Video Article

In Vitro Imaging and Quantification of the Drug Targeting Efficiency of Fluorescently Labeled GnRH Analogues

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Abstract

GnRH analogues are effective targeting moieties and able to deliver anticancer agents selectively into malignant tumor cells which highly express GnRH receptors. However, the quantitative analysis of GnRH analogues' cellular uptake and the investigated cell types in GnRH-based drug delivery systems are currently limited. Previously introduced, selectively labeled fluorescent GnRH I, -II and -III derivatives provide great detectability, and they have suitable chemical properties for reproducible and robust experiments. We also found that the appropriate up-to-date methods with these labeled GnRH analogues could offer novel information about the GnRH-based drug delivery systems. This manuscript introduces some simple and fast experiments regarding the cellular uptake of [D-Lys^6(FITC)]-GnRH-I, [D-Lys^6(FITC)]-GnRH-II and [Lys^8(FITC)]-GnRH-III on the EBC-1 (lung), the BxPC-3 (pancreas) and on the Detroit-562- (pharynx) malignant tumor cells. In parallel with these GnRH-FITC conjugates, the cell surface level of GnRH-I receptors was also examined on these cell lines before and after the GnRH treatment by confocal laser scanning microscopy. The cellular uptake of GnRH-FITC conjugates was quantified by fluorescence-activated cell sorting. In these experiments minor differences among GnRH analogues and major differences among cell types was observed. The significant differences among cell lines are correlated with their distinct level of cell surface GnRH-I receptors. The introduced experiments contain practical methods to visualize, quantify and compare the uptake efficiency of GnRH-FITC conjugates in a time- and concentration-dependent manner on various adherent cell cultures. These results could predict the drug targeting efficiency of GnRH conjugates on the given cell culture, and offer a good basis for further experiments in the examination of GnRH-based drug delivery systems.

Video Link

The video component of this article can be found at https://www.jove.com/video/55529/

Introduction

Peptide based targeted drug delivery systems have become a fast developing and promising area in cancer therapy over the past few years[1,2]. Human gonadotropin releasing hormone receptor type I (GnRH-I-R) is primarily located in the pituitary gland but is also present in several other tissues which are responsible for self-reproduction[3]. GnRH-I-R is also expressed in a number of cancer tissues, related or unrelated to the reproductive system[4,5]. The high expression of GnRH-I-R on several malignant tumor cells compared with healthy tissues provides an opportunity for targeted therapy[5,6].

Many gonadotropin-releasing hormone (GnRH) analogues have been developed in the last few decades, which could be used as targeting moieties[7,8,9]. These peptides are able to deliver anticancer agents with high selectivity into malignant tumor cells which over-express GnRH-I-R[6]. Several GnRH conjugated anti-tumor drugs with higher selectivity and better efficiency than the corresponding unconjugated free drug have been reported[7,8,9].

Previous publications about GnRH peptides and their receptors reported that the GnRH-I-R can assume various conformations which have different selectivity for GnRH analogues[10]. The highly variable GnRH-I-R has complex and various signaling pathways are endowed with different activity against their natural and artificial ligands[11]. These facts make investigation of GnRH-based systems challenging. On the other hand, they possess promising therapeutic potential. Several experiments with radiolabeled GnRH peptides were previously reported[12,13,14,15], but experiments in which fluorescently labeled GnRH analogues were used are still limited. While radioactive labeling offers high sensitivity, fluorescent labeling has several other advantages, for example the easier handling, and the ability to counterstain with different fluorophores. Three common GnRH analogues which have successfully been used for drug delivery are the [D-Lys^6]-GnRH-I, [D-Lys^6]-GnRH-II and GnRH-III, but the effectiveness of these peptides as targeting moieties is rarely compared[16,17]. On the other hand, results from separate experiments in which different cancer cells and GnRH analogues were used is diverse.

Based on these considerations, we focused on the tumor targeting and drug delivery potential of these GnRH peptides, and thereby synthesized and characterized the [D-Lys^6(FITC)]-GnRH-I, [D-Lys^6(FITC)]-GnRH-II and [Lys^8(FITC)]-GnRH-III peptide conjugates[18]. These analogues are

selectively labeled with FITC on the side chain of their Lys or D-Lys (peptide-FITC ratio 1:1 at each conjugate). The idea was that the selective fluorescent labeling can offer novel information about these peptides, and allows their good tracking and reliable quantification. These conjugates have safe handling and reliable detectability, which make it easier to compare their tumor targeting efficiency, and the screening of numerous types of malignant tumor cells. We hope that up-to date experiments with these peptide conjugates could contribute to the development of novel cancer targeting GnRH-drug conjugates, and help to identify new therapeutic targets as well.

The present manuscript demonstrates some well reproducible and fast experiments with GnRH-FITC conjugates. The cell surface expression of GnRH-R is a determinative condition regarding GnRH uptake, therefore we simultaneously investigated the cell surface level of GnRH-I-R on the tested cell lines. We visualized the GnRH-I-R and GnRH-FITC conjugates by confocal laser scanning microscopy (CLSM) and quantified the cellular uptake of GnRH-FITC conjugates using fluorescence-activated cell sorting (FACS).

Protocol

1. Preparation of Cell Cultures and Reagents

1. Maintain the cell cultures in the manufacturer's recommended medium, supplemented with 10% (v/v) fetal bovine serum and antibiotics (called complete medium). Keep the cell culturing flask in a humidified, 5% CO_2 atmosphere incubator at 37 °C. Follow the proliferation and confluency of cells by inverted microscope (using 10X phase contrast objective).
2. When cells reach adequate confluency, remove the medium, and wash the culture with 2-3 mL, sterile phosphate-buffered saline (PBS). Remove the PBS and add 0.5 mL, sterile 0.25% trypsin-EDTA solution to the cell culture and incubate at 37 °C until cells detach (approximately 10 min).
3. Suspend the cells in 3-4 mL sterile complete medium to stop trypsin and transfer them into a sterile centrifuge tube. Centrifuge the cells at 150 x g for 4 min at room temperature (RT). Discard the supernatant carefully and suspend the pellet in 2-3 mL sterile complete medium.
4. Take out 100 µL of the cell suspension and mix with 100 µL 0.4% (m/V) trypan blue solution, to stain the dead cells. Load 10 µL of this mixture into a hemocytometer and determine the number of viable cells by inverted microscope.
5. Dilute the required amount of cell suspension prepared in step 1.3 to 10 mL with sterile complete medium, containing 4 x 10^4 cell/mL.
6. Add 250 µL of cell suspension (prepared in step 1.5) per well (10^4 cell/well) into seven wells of the first glass bottom 8-well microscopic slide. Use this slide in the GnRH uptake method.
7. Add 250 µL of cell suspension (prepared in step 1.5) per well (10^4 cell/well) into five wells of the second 8-well microscopic slide. This slide will be used in the GnRH-I-R expression method.
8. Add 1 mL of cell suspension (prepared in step 1.5) per well (4 x 10^4 cell/well) into seven wells of a 12-well plate. This plate will be used in the GnRH quantification method.
9. Let the cells adhere on the two microscopic slides and the plate. Incubate them in a humidified, 5% CO_2 atmosphere incubator at 37 °C for 48 h.
10. After the incubation check the cells by inverted microscope (using 10X phase contrast objective). If the cells are healthy and attached, continue with the following steps.
11. Prepare 10 mM GnRH-FITC stock solution in dimethyl sulfoxide (DMSO) from each of the three conjugates[18]. (Use these dilution concentrations: 16.43 µg/µL [D-Lys^6(FITC)]-GnRH-I, 16.97 µg/µL [D-Lys^6(FITC)]-GnRH-II and 16.47 µg/µL [Lys^8(FITC)]-GnRH-III) Keep these stock solutions in a dark place at RT and use them up within a few weeks.
12. Dilute 1.7 µL each of the three 10 mM GnRH-FITC stock solutions in 1.7 mL complete medium. Shake these solutions. (These are the 10 µM GnRH-FITC treating media.) Dilute 150 µL each of the three 10 µM GnRH-FITC treating medium in 1.35 mL complete medium. Shake these solutions as well (these are the 1 µM GnRH-FITC treating medium).
    NOTE: All GnRH-FITC containing treating medium should be protected from light and used up within a few h.
13. Preheat the six GnRH-FITC treating medium (prepared in step 1.12) and 4 mL complete medium to 37 °C.
14. Dilute 3 µL of 5 mM fluorescent probe solution (nuclear counterstain mentioned in the Materials List) in 3 mL PBS to 5 µM. This solution should be protected from light, keep it at RT and use it up within a few hours.

2. Confocal Laser Scanning Microscopy (CLSM)

1. **GnRH uptake method**
    1. Take the first microscopic slide prepared in step 1.6 and pipette out the complete medium from each of the seven well. Add 250 µL of preheated complete medium into the first well. Use this as a negative control. Add 250 µL of the preheated GnRH-FITC treating media (from 1.13) to each of the next six wells (3 wells with 1 µM and 3 with 10 µM). Incubate the slide in a humidified, 5% CO_2 atmosphere incubator at 37 °C for 5 h.
    2. Pipette out the medium from each well and wash the cells with 250 µL PBS. Add 250 µL of fixing solution (10% neutral buffered formalin) into each well and incubate the slide at RT, for 10 min.
    3. Pipette out the fixing solution from each well and wash the cells with 250 µL PBS. Add 250 µL of PBS containing 5 µM fluorescent probe solution prepared in step 1.14 into each well and incubate the slide at RT, for 10 min.
    4. Pipette out the fluorescent probe solution from each well, and wash the cells twice with 250 µL PBS carefully. Add 3-4 drops of mounting medium into each well, finally. Keep the slide in dark at RT, until the imaging.

2. **GnRH-I-R expression method**
    1. Take the second 8-well microscopic slide prepared in step 1.7 and pipette out the complete medium from each of the five wells.
    2. Add 250 µL of preheated complete medium into the first well, use this as negative control. Add 250 µL of preheated complete medium into the second well too, and use this to examine GnRH-I-R expression before the GnRH treatment.
    3. Add 250 µL each of the three preheated 10 µM GnRH-FITC treating media into the next three wells. Use these wells to pretreat the cells with GnRH-FITC conjugates before examining the GnRH-I-R expression. Incubate the slide in a humidified, 5% CO_2 atmosphere incubator at 37 °C for 1 h.

4. Pipette out the treating medium from each of the three wells which will be used to examine GnRH-I-R expression after the GnRH treatment and wash these wells with 250 µL of preheated complete medium. Add 250 µL of preheated complete medium into each of the three wells. Incubate the slide in a humidified, 5% CO_2 atmosphere incubator at 37 °C for 1 h.
5. Pipette out the medium from each of the five wells and wash the cells with 250 µL PBS. Add 250 µL of fixing solution into each well and incubate the slide at RT, for 10 min.
6. Pipette out the fixing solution from each well and wash the cells with 250 µL of PBS. Add 250 µL of PBS containing 5% bovine serum albumin (BSA) blocking solution into each well and incubate the slide at RT, for 1 h.
7. Dilute 10 µL of GnRH-I-R primary antibody in 1 mL PBS (1:100 ratio). Keep it at RT and use it up within a few hours.
8. Pipette out the BSA blocking solution from each well except the negative control (first well). Wash the cells with 250 µL PBS (except the negative control well). Add 250 µL of PBS containing GnRH-I-R primary antibody prepared in step 2.2.7 into each well (except the negative control). Incubate the slide at RT, for 1 h, in a dark place.
9. Dilute 2.5 µL of Alexa 546 labeled secondary antibody in 1.25 mL PBS (1:500 ratio).
   NOTE: This solution should be protected from light, keep it at RT and use it up within a few hours.
10. Pipette out solutions from each of the five wells and wash the cells with 250 µL PBS. Add 250 µL of PBS containing AF 546 labeled secondary antibody prepared in step 2.2.9 into each well. Incubate the slide at RT, for 1 h in dark.
11. Pipette out the solution from each well and wash the cells with 250 µL PBS. Add 250 µL of PBS containing 5 µM fluorescent probe solution prepared in step 1.14 into each well and incubate the slide for 10 min, at RT.
12. Pipette out the fluorescent probe solution from each well and wash the cells twice with 250 µL PBS carefully. Add 3-4 drops of mounting media into each well, afterwards. Keep the slide in dark at RT until the microscopic imaging.

3. **Imaging**
   1. Image the cells by inverted confocal laser scanning microscope (using 63X oil immersion objective)
      1. Use the following excitation/emission wavelengths. GnRH conjugates: 488/514 nm, Alexa 546 labeled antibody: 488/546 nm (use only for the GnRH-I-R expression method), Draq5 fluorescent probe: 633/680 nm.
      2. Set up the imaging parameters using the negative control cells. Use these parameters to image treated cells (**Figure 3**). Re-adjust imaging parameters on each microscopic slide at the beginning of the analysis. Fine-tune and export images with the software provided by the manufacturer if necessary.

3. Fluorescence-activated Cell Sorting (FACS)

1. **GnRH quantification method**
   1. Take the plate prepared in step 1.8 and pipette out the complete medium from each of the seven wells. Add 1 mL of preheated complete medium into the first well. Use this as a negative control. Add 1 mL each of the six preheated treating media (3 wells with 1 µM and 3 wells with 10 µM) into the next six wells. Incubate the plate in a humidified, 5% CO_2 atmosphere incubator at 37 °C for 5 h.
   2. Pipette out the medium from each well. Wash the cells twice with 2 mL PBS carefully. Add 500 µL of trypsin-EDTA solution into each wells. Incubate the plate at 37 °C until cells detach (approximately 10 min).
   3. Add 1 mL of complete medium into each well to stop trypsin. Shake the plate. Suspend the cells gently with a pipette, and transfer the suspension from each well into FACS tubes. Centrifuge the tubes at 150 x g for 4 min, at 4 °C.
   4. Pour out the supernatants carefully, with one move. Add 500 µL of ice-cold PBS into each FACS tube and gently re-suspend the cells. Keep the tubes on ice until the end of the experiment.

2. **Analysis and calculation**
   1. Analyze the cells by flow cytometry. For excitation, use 488 nm argon laser wavelength and for detection use 530 nm wavelength. Set up the flow cytometer parameters by running the negative control cells. Use these parameters to analyze treated cells (**Figure 4A**). Evaluate the data with the manufacturer's software, determine median fluorescent intensity (MFI) values, and calculate the relative MFI values.

Representative Results

Images obtained by confocal laser scanning microscopy (CLSM) offer spectacular information about the uptake of GnRH-FITC conjugates on the given cell culture in a time and concentration dependent manner. In parallel with these GnRH-FITC conjugates, the presence of the GnRH-I-R on the cell surface is also verifiable by the CLSM experiment. Furthermore, by using a far-red DNA staining fluorescent probe, it is possible to counterstain the nuclei of cells besides GnRH and GnRH-I-R. However, while the confocal image is not quantifiable, the simple "GnRH uptake method" can easily estimate whether the tested cells contain higher or lower amount of GnRH-FITC conjugates. The applied GnRH-FITC conjugates, their concentrations, and the time of the treatment are variable in this method, as needed. As shown in **Figure 1** different cancer cells contain different levels of GnRH-FITC in a concentration dependent manner.

The advanced "GnRH-I-R expression method" describes the visualization of the cell surface GnRH-I-R by immunocytochemistry, before and after the GnRH-FITC treatment. In the first part of this method (before the GnRH-FITC treatment), we visualize GnRH-I-R by immunocytochemistry, without GnRH-FITC treatment. In the second part of this method (after the GnRH-FITC treatment), we treat cells for 1 h with 10 µM GnRH-FITC. After the treatment, we incubate the cells in complete medium for further 1 h and visualize GnRH-I-R by immunocytochemistry. As shown in **Figure 2A**, different cancer cells exhibit different levels of GnRH-I-R on their surface before the GnRH-FITC treatment. As shown in **Figure 2B**, after the GnRH-FITC treatment, the GnRH-I-R expressing cells maintain (EBC-1) or increase (Detroit-562) GnRH-I-R level on their membranes. At this point, note that we previously confirmed that BxPC-3 cells also express GnRH-I-R, but do not present it on their membrane[18]. GnRH-I-R can be visualized inside the BxPC-3 cells by immunocytochemistry if their membranes are permeabilized. This phenomenon explains the relatively lower GnRH uptake efficiency of BxPC-3 cells. Based on these statement we focus only on cell surface GnRH-I-R here. Comparing **Figure 1** to **Figure 2** confirms that the level of cell surface GnRH-I-R correlates with the amount of GnRH-FITC in the corresponding cells. Furthermore, **Figure 3C** clarifies that GnRH-FITC is internalized into cells because the localization of GnRH-FITC is separated from the signal of cell surface GnRH-I-Rafter 1 h of the GnRH-FITC treatment. We conclude that "GnRH-I-R expression method" supports the information obtained from the "GnRH uptake method".

It is important to use well-adjusted settings during the imaging. As illustrated in **Figure 3A** at the emission wavelength of FITC (514 nm) and the fluorophore labeled secondary antibody (546 nm), the untreated negative control cells also have a slight autofluorescence. This undesired fluorescence can provide false positive results. Thereby, at the beginning of the imaging it is important to adjust the fluorescence intensity on the control cells as shown in **Figure 3B**. It is expected that one will observe clear signal of the nuclei at the emission wavelength of the nuclear stain (680 nm), with the fluorescent signal at the other two wavelengths is just visible or near zero. All the other cell samples should be imaged with these well-adjusted and fixed parameters. This way, the signal observed at 514 nm is derived from the signal of FITC-GnRH only, at 546 nm from the signal of GnRH-I-R particularly and at 680 nm from the signal of nuclei as shown in **Figure 3C**. Images could be fine-tuned after the imaging using software if necessary.

Flow cytometry experiments offer quantifiable information about the amount of GnRH-FITC conjugates which were taken up by the cells. The applied GnRH-FITC conjugates, their concentrations and the time of the treatment are also variable in this method, as needed. The untreated cells are used as negative control for adjusting the flow cytometry parameters and to determine their median fluorescent intensity (MFI) at the beginning of the analysis. As illustrated on **Figure 4A** the MFI was determined for each sample using the same parameters. The relative MFI values are calculated from the MFI values using the calculation formula in **Figure 4B**. Using this formula, the relative MFI value of control cells is always zero. The calculated relative MFI values quantify the fluorescent intensity of GnRH-FITC conjugates, which are proportional to their average concentrations in the cells. As illustrated in **Figure 5**, with these data, one can demonstrate and compare how efficiently these cancer cells take up the GnRH-FITC conjugates. Thus, results obtained by flow cytometry complement and support the images acquired by confocal microscopy.

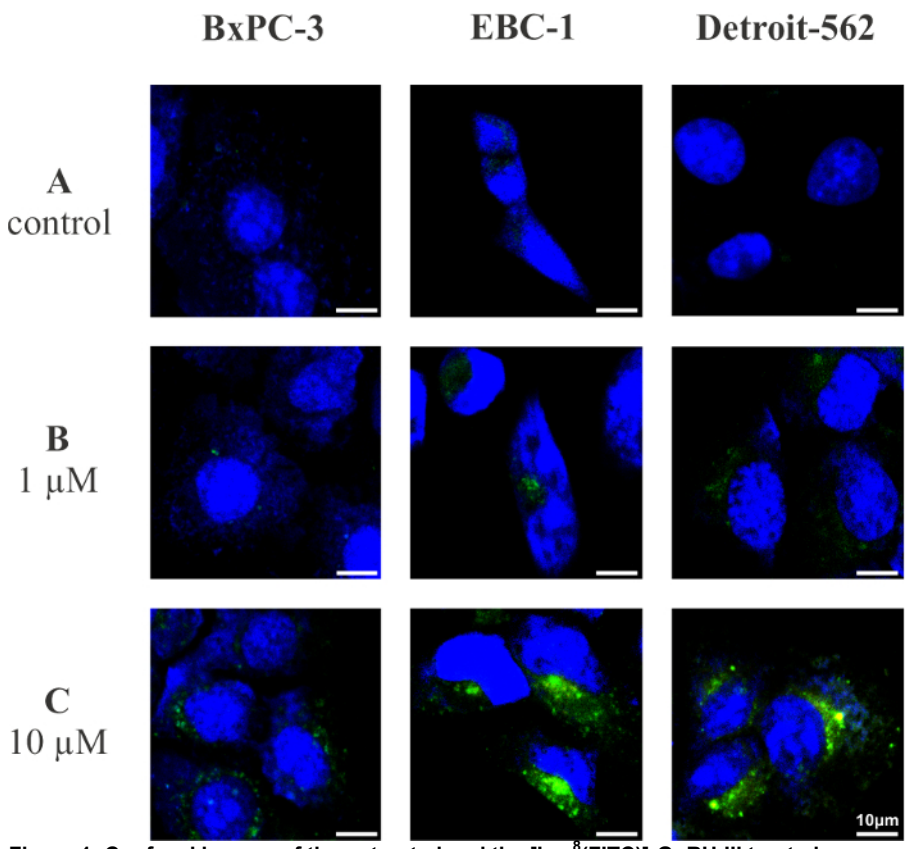


Figure 1: Confocal images of the untreated and the [Lys^8(FITC)]-GnRH-III treated cancer cells. Blue stain: nuclei; green stain: [Lys^8(FITC)]-GnRH-III. These images are obtained using the "GnRH uptake method". (**A**) The fluorescence signals of the untreated cells are adjusted to near zero at 514 nm (green) on each cell line. The Draq5 far-red fluorescent probe is used to stain the nuclei of cells. (**B**) After applying the settings, the EBC-1 lung cancer and the Detroit-562 human pharynx cancer cells show detectable, but low signal at 514 nm (green) after 5 h treatment with 1 μM [Lys^8(FITC)]-GnRH-III. (**C**) After 5 h treatment with 10 μM [Lys^8(FITC)]-GnRH-III, each of the three cell lines present higher fluorescent signal. Results from this experiment suggest that the BxPC-3 pancreatic cancer cells took up GnRH-FITC with much lower efficiency as compared to the other two cell lines that are easily targetable by GnRH conjugates. Please click here to view a larger version of this figure.

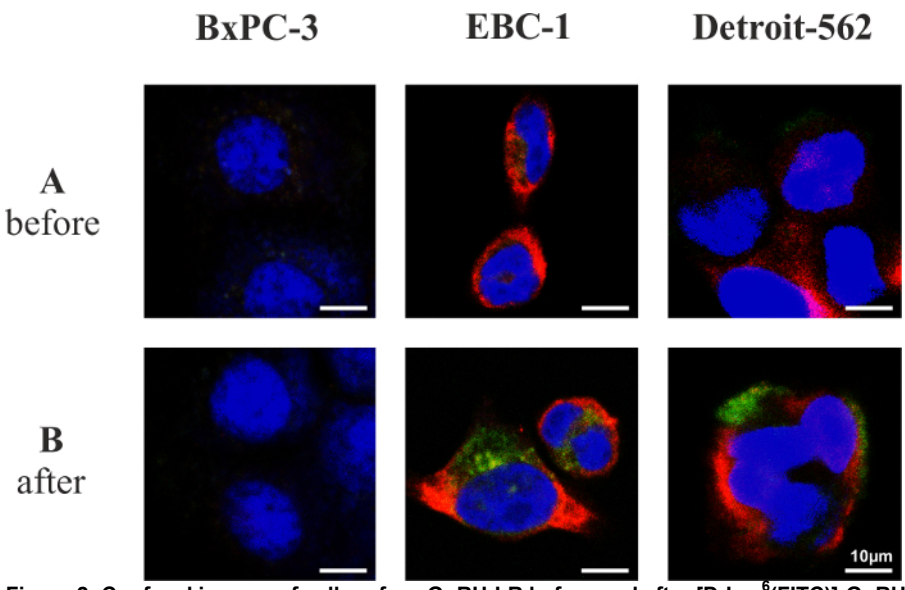


Figure 2: Confocal images of cell surface GnRH-I-R before and after [D-Lys^6(FITC)]-GnRH-I treatment. Blue stain: nuclei; green stain: [D-Lys^6(FITC)]-GnRH-I; red stain: GnRH-I-R. These images are generated using the "GnRH-I-R expression method". (**A**) Before the GnRH-FITC treatment, BxPC-3 cells do not have GnRH-I-R on the membrane, however EBC-1 cells exhibit high, and certain Detroit-562 cells exhibit moderate level of GnRH-I-R. (**B**) After the GnRH-FITC treatment, BxPC-3 cells still do not exhibit GnRH-I-R on their membrane. EBC-1 cells maintain their GnRH-I-R expression and GnRH-FITC appears inside of them. Detroit-562 cells seem to be exhibiting higher level of GnRH-I-R after treatment and they take up GnRH-FITC, as well. This experiment reveals that different types of malignant tumor cells exhibit different level of GnRH-I-R on their surface, and the GnRH-FITC uptake seems to be closely related to the cell surface GnRH-I-R expression. Please click here to view a larger version of this figure.

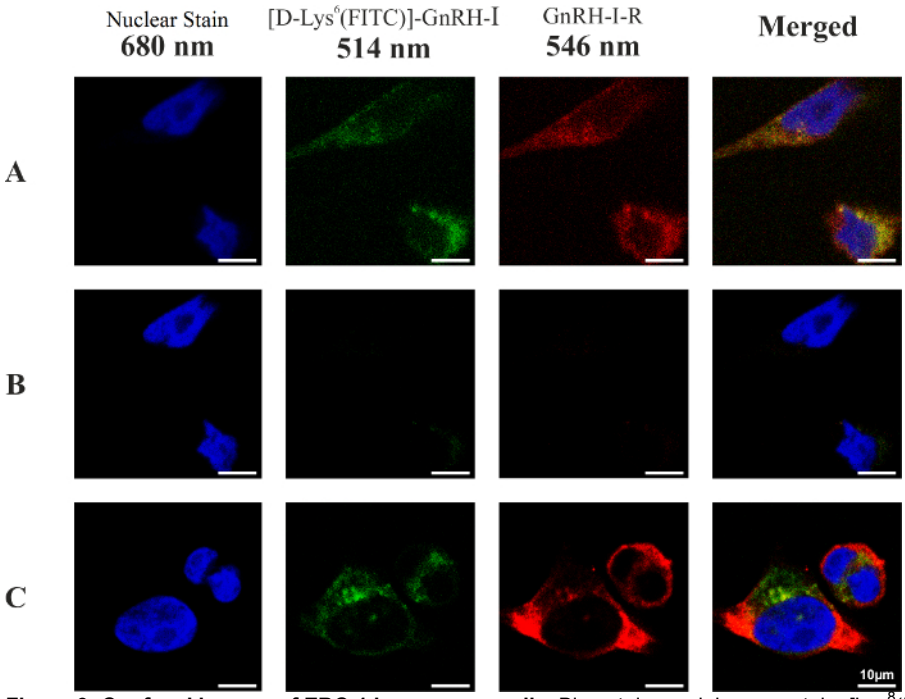
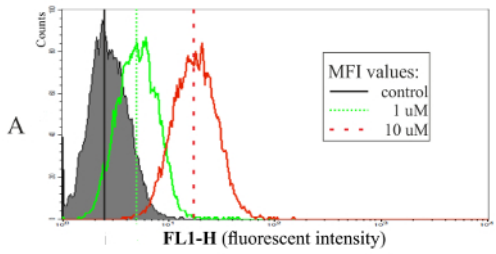


Figure 3: Confocal images of EBC-1 lung cancer cells. Blue stain: nuclei; green stain: [Lys^8(FITC)]-GnRH-III; red stain: GnRH-I-R. These images are made by the "GnRH-I-R expression method". (**A**) Untreated negative control cells (without GnRH-FITC and Alexa 546) have detectable and undesired fluorescence at 514 nm and 546 nm when using over-amplified instrument settings. (**B**) Adjusting the instrument settings on the untreated negative control cells, the green (514nm) and red (546 nm) signal is near zero. (**C**) Adjusted parameters result in clear and reliable signals for the GnRH-FITC treated and GnRH-I-R stained cells. After the GnRH-FITC treatment, localization of GnRH-FITC (green) is separated from the cell surface GnRH-I-R (red). Please click here to view a larger version of this figure.



B $$rel.MFI = \frac{MFI\ (treated; [x(h);\ y(\mu M)])}{MFI\ (control)} - 1$$¶

Figure 4: Evaluation of flow cytometry data. (**A**) Histogram of the untreated and the treated Detroit-562 cells. MFI values of the cell samples are determined from the histogram. The x-axis depicts the fluorescent intensity of cells, and the y-axis depicts the counts. Black line: MFI before the GnRH-FITC treatment; green dotted line: MFI after the 5 h treatment with 1 µM [Lys^{8}(FITC)]-GnRH-III; red dashed line: MFI after the 5 h treatment with 10 µM [Lys^{8}(FITC)]-GnRH-III. (**B**) Calculation formula of relative MFI values. The relative MFI values are calculated from the MFI values. Using this calculation, the relative MFI value of the untreated control sample is always zero. The relative MFI values of the treated samples represent the amount of GnRH-FITC conjugates which are inside the cells. Please click here to view a larger version of this figure.

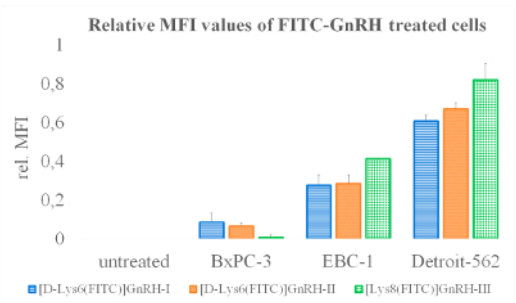


Figure 5: Relative MFI values of cancer cells after 5 h treatment with 1 µM of GnRH-FITC conjugates. Relative MFI values are comparable and define how efficiently the given cell types could take up GnRH analogues. The GnRH-I-R non-exhibiting BxPC-3 cells contain only traces of these GnRH analogues. The GnRH-I-R expressing EBC-1 lung cancer cells contain moderate and the Detroit-562 pharynx cancer cells contain high amount of GnRH analogues. Results are presented as mean ± SD, N = 3. Please click here to view a larger version of this figure.

Discussion

Experiments described herein use selectively labeled GnRH analogues for screening adherent cell cultures *in vitro*. Confocal microscopy and flow cytometry methods are suitable for tracking and quantifying the cellular uptake of these GnRH-FITC conjugates in a time and concentration dependent manner. These experiments have the following critical steps: 1) maintain a sterile, healthy cell culture; 2) GnRH peptides must be of high quality; 3) reasonable concentrations and incubation times must be followed; 4) treating and washing steps; 5) well-adjusted instrument settings.

Cells are maintained in the manufacturer's recommended medium supplemented with 10% (V/V) fetal bovine serum and antibiotics (complete medium). Cells should be kept and handled in a sterile environment until the treatment steps (with GnRH-FITC conjugates). Before the experiments, check the cells using an inverted microscope. They should be healthy, attached and must reach the required quantities.

It is important to use GnRH peptides with high quality and purity. We synthesized these peptides and purified them to over 98%[18]. The peptide can be stored at -25 °C in a refrigerator as a lyophilized powder. The 10 mM GnRH-FITC stock solutions in DMSO should be kept in a dark place at room temperature and used up within a few weeks. We investigated the stability of these conjugates and found that they are stabile in DMSO and their fluorescence intensity are maintained after 1 month of proper storage. The concentration of GnRH-FITC conjugates and the time of treatments are variable in these experiments, which offers great advantages, but with certain limitations (see below). The applied concentrations and incubation times in this protocol are only recommendations, but they provide a good basis for the initial experiments.

CLSM experiments require several washing steps. These steps should be carried out carefully. Do not transfer liquid directly onto the cells. Rather, use the side or the corner of the wells for this purpose. This can minimize washing off and losing the adhered cells. It is also recommended to avoid direct light during the whole experiment, as it can desensitize the fluorescent samples (photobleaching effect). Keep the treated samples in a dark place or covered with a piece of aluminum foil during the experiment. Based on previous findings, we noticed that the final DMSO concentration in the applied treating media is negligible (0.1% (V/V) DMSO in the 10 µM treating media) and has no effect on the results, thereby DMSO-free complete medium is also suitable as negative control.

It is important to use well-adjusted instrument parameters during the CLSM and FACS experiments. These parameters need to be re-calibrated on every cell type, before both analyses. In the CLSM experiment, the signal intensity of confocal images depends on the following parameters: laser intensity, detector gain and offset, and pinhole size. When adjusting these parameters, use the lowest laser power to produce an acceptable image and avoid photobleaching. Set the imaging parameters to adjust the background fluorescence of unstained negative control cells to near zero (**Figure 3B**). Image treated cells with these adjusted parameters to avoid false positive results. Additional signals emitted from the treated cells at 514 nm and 546 nm contain the required information about GnRH or GnRH-I-R. These steps require experience in the

CLSM experiments. In the FACS experiment, create a standard forward scatter-side scatter plot (linear scale) and set voltages to adjust the main living cell population in the middle of the plot. Draw a region around living cells. Create a histogram, set abscissa to channel one (FL1-530 nm, logarithmic scale) and set the previously defined region as a gate for this histogram. Set FL1 voltage to bring the signal of unstained negative control cells under 10^1 (**Figure 4A**). Analyze treated cells with these adjusted parameters.

Further, it is important to check the mycoplasma status of the cell cultures from time to time. Various assays are available for this purpose. In case of mycoplasma positivity, discard cells and start working with a new culture. It is recommended to use an antibiotic mixture in the culture medium which is optimized to prevent mycoplasma. The applied concentration of GnRH-FITC conjugates, and the time of treatments are variable in these experiments, however applying low concentrations and short incubation times could result in too weak signals for fluorescence detection.

When imaging the GnRH-I-R in parallel with GnRH analogues, higher GnRH-FITC concentration is recommended (10 μM is optimal). The reason for the higher concentration is the short incubation time (1 hour) and the relatively high fluorescence signal of the labeled antibody as compared to GnRH-FITC conjugates. On the other hand, a minor overlap between the two fluorescent dye emissions (514 nm and 546 nm) was found, which occurs only at 546 nm and is derived from the signal of GnRH-FITC conjugates. However, the relatively higher fluorescent signal of Alexa 546 and the well-adjusted parameters could minimize this effect, as demonstrated in **Figure 3**. Another possible solution to avoid the overlap, is to apply labeled secondary antibody which has different excitation wavelength and/or better separated emission spectrum compared to FITC (if the CLSM technical parameters allow this). This possibility offers great advantage for the CLSM method, which enables using optional fluorescent probes in parallel with GnRH-FITC conjugates. For example, these alternative fluorescent probes could be used for counterstaining other organelles as needed.

The applied concentration of GnRH-FITC conjugates has the following limitations. Maximum concentration limit of GnRH-FITC conjugates is based on their solubility[18]. These values are the following in PBS at room temperature: GnRH-I: 90 μM; GnRH-II: 40 μM; GnRH-III: 200 μM. On the other hand, in previous experiments we supposed that the uptake of these GnRH peptides could take place by receptors other than human GnRH-I receptors; this uptake seems to be more significant at higher concentrations (above 10 μM)[18]. The lower detection limit of GnRH-FITC conjugates is based on a variety of parameters, but in ideal settings it is approximately 1 μM for CLMS experiments. We found that flow cytometry offers better sensitivity than confocal microscopy. In ideal settings, the quantification limit of GnRH-FITC conjugates could be lower than 1 μM. The limit of detection or quantification could be different in every experiment, because they are dependent on cell type and incubation time. Note that data obtained by FACS is more reliable than CLSM, because it detects thousands of cells per sample and the average signal of cells is calculated. However, in the protocol, FACS does not provide information about the cellular localization of GnRH-FITC conjugates and the cell surface expression of GnRH-I-R. Based on these considerations we conclude that, the CLSM and FACS experiments have distinct advantages, and they complement each other. On the other hand, high content image analysis could be an interesting and a good alternative for FACS and CLSM analysis.

In summary, the data obtained from these experiments confirm that each of the three GnRH-FITC conjugates can enter into cells which express cell surface GnRH-I-R. These GnRH analogues have similar targeting potency at the same concentrations. However, the GnRH-I-R level on the surface of different cancer cells is highly variable. Results calculated from the data of flow cytometry analysis enables the comparison of cell lines or GnRH analogues. At the same time, images obtained by confocal microscopy could reveal the level of cell surface GnRH-I-R expression and confirm that these conjugates are internalized into the cells. Based on these results, one can confirm that the introduced experiments contain practical and variable methods for the investigation of GnRH based drug delivery systems and offer a good basis for further experiments.

Disclosures

The authors have nothing to disclose.

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