantly increased (3 fold) on MCF-7 cells. However the proapoptotic peptide can positively alter the *in vitro* long-term cytotoxic activity of the drug containing GnRH conjugates on MCF-7 cells, this tendency was not observed on HT-29 cells. This discrepancy between the MCF-7 and HT-29 cells might be explained by the differences in the expression level of the antiapoptotic proteins.

The regulation of apoptosis is a very complex process; not only the pro- and anti-apoptotic proteins play a role in it, but the other molecules also attend. Casein kinase II (CK2) enzyme is an ubiquitous kinase, which play key roles in several physiological and pathological processes, such as cell proliferation and apoptosis. The activity and/or expression of this enzyme are found to be increased in cancers (*e.g.* breast, colon). Overexpression of CK2 attenuates apoptosis induced by chemotherapeutic drugs and its antiapoptotic effect is

attributed to its ability to phosphorylate proapoptotic proteins. In agreement with this, the down-regulation of CK2 results apoptosis in tumor cells, therefore inhibition of CK2 promise to pave the way for a more targeted cancer treatment.
According to the literature, potential CK2 enzyme inhibitor peptide containing conjugates were designed, prepared and characterized by chemically. In the conjugates, the targeting units are GnRH-I and GnRH-II agonist derivatives, drug molecule is the daunomycin, and the potential CK2 inhibitor peptide is RRRDDDADDD sequence, which was derived from a peptide substrate (RRRDDDSDDD) of CK2 enzyme. In our case, the obligate serine (obligate phosphorylation site) of the substrate was replaced by alanine. The structure of the CK2 inhibitor containing conjugates and the proapoptotic peptide containing conjugates was similar.

Similar to the Bak proapoptotic conjugates, CK2 inhibitor peptide containing ones were also tested *in vitro*. These conjugates have been taken up in a concentration dependent manner, but the presence of CK2 inhibitor peptide sequence resulted in lower fluorescence intensity than the control peptides (Dau-GnRH peptides) on MCF-7 and HT-29 cells. These conjugates resulted in similar *in vitro* long-term cytotoxic activity, than the control Dau-GnRH peptides (Table 2), which is in correlation with the cellular uptake rate of the conjugates on MCF-7 and HT-29 cells (Figure 3).

Table 2. *In vitro long-term cytotoxic activity of GnRH and CK2 inhibitor peptides (as controls) and their daunomycin conjugates on MCF-7 and HT-29 cells*

	IC_{50} values (μM±SD)	
	MCF-7	**HT-29**
Dau	0.4±0.05	0.4±0.08
CK2 inhibitor (H-RRRDDDADDD-OH)	**67.9±10.6**	**62.1±4.5**
[D-Lys^6]GnRH-I-CK2 inhibitor	**61.5±10.2**	**38.7±9.0**
[D-Lys^6]GnRH-II-CK2 inhibitor	**50.9±10.2**	**58.4±12.6**
[D-Lys^6(Dau=Aoa)]GnRH-I-CK2 inhibitor	2.1±0.7	1.9±0.7
[D-Lys^6(Dau=Aoa)]GnRH-II-CK2 inhibitor	1.3±0.4	1.8±0.3

However the presence of CK2 inhibitor peptide sequence cannot be significantly increased the *in vitro* efficacy of this conjugates, but the *in vitro* apoptotic effect of them was pronounced on MCF-7 cells. Unfortunately on HT-29 cells, this increased apoptotic effect was not observed, similarly to Bak-BH3 proapoptotic peptide conjugates. This diverse effect on the two types of cell cultures confirm our suggestion, there is significant difference in the expression profile of the apoptotic proteins in the MCF-7 and HT-29 cells (Figure 3).

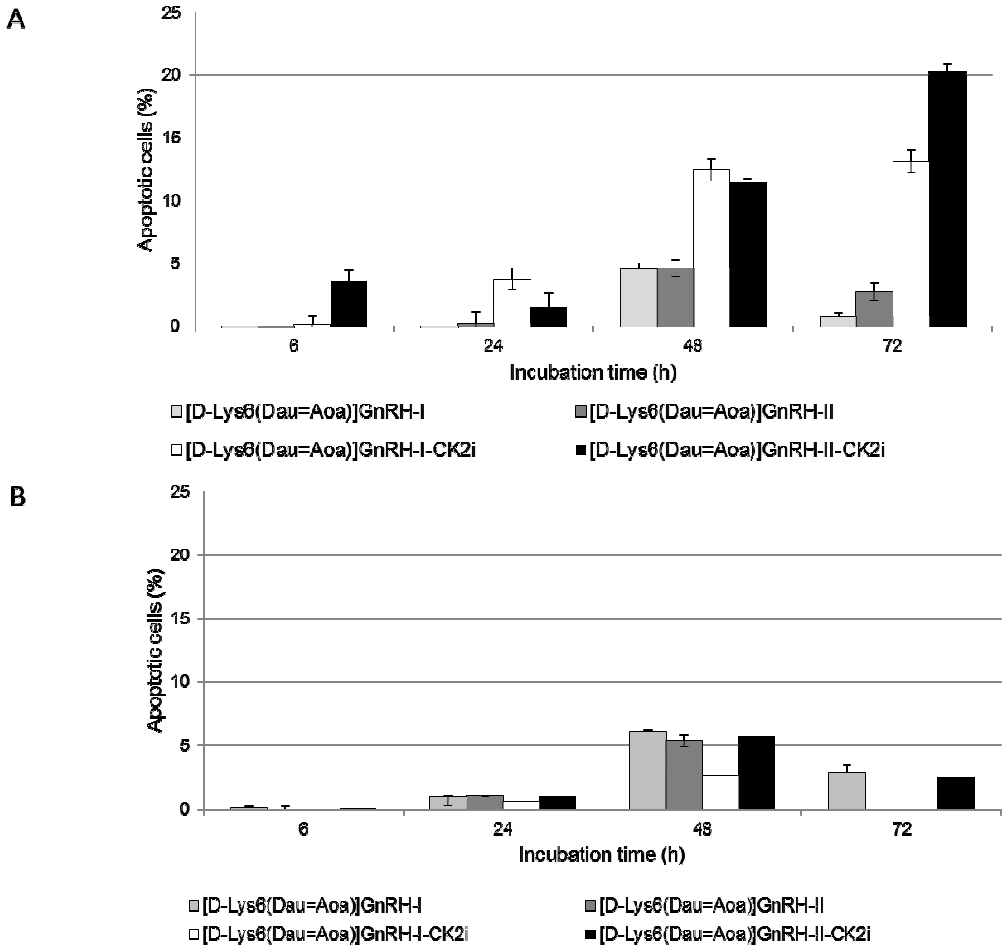

Figure 3. Determination of apoptotic effect of the daunomycin and CK2 inhibitor peptide containing GnRH conjugates on MCF-7 (A) and HT-29 (B) cells

In the third year localization assays were performed, which can be helped to understand the mechanism of action of these conjugates. In the first step, mitochondrial localization was studied using intracellular marker – MitoTracker - staining. Unfortunately, these experiments were not successful. The reason of it, the chemotherapeutic agent daunomycin, not only as a drug, but also as a fluorophore unit can be used in these conjugates. This fluorescent feature of drug molecule is the main advantage of it (we can used this conjugates without any other modification in the cellular uptake studies), but this is also caused several difficulties in the *in vitro* tests. The fluorescent spectrum of daunomycin is more or less overlapped the whole range of fluorescence, therefore the usage of daunomycin in the apoptosis or localization tests is very limited. Most of the commercially available fluorescent labeled special extra- and intracellular markers are unusable in the biological tests with the daunomycin. Deep red labelled MitoTracker was selected, as intracellular marker, in order to determine the intracellular localization of the proapoptotic GnRH conjugates. But unfortunately, the presence of daunomycin in the conjugates significantly renders more difficult the investigation of intracellular localization, because the fluorescence spectra of daunomycin and MitoTracker overlap. Therefore this unexpected difficulty interferes the performance of the *in vitro* localization assays.

During the three years period of this project several unexpected results lead to the modification of the original work plan. Based on this observation carrier peptide or the drug molecule was altered.

In the first step, GnRH agonist carrier peptides were changed to antagonist ones, because the apoptotic effect of the conjugates was lower than expected. In this case drug containing GnRH antagonist conjugates were synthesized, chemically characterized, and *in vitro* assays were performed. The long-term cytotoxicity of these antagonist conjugates was lower than the agonist ones had (Table 3), but their cellular uptake (Figure 4) and apoptotic activity (Figure 5) was significantly pronounced. According these findings, GnRH antagonist peptides, as carriers will be tested in the future.

Table 3. *In vitro long-term cytotoxic activity of GnRH antagonist peptides (as controls) and their daunomycin conjugates on MCF-7 and HT-29 cells*

	IC_{50} values (μM±SD)	
	MCF-7	HT-29
[D-Lys^6]Cetrorelix	>100	31,3±15,3
[D-Lys^6]GnRH-II antagonist	>100	50,7±6,2
[D-Lys^6(Dau=Aoa)]Cetrorelix	8.7±1.8	4.4 ±0.4
[D-Lys^6(Dau=Aoa)]GnRH-II antagonist	4.6 ±0.7	16.7±1.8

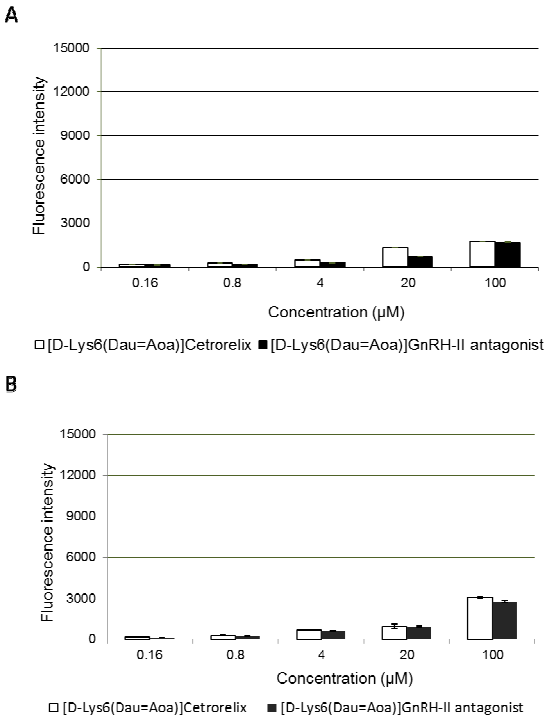

Figure 4. Determination of cellular uptake profile of daunomycin containing GnRH antagonist conjugates compared to daunomycin-GnRH agonist conjugates on MCF-7 (A) and HT-29 (B) cells

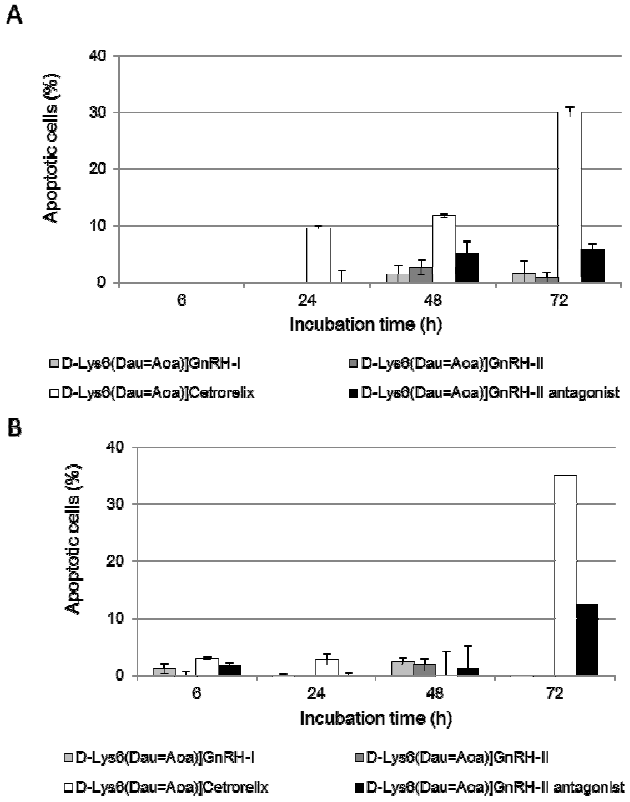

Figure 5. Determination of apoptotic effect of the daunomycin containing GnRH antagonist conjugates on MCF-7 (A) and HT-29 (B) cells

Based on these *in vitro* data, GnRH antagonists are better candidates as targeting moiety, than the GnRH agonist ones are.

On the other hand, daunomycin caused difficulties in the *in vitro* tests require developing new type of drugs with *in vitro* cytostatic and apoptotic activity. Therefore different types of metal containing drug candidates (ferrocene-or rhodium containing complexes) were tested *in vitro*. These compounds were synthesized by national and international collaborations. Ferrocene containing compounds have pronounced *in vitro* efficacy with significant apoptotic effect, therefore they will be good candidates, as drugs for conjugation to peptide carriers and the conjugates will be tested in the future.

During the three years, two BSc and one MSc students (in all cases the expected date of the diploma is in 2016) were involved in the experimental research. The results have been published in three prestigious international journals (Journal of Peptide Sciences, Journal of Organometallic Chemistry, Microchemical Journal) and they were shown international conferences (three poster- and three oral presentations).