DEVELOPMENT OF BIOCONJUGATES AND THEIR MODUL CONSTRUCTS FOR TARGETED THERAPY OF CANCERS WITH HIGH MORTALITY

Excerption from the results obtained in frame of the grant

NVKP_16-1-2016-0036

supported by the National Research, Development and Innovation Office

ISBN 978-963-489-286-1

Budapest, 2020

Comparison of the apoptotic effects of different GnRH-based conjugates with or without butyrated Lys in position 4 on colon carcinoma cells

Eszter Lajkó[1], Rózsa Hegedüs[2], Gábor Mező[2,3], László Kőhidai[1]

[1]Department Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary

[2]Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Budapest, Hungary

[3]Institute of Chemistry, Eötvös L. University, Budapest, Hungary

Introduction

Targeted tumor therapy represents a promising strategy to improve the selectivity and efficacy of chemotherapy, by delivering a cytotoxic drug covalently linked to a targeting unit which is selective to a tumor's overexpressed receptors. Gonadotropin-releasing hormone (GnRH) is one of the hormone peptides extensively studied and used for drug delivery. As for targeting units, several native and synthetic GnRH analogs were shown to efficiently affect only the tumor cells with GnRH receptor (GnRH-R) and spare the healthy cells with no or limited number of GnRH-R.[1,2]

There are three main types of the decapeptide GnRH: GnRH-I (<EHWSYGWLPG-NH_2, where <E is pyroglutamic acid) and GnRH-II (<EHWSHGWYPG-NH_2) can be found in the human body,[3,4] while GnRH-III (<EHWSHDWKPG-NH_2) is a non-human isoform; however, it can specifically bind to different human GnRH receptor-expressing tumor cells.[5] GnRH peptides, as part of drug-delivery systems, have some valuable properties such as (i) having a tumor growth inhibitory effect on their own, (ii) providing an easy way of modification and conjugation due to the well-studied structure-activity relationships.[6] One of the fundamental limitations of GnRH-based targeting is the relatively rapid proteolytic degradation of the peptide part.[6,7] The modification of GnRH-I and GnRH-II in position 6 with D-amino acid could increase their affinity to the GnRH-R,[4] their enzymatic stability as well as influence their antitumor activity.[8,9] One of the most effective strategies to improve the antitumor activity and other biochemical properties (*e.g.* enzymatic stability) of GnRH-III and its conjugate was the replacement of Ser in position 4 with butyrated Lys.[7,10]

It has been suggested that the apoptosis is involved in the antitumor activity of different anthracycline-GnRH conjugates.[11,12] Depending on the target cells and the type of GnRH built in the anthracycline-GnRH conjugates, they could exhibit apoptotic activity with different extent.[9,13] Our recent results on melanoma cells indicated that in the case of the GnRH-III conjugate modified with butyrated ^{4}Lys (GnRH-III[^{4}Lys(Bu),^{8}Lys(Dau=Aoa)],

where Dau is daunomycin and Aoa is aminooxyacetyl moiety providing an oxime linkage between the drug and homing peptide), its antitumor effect was rather attributed to apoptotic activity than, in contrast to the GnRH-III[^{8}Lys(Dau=Aoa)] possessing ^{4}Ser, where the cell cycle blocking effect (arrest in G2/M phase) was shown to be more prominent.[14] Although an increasing number of studies have focused on the determination of the apoptosis induced by different cytotoxic drug-containing GnRH conjugates, there are only a few data on the underlying molecular mechanism.

The aim of our work was to compare the apoptotic activity of different GnRH-based, Dau-containing conjugates by impedimetry (xCELLigence SP System), flow cytometry and quantitative real-time RT-PCR in HT-29 human colon carcinoma cell line.

Results

Three GnRH analogs (GnRH-I-[^{6}D-Lys], GnRH-II-[^{6}D-Lys] and GnRH-III), previously proven to be effective in Dau-delivery, were selected to compare the apoptotic activity of their conjugates. Two sets of conjugates were synthesized by attaching Dau directly to the ^{6}D-Lys or ^{8}Lys (depending on the GnRH analog) *via* oxime linkage; one group with Ser in position 4 and a second group, where this Ser was replaced with butyrated Lys (Table 1).

First, the cytotoxic/antiproliferative effect of the conjugates with different native GnRH isoforms and the effect of the substitution of butyrated Lys in position 4 were studied by using a more sophisticated, impedance-based method (xCELLigence SP System). In case of the conjugates built on native GnRH conjugates, the II-[^{4}Ser,^{6}D-Lys(Dau)] proved to be the most potent one followed by the I-[^{4}Ser,^{6}D-Lys(Dau)] and III-[^{4}Ser,^{8}Lys(Dau)] (the applied codes can be seen in Table 1). The substitution with ^{4}Lys(Bu) proved to modify the cytotoxic effect of the conjugates depending on the type of GnRH analog. In the case of the GnRH-III based conjugates, this modification led to a more than one order of magnitude smaller IC_{50} value (Table 1) and a stronger antitumor activity with an earlier onset. In the case of GnRH-I conjugates, the replacement of ^{4}Ser by ^{4}Lys(Bu) could cause only a slight increase in the potency (smaller IC_{50} value) after 72 h, but the onset of the cytotoxic activity took less time. On the contrary, IC_{50} values of GnRH-II conjugate with ^{4}Lys(Bu) (II-[^{4}Lys(Bu),^{6}D-Lys(Dau)]) were more than two times higher than that of II-[^{4}Ser,^{6}D-Lys(Dau)] (Table 1).

In general, the conjugates had minor or no apoptotic effect. The apoptotic cell death induced by 24 h incubation with GnRH conjugates was measured by detecting the binding of FITC-conjugated Annexin V. In the case of conjugates with Ser^{4} only the GnRH-II conjugate

could elicit a slight, but significant apoptotic effect and the incorporation of ^{4}Lys(Bu) diminished this activity (Table 1). Among the tested conjugates, III-[^{4}Lys(Bu),^{8}Lys(Dau)] had the maximal apoptotic effect (Table 1).

Table 1. IC_{50} values and apoptotic effects of Dau-GnRH-[^{4}Ser/^{4}Lys(Bu)] conjugates determined on HT-29 cell line

Conjugate	Code	IC_{50} [1] (μM)	Ratio of apoptotic cells[2] [%] (cont.: 8.91 ± 0.58)
		72 h	24 h
GnRH-I-[^{4}Ser,^{6}D-Lys(Dau=Aoa)]	I-[^{4}Ser,^{6}D-Lys(Dau)]	21.94 ± 1.54	12.46 ± 1.1
GnRH-II-[^{4}Ser,^{6}D-Lys(Dau=Aoa)]	II-[^{4}Ser,^{6}D-Lys(Dau)]	19.73 ± 2.51	15.05* ± 0.94
GnRH-III-[^{4}Ser,^{8}Lys(Dau=Aoa)]	III-[^{4}Ser ,^{8}Lys(Dau)]	56.82 ± 5.44	9.21 ± 0.76
GnRH-I-[^{4}Lys(Bu),^{6}D-Lys(Dau=Aoa)]	I-[^{4}Lys(Bu),^{6}D-Lys(Dau)]	16.18 ± 1.75	12.08 ± 1.36
GnRH-II-[^{4}Lys(Bu),^{6}D-Lys(Dau=Aoa)]	II-[^{4}Lys(Bu),^{6}D-Lys(Dau)]	48.08 ± 6.89	9.52 ± 1.06
GnRH-III-[^{4}Lys(Bu),^{8}Lys(Dau=Aoa)]	III-[^{4}Lys(Bu),^{8}Lys(Dau)]	4.56 ± 0.27	17.00** ± 1.25

[1] IC_{50} values represent the mean ± SD of three parallel measurements and were calculated by fitting a sigmoidal dose-response curve with OriginPro 2016 software.
[2] For the treatment, the conjugates were applied at 10^{-4} M concentration for 24 h. Only the viable cells were taken into consideration to determine the ratio of apoptotic cells (percentage of Annexin V positive cells). Data shown are mean of two parallels ± SD. The significance levels are the followings: *: $p < 0.05$, **: $p < 0.01$.

To investigate the molecular background of the HT-29 cell death induced by GnRH conjugates, a human apoptosis gene PCR array (RealTime ready Custom panel, Roche Applied Science, Mannheim, Germany) containing 23 apoptosis-related genes was used. GnRH-I and GnRH-III conjugates with ^{4}Ser increased the expression of *TP53* after 24 h incubation (Figure 1/A and C). All of the conjugates containing ^{4}Ser could increase the expression of genes involved in the intrinsic pro-apoptotic pathway, but with a different activity. The ^{4}Ser conjugates caused the most remarkable increase in case of the *TNF* expression, especially after 24 h (I-[^{4}Ser,^{6}D-Lys(Dau)]: 40.57 fold, II-[^{4}Ser,^{6}D-Lys(Dau)]: 18.33 fold, III-[^{4}Ser,^{8}Lys(Dau)]: 50.45 fold) (Figure 1). Only the I-[^{4}Ser,^{6}D-Lys(Dau)] could influence (increase) the expression of *CASP9, 7* and *3*. All of the ^{4}Ser conjugates upregulated the expression of *FASL* and *STAT1* with comparable activity. The expression of other tested genes involved in the growth factor signaling pathway was reduced while the expression of

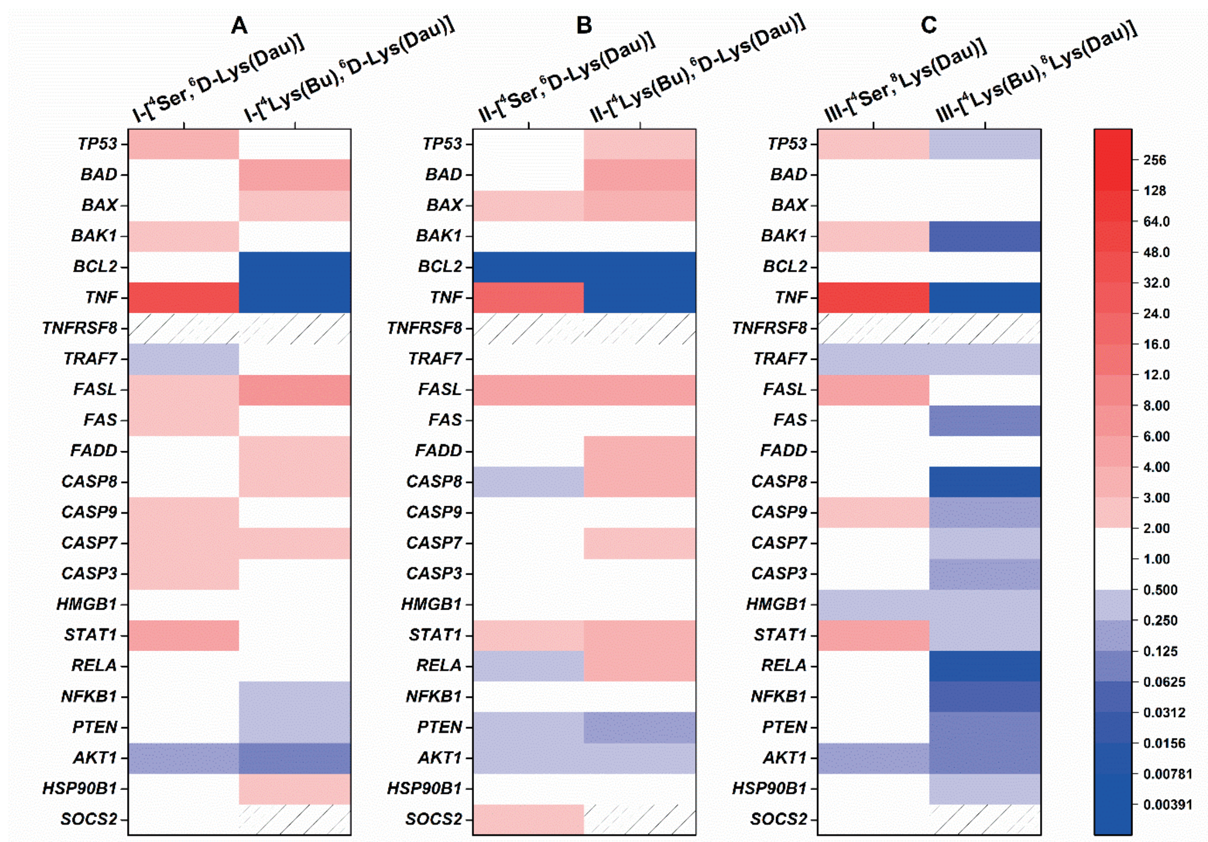

Figure 1. Comparison of the expression of human apoptosis-related genes in HT-29 cells treated with GnRH-[^{4}Ser] and GnRH-[^{4}Lys(Bu)] conjugates for 24 h. Effects of the conjugates pairs of GnRH-I (A), GnRH-II (B), GnRH-III (C) on gene expression were analyzed by a human apoptosis gene PCR array (RealTime ready Custom panel).

Colors of heatmap showed significant fold changes in gene expression compared to control. Fold changes ≥ 2 and $p < 0.05$ were considered as significant. Hashed zone means invalid PCR results.
TP53: tumor protein p53 data; *BAD*: BCL2-associated agonist of cell death; *BAX*: BCL2-associated X protein; *BAK1*: BCL2-antagonist/killer 1; *BCL2*: B-cell CLL/lymphoma 2; *TNF*: TNF-alpha, Tumor necrosis factor ligand superfamily member 2; *TNFRSF8*: Tumor necrosis factor receptor superfamily, member 8; *TRAF7*: TNF receptor-associated factor 7; *FASL*: Fas ligand, TNF superfamily member 6 (*TNFSF6*); *FAS*: TNF receptor superfamily member 6 (*TNFRSF6*); *FADD*: Fas (*TNFRSF6*)-associated via death domain; *CASP7*: caspase 7; *CASP3*: caspase 3; *CASP9*: caspase 9; *CASP8*: caspase 8; *HMGB1*: high-mobility group box 1; *NFKB1*: nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; *RELA*: v-rel reticuloendotheliosis viral oncogene homolog A; *AKT1*: v-akt murine thymoma viral oncogene homolog 1; *PTEN*: phosphatase and tensin homolog; *STAT1*: signal transducer and activator of transcription 1; *SOCS2*: suppressor of cytokine signaling 2; *HSP90B1*: heat shock protein 90kDa beta (Grp94) member 1.

anti-apoptotic genes was elevated by II-[^{4}Ser,^{6}D-Lys(Dau)]. The replacement of ^{4}Ser by ^{4}Lys(Bu) led to significant changes in the expression of apoptosis-related genes as shown in Figure 1. When the cells were treated with ^{4}Lys(Bu) containing GnRH-I or GnRH-III, the expression of the *TP53* was decreased to the control level or below in comparison with ^{4}Ser derivatives (Figure 1/A and C). Similar to ^{4}Ser conjugates, all of the conjugates with ^{4}Lys(Bu) increased the level of *BAD* and *BAX* as well as abolished *BCL2* expression. The most

remarkable difference between the ^{4}Ser and ^{4}Lys(Bu) conjugates were detected in the expression of *TNF*. Due to the 24 h treatment with the ^{4}Lys(Bu) conjugates the expression of *TNF* was not detectable. The cells treated with ^{4}Lys(Bu) derivatives could also overexpress the *FASL* (Figure 1). Gene expression of the effector proteins and members of the growth factor signaling pathway were greatly reduced by III-[^{4}Lys(Bu),^{8}Lys(Dau)] (Figure 1/C). Contradiction with the ^{4}Ser counterparts, GnRH-I and GnRH-II conjugates with ^{4}Lys(Bu) caused an increase in the expression of *CASP8* and *CASP7* after 24 h incubation (Figure 1/A and C). I-[^{4}Lys(Bu),^{6}D-Lys(Dau)] and III-[^{4}Lys(Bu),^{8}Lys(Dau)] downregulated *HMGB1* expression by *ca.* 3-fold change (Figure 1/A and C). The expression of *STAT1* was changed in parallel with the *TP53*. Except for *RELA* and *NFKB1* in the II-[^{4}Lys(Bu),^{6}D-Lys(Dau)] treated group, all of the genes involved in the growth factor signaling pathway were reduced by the ^{4}Lys(Bu) conjugates (Figure 1).

In summary, we demonstrated that modification with Lys(Bu) in position 4 increased the cytotoxic and apoptotic effect of GnRH-I and GnRH-III conjugates containing an oxime-linked Dau. Although the conjugates had a minor apoptotic effect, they could regulate the expression of several apoptosis-related factors, and this activity proved to be sensitive to the GnRH isoforms and the presence of the ^{4}Lys(Bu), especially in case of *TNF*, *TP53* and members of the growth factor signaling pathway. Nevertheless, it is worth mentioning that independently of this modification, all of the investigated conjugates could increase the expression of *FASL* and the mitochondrial pro-apoptotic factors (e.g. *BAD* and *BAX*), which indicates the general importance of the FAS-dependent pathway and the mitochondrial apoptotic pathway in the antitumor effect of different GnRH conjugates. By detecting the expression of 23 apoptosis-related genes we could find further evidence that the GnRH-I and GnRH-III conjugates acted in a more or less similar way. Our comprehensive PCR results could show that the stronger cytotoxic activity of I-[^{4}Lys(Bu),^{6}D-Lys(Dau)], III-[^{4}Lys(Bu),^{8}Lys(Dau)] and II-[^{4}Ser,^{6}D-Lys(Dau)] was associated with a stronger and a more immediate inhibitory effect on the expression of elements of growth factor signaling comparing to their counterparts, where the upregulation of the expression *TP53*, *TNF* and caspases (*e.g. CASP9*) probably had a more important role. Our results also suggest the significance of ^{4}Lys(Bu) in the anti-tumor activity of GnRH-I and GnRH-III conjugates, while in case of GnRH-II conjugates, the native Ser in position 4 appeared to be more important.

References

1. Engel JB, Tinneberg HR, Rick FG, Berkes E, Schally AV, *Curr Drug Targets* **17:** 488-494 (2016)
2. Szabó I, Manea M, Orbán E, Csámpai A, Bősze S, Szabó R, Tejeda M, Gaál D, Kapuvári B, Przybylski M, Hudecz F, Mező G, *Bioconjug Chem* **20:** 656-665 (2009)
3. Cheng CK, Leung PCK, *Endocr Rev* **26:** 283-306 (2005)
4. Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR, *Endocr Rev* **25:** 235-275 (2004)
5. Manea M, Mező G, *Protein Pept Lett* **20:** 439-449 (2013)
6. Mező G, Manea M, *Expert Opin Drug Deliv* **7:** 79-96 (2010)
7. Manea M, Leurs U, Orbán E, Baranyai Z, Ohlschlager P, Marquardt A, Schulcz A, Tejeda M, Kapuvári B, Tóvári J, Mező G, *Bioconjug Chem* **22:** 1320-1329 (2011)
8. Sealfon SC, Weinstein H, Millar RP, *Endocr Rev* **18:** 180-205 (1997)
9. Szabó I, Bősze S, Orbán E, Sipos E, Halmos G, Kovács M, Mező G, *J Pept Sci* **21:** 426-435 (2015)
10. Hegedüs R, Manea M, Orbán E, Szabó I, Kiss E, Sipos E, Halmos G, Mező G, *Eur J Med Chem* **56:** 155-165 (2012)
11. Moretti RM, Montagnani Marelli M, Taylor DM, Martini PG, Marzagalli M, Limonta P, *PLoS One* **9:** e93713 (2014)
12. Zhang N, Qiu J, Zheng T, Zhang X, Hua K, Zhang Y, *Oncol Rep* **39:** 1034-1042 (2018)
13. Gunthert AR, Grundker C, Bongertz T, Schlott T, Nagy A, Schally AV, Emons G, *Am J Obstet Gynecol* **191:** 1164-1172 (2004)
14. Lajkó E, Spring S, Hegedüs R, Biri-Kovács B, Ingebrandt S, Mező G, Kőhidai L, *Beilstein J Org Chem* **14:** 2495-2509 (2018)