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Synthesis, in vitro progesterone receptors affinity of gadolinium containing mifepristone conjugates and estimation of binding sites in human breast cancer cells

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ABSTRACT

Novel gadolinium-based mifepristone conjugates were synthesised using various synthetic routes. Moderate antiprogestagenic activity of the new conjugates was observed in human breast cancer cells (T47-D cells) using AP (alkaline phosphatase) assay. The amount of incorporated Gd determined by inductively coupled plasma mass spectroscopy (ICPMS) indicates the number of binding sites per cell. These conjugates might be important compounds to develop receptor-targeted MRI contrast agents as well as other anti-breast cancer therapeutics.

1. Introduction

In continuation of our studies of novel, high affinity PR (Progesterone Receptor) antagonists, which are selectively enriched in human breast cancer cells, we synthesised mifepristone conjugates containing Gd.[1] Such conjugates could be used as contrast agents for magnetic resonance imaging (MRI) or can be model compounds for radioligand containing conjugates.[2,3] Approximately more than 50% of all MRI contrast agents used for scanning are based on Gd complexes.[4] Over the last two decades in vivo application of paramagnetic gadolinium complexes as MRI contrast agents has become an important tool in modern medical diagnostics.[5,6] Complexes of the highly paramagnetic Gd(III) ion with polyamino carboxylate ligands are the most widely utilised MRI contrast agents and complexes with DOTA [1,4,7,10-tetraaza-1,4,7,10-tetrakis(carboxymethyl)cyclododecane] and DTPA (diethylene triamine pentaacetic acid) are currently used in clinical practice.[5]

Paramagnetic metals have also been attached to antibodies or other tissue-specific molecules to impart disease-specific MRI agents.[6] It is however important to deliver sufficient quantity of paramagnetic label and several reports have been published describing such agents that contain a significant number of gadolinium atoms.[7–9] Gd(III) accelerates proton relaxation in the surrounding water through dipolar interactions between the unpaired electron spin of the metal ion and the proton nuclei of the water molecules resulting MRI contrast enhancement.[10] Also numerous conjugates based on radiometals for diagnostic and therapeutic purposes have been described.[11–14] Present development of drugs selectively interacting with the PR in human tumour cells include: (i) selective PR antagonists[15,16] expected to be useful for the treatment of chronic conditions, for example, endometriosis or leiomyomas and (ii) radio ligands[17–19] to enable determination of PR levels in vivo for assessing the hormone responsiveness of breast cancer without tissue biopsy. PR status of breast cancer was found to be an independent predictive factor for benefit from adjuvant endocrine therapy. Patients with steroid hormone receptor-positive tumours, expressing ER (Estrogen Receptor) as well as PR, have the most favourable prognosis.[20] Therefore for reliable determination of PR status as well as getting clear picture of the breast tumour needs a high selective and harmless MRI contrast agent.

Nitrobenzyl-DOTA has been used as a precursor for the synthesis of a new chelate conjugate for pre-targeted diagnosis and therapy for its superior stability with radiometals as well as lan-

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thanides.[12,21,22] Lee et al. published the synthesis of a conjugate MRI contrast agent with Gd-containing residue attached to the 3-keto position of mifepristone.[23] They however determined in vitro a very small influence on proton relaxation at extremely high concentrations, which are far beyond the limit of therapeutic or diagnostic useful concentrations. This might be due to the fact that attachment of the Gd-cage at the 3-keto-position reduces considerably the PR affinity. We have reported a novel high affinity fluorescent PR antagonist that is accumulated in the nucleus of tumour cells.[1] This conjugate successfully crossed cell membranes and modulated gene transcription. It has been used for the fluorescence microscopic investigation of progesterone receptors in T47-D tumour cells. Aim of this work was to retain this selectivity and synthesise MRI agents, which will analogously selectively accumulate in PR positive breast cancer as well as other PR positive tumours such as uterine fibroid tumours and their metastatic tumours.[24]

2. Results and discussion

2.1. Chemistry

Scheme 1 shows the synthesis of the Gd chelating part, (*p*-NH_2-Bn-DOTA) via seven steps starting from the nitration of *S*-phenylalanine (**1**) and concluded with hydrogenation of tetrabenzyl ester **7** by 5%Pd/C/H_2 gas as described in the literature.[21,25–30]

Scheme 2 illustrates the synthetic pathway[1,31,32] of attaching linkers to the antiprogestin mifepristone (**10**, RU486). Mifepristone was converted to desmethylmifepristone (**11**) by demethylation with CaO and I_2 in MeOH/THF mixture. Alkylation of the methylamino group of **11** was done with 3-bromopropionic acid and 6-bromohexanoic that afforded the mifepristone derivatives **12** and **13** with 51% and 39% yield, respectively.

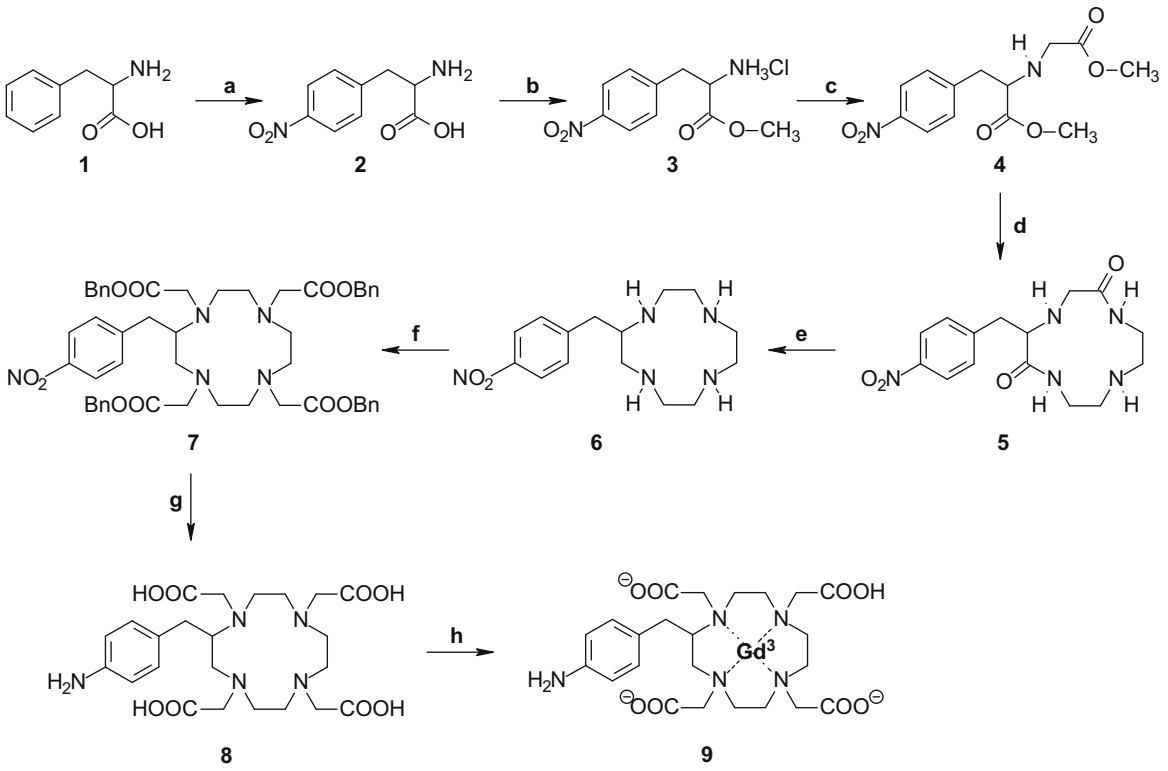
Scheme 3 shows the synthesis of conjugates **16** and **17** from the coupling reaction of **9** with **12** and **13** respectively. Mifepristone derivatives **12** or **13** were activated with isobutyl chloroformate and triethylamine in THF. Coupling the gadolinium complex **9** afforded the desired conjugates **16** and **17**.

Structures of all compounds were established by NMR except for conjugate **16** and **17**. Due to paramagnetic nature of Gd(III), it was not possible to characterise the conjugates by NMR spectroscopy and the structure of **16** and **17** was confirmed by high resolution mass spectroscopy. In addition, the structure of compounds **8** and **9** was also established by high resolution mass spectroscopy.

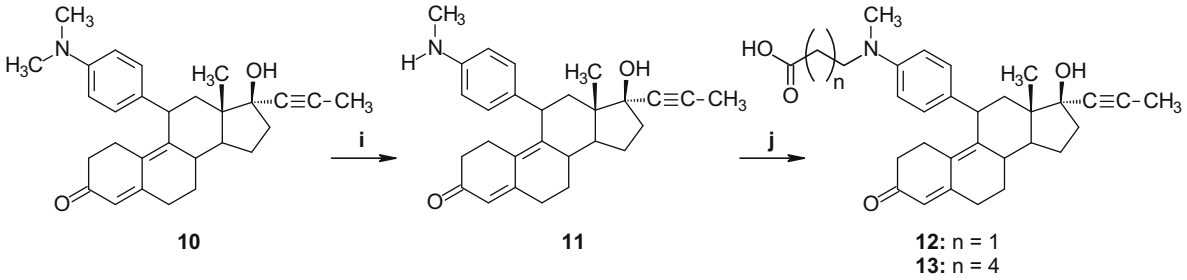
The mass spectrum of compound **8** in positive mode showed signals corresponding to $[M+H]^+$ and $[M+Na]^+$ ions as expected from the proposed structure. Further signals can be attributed to sodium chloride adducts as $[M+NaCl+H]^+$ and $[M+NaCl+Na]^+$, probably due to presence of a small amount of NaCl in the sample. At *m/z* 452.0 a signal corresponding to the trialkylated derivative (proposed as a by-product) was observed but seems to be only of minor abundance. In negative mode, all major signals can be assigned to the main compound: $[M-H]^-$, $[M+Na-2H]^-$, $[M+NaCl-H]^-$ and $[M+NaCl+Na-2H]^-$.

For the compound **9**, the unique isotope distribution of gadolinium facilitates the identification of Gd-containing species in the sample. In positive mode, the most abundant signal corresponds to the $[M+2Na]^+$ ion at *m/z* 708.9 (most abundant isotope). The signal at *m/z* 766.9 corresponds to $[M+NaCl+2Na]^+$, while the trialkyl impurity only appears at very low abundance at *m/z* 628.9.

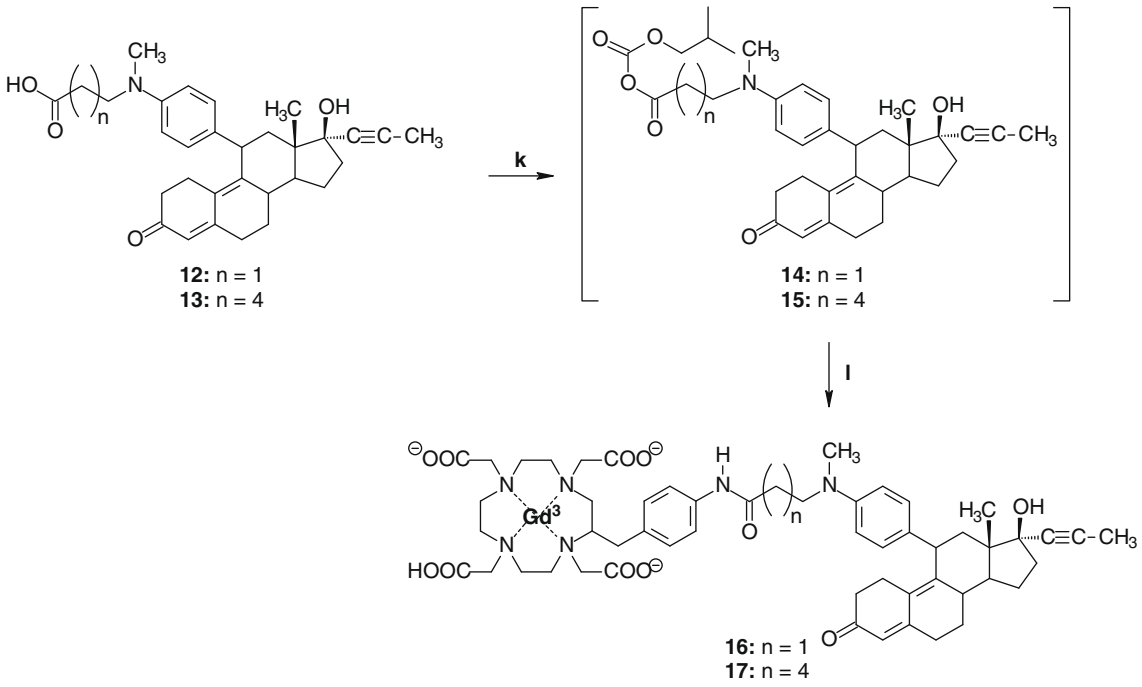
For conjugate **16**, signals were observed in negative mode only. The signal corresponding to $[M]^-$ was the most abundant one. It was accompanied by a peak at 14 units lower; this could result from partial demethylation of the *N*-methyl group during ionisation step.



Scheme 1. Synthesis of *p*-NH_2-Bn-DOTA and its Gd-complex. Reagents and conditions: (a) concd H_2SO4/concd HNO_3; (b) CH_3OH/HCl gas; (c) DMF/TEA/$BrCH_2COOCH_3$; (d) CH_3OH/diethylenetriamine/reflux for 11 days; (e) THF/BH_3 in THF/concd HCl; (f) CH_3CN/$BrCH_2CO_2Bn$/Na_2CO_3/70 °C; (g) 5% Pd/C/H_2 gas at 30 psi/CH_3OH/H_2O; (h) $GdCl_3·6H_2O$/100 mM Na_2CO_3 solution/pH 8/80 °C.



Scheme 2. Attaching linker groups to desmethylmifepristone. Reagents: (i) CaO/I_2; (j) $Br(CH_2)_n$+1COOH/$NaHCO_3$ (n = 1 for **12** and 4 for **13**).



Scheme 3. Syntheses of bioconjugates **16** and **17**. Reagents: (k) THF/TEA/isobutylchloroformate; (l) DMF/compound **9**.

For conjugate **17**, the spectrum in positive mode showed abundant signals corresponding to the $[M+2Na+H]^{2+}$ ion of the proposed structure, as well as corresponding to the $[M+Na+H]^{+}$ ion of the same compound. In negative mode, only a single dominant signal was visible corresponding to the $[M]^{-}$ ion of the proposed structure. All other signals were below 2% in relative intensity and resulted from the addition of Na or NaCl.

In TLC every conjugate showed two very close spots [for **16** R_f value = 0.46 and 0.39 in MeCN/H_2O (20:7); for **17** R_f = 0.39 and 0.32 in MeCN/H_2O (10:3)] and the two spots were separated by TLC from each conjugate. MS displayed almost identical spectra for the two separated spots from **16** and **17** and in both cases the most abundant signals were corresponded to $[M]^{-}$ ion of the expected conjugates. As the mass spectra for both fractions are practically identical we conclude that the two main compounds represent stereo-isomers, most probably related to the non-complexing molecular part. The isomerisation (epimerisation) might also be taken place at the condensation reaction conditions at which the gadolinium ion is already present. An induction of isomerisation due to the presence of a metal ion or caused by the reaction conditions need to be considered. However, further thorough investigation is suggested in this regard. For all biological experiments we used the mixture of these two isomers.

It should be noted that the highest yield of **16** and **17** was obtained if the coupling reaction was done after preparation of the gadolinium complex **9**. Furthermore Gd complex formation diminishes the risk of contamination from trace metals during purification of the conjugates. Hüber et al.[30] reported that they were not able to make the conjugate complex (rhoda-DOTA) unless Gd^{+3} was introduced into p-NH_2-DOTA prior to the addition of tetramethyl rhodamine (TRITC) due to sterically shielding the binding cavity of the macrocycle that prevents Gd^{+3} coordination.

2.2. Antiprogestagenic activity

Antagonistic activity of conjugates **16**, **17** and intermediates **11**, **12** and **13** was determined in cell culture using the AP (alkaline phosphatase) assay in human T47-D tumour cells. Inhibition of progesterone-induced AP activity was found for all compounds. IC_{50} values were estimated from dose–response curves and the relative potencies (defined as the ratio of IC_{50} value of the reference antagonist **10** to the IC_{50} value of the individual test compound and multiplied by 100) are summarised in Table 1. Antiprogestagenic activity of the intermediates **11** and **13** was 3–9 whereas **12** was more than 100-fold lower than that of the reference antagonist mifepristone (**10**). Conjugates **16** and **17** exhibited

Table 1
Antiprogestinic activity of the new conjugates, intermediates and mifepristone from alkaline phosphatase assay[a]

Compound	IC_{50} values[b]	Relative potency[c]
10	0.05	100
11	0.17	29
12	34	0.15
13	0.44	11
16	45	0.11
17	47	0.11

[a] Inhibition of progesterone-induced (10^{-9} mol/L) alkaline phosphatase activity in T 47-D cells.
[b] IC_{50} values were determined from dose–response curve and are given in nmol/L.
[c] Relative potency is defined as the ratio of IC_{50} (**10**): IC_{50} (**x**) and multiplied by 100 (**x** = **11**, **12**, **13**, **16**, **17**).

considerable antiprogestagenic activity with IC_{50} values in the nano molar range although the relative potencies of both conjugates were approximately 900-fold lower compared with the parent compound **10**. Conjugates showed no antagonistic activity at 10 nM but displayed almost complete antagonistic activity at 100 nM concentration.

To assess ligand-receptor interaction of conjugates **16**, **17**, intermediates **11**, **12** and **13** in cell culture, antiprogestagenic activity of all compounds was determined using AP Assay which was compared with that of parent compound **10**, mifepristone, as reference antagonist. Nevertheless after attachment of Gd-chelate significant antiprogestagenic activity was observed. Since functional activity is approximately in agreement with receptor binding affinity[33] hence considerable affinity of conjugates **16** and **17** to PR is assumed.

2.3. Proton relaxation experiments

T47D human breast cancer tumour cells have been incubated with different concentrations (10^{-7}, 10^{-8} and 10^{-9} M) of compound **17**. After washing the cells and transferring the same number of cells into NMR tubes, no influence on the relaxation behaviour could be observed. Therefore, we washed the cells and investigated the relaxation of the water molecules of the cell lysate. The cell lysate with the same number of cells was dispersed in DMSO and the T_1-relaxation rate of the water molecules was determined. However, the T_1 values (about 1.4 s) obtained for different incubation times did not show any dependency on incubation time. We therefore concluded that the Gd-concentration in the cells was too small to reduce the relaxation time of water considerably and decided to determine the mean Gd concentration in the tumour cells to gain information about the number of progesterone binding sites per cell.

2.4. Determination of progesterone binding sites

A sample of 10 Mio. T47D cells was incubated with **17** at different time periods and after washing, the cells were destroyed and Gd concentration was determined with inductively coupled plasma mass spectrometry. As there is usually almost no Gd present in tissues (ultra)traces of Gd or Gd-containing molecules are easily quantifiable with ICPMS. The precision is <5% at a concentration of 1.0 ng Gd/L. From the amount of pg Gd and the number of cells one can calculate the mean value of Gd atoms in a cell, thus giving an estimate of progesterone binding sites per cell.

Equation for calculation of binding sites:

$x =$ pg Gd, $y =$ Mio. cells; $R =$ number of binding sites

$$R = (x \times 10^{-12} \times 6.02 \times 10^{23}/y \times 10^{6} \times 157) = (x/y) \times 3834$$

Table 2 shows the results. At a 'physiological' concentration of 10^{-7} mol/L the number R of binding sites at increasing incubation time increases from 6.2×10^3 at 0 h to 25.8×10^3 at 48 h. These numbers are in good agreement with the number of binding sites per cell which was determined for T47D breast cancer cells by radioligand assay as reported by Gunnet et al.[34] The number given in this paper was 32,947 ± 14,517 per cell, resp. 3296 ± 947 fmol/mg protein. To see if mifepristone (**10**) interferes with the binding of compound **17** we have added **10** in a concentration of 10^{-6} mol/L during a period of 48 h and again determined R. The result is a considerable drop of R from 25,800 to 10,800. From this we conclude that compound **17** selectively binds to the PR binding sites in T47D tumour cells. If the incubation concentration is enhanced to 10^{-6} mol/L, the calculated number of binding sites is greatly enhanced in the region from 8100 at 0 h to 259,000 at 48 h, probably because at this concentration compound **17** is not only PR bound, but also free dissolved in the cytoplasm.

3. Conclusion

The newly synthesised conjugates bind selectively to the progesterone binding sites of T47D human breast cancer cells. However the concentration one can achieve in the cells by the application of physiological useful incubation concentration is not high enough to use the conjugates as a diagnostic tool in MRI. It however can be used to determine the number of progesterone binding sites in these breast tumour cells. In addition the present conjugates might be the lead compounds to design receptor-targeted MRI contrast agents as well as a series of steroid conjugates holding therapeutic or diagnostic agents with high affinities for their cognate receptors for potential use for therapeutic, mechanistic and diagnostic purposes. It is also predictable that PR-binding specificity can be enhanced by using highly selective antiprogestins thus minimising binding to other steroid hormone receptors.[15,16,35,36]

4. Experimental

All reagents and solvents for syntheses were purchased from Sigma–Aldrich, Fluka or Merck and used without further purification. Reagent-grade solvents were purified and dried using standard methods. DMF was purified by azeotropic distillation and dried over 4 Å molecular sieve. Trietylamine and diethylenetriamine were dried over KOH. For preparation of absolute methanol, 98% methanol was first treated with fresh calcinated CaO and distilled; subsequently, it was refluxed with magnesium splinters and iodine and distilled again. THF and acetonitrile were dried over

Table 2
Determination of progesterone binding sites using conjugate 17 in T47D cells

Incubation time in hour	Number of binding site		
	R^a	R^b	R^c
0	8.1×10^3	6.2×10^3	
3	8.8×10^3	5.87×10^4	
6	6.22×10^4	1.44×10^4	
24	9.18×10^4	1.77×10^4	
48	2.59×10^5	2.58×10^4	1.08×10^5

[a] Number of binding sites estimated using the concentration 10^{-6} mol/L of conjugate **17**.
[b] Number of binding sites estimated using the concentration 10^{-7} mol/L of conjugate **17**.
[c] Number of binding sites estimated using the concentration 10^{-7} mol/L of conjugate **17** plus 10^{-6} mol/L of mifepristone during an incubation period of 48 h.

sodium wire and P_2O_5 (0.5–1% w/v) respectively. Solvents of analytical and spectroscopic grade as well as deuterated NMR solvents were purchased from Merck and Chemische Fabrik Uetikon, respectively.

UV spectra were recorded on a Shimadzu UV-160 Å spectrometer and absorption maxima λ_{max} are given in nm. IR spectra were taken on a Perkin–Elmer FT-IR 2000 spectrometer. Absorption maxima ν_{max} are given in cm^{-1} and referred to as s (strong), m (medium), w (weak) and br (broad). NMR spectra were recorded on a Varian Unity Inova 400/600 NMR spectrometer equipped with a tuneable broadband probe. Inverse detected gradient selected 2D experiments (gCOSY, HSQC, HMBC) were done using standard pulse sequence programs. HMBC experiments were optimised for a long range-coupling constant of 8 Hz. Tetramethylsilane (TMS) was used as internal reference standard. Chemical shifts δ are given in ppm, coupling constants (J values) are expressed in Hz and multiplicities are referred to as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad).

The T_1-relaxation time was determined by inversion recovery experiments with standard experiments provided in the NMR software VNMR (Varian). For these experiments, lysates of the cells (1.7 million cells) were dissolved in 690 μL DMSO-d_6 and 30 μL H_2O.

Column chromatography was carried out on Silica Gel 60 (0.063–0.200 mm). Flash chromatography was done by Flash Master Personal, Jones Chromatography. Column width 27 mm, length 150 mm, silica gel Merck 60 (0.063–0.200 mm; 70–230 mesh ATSM). Preparative HPLC was done using a Labomatic HD-200 pump equipped with a Labocord-700 UV/vis detector (absorption recorded at λ = 254 nm); column 1: YMC-Pack™ ODS-A™ (C18), 10 μm, 250 × 20 mm and column. Analytical HPLC was done using a HP 1050 autosampler and pump equipped with an Agilent 1100. G1314A VWD UV/vis detector; column 2: YMC-Pack™ ODS-A™ (C18), 5 μm, 250 × 4.6 mm. Elemental analyses were performed at the Institute of Organic Chemistry, University of Graz.

EI-mass spectra were measured with a Finnigan MAT 212 or Varian MAT 312 spectrometer at 70 eV ionising voltage. GC–MS were measured on a HP-GC 6890-HP-MSD 7890. Intensities are given in % of the base peak. High resolution mass spectra were obtained in the positive and negative mode on an Agilent 1100 Trap SL ion trap in the range of 100–1000 m/z or 100–2000 m/z using electrospray ionisation.

4.1. Synthesis of bifunctional chelating agent (*p*-NH_2-Bn-DOTA) and its gadolinium complex

4.1.1. (*S*)-*p*-Nitrophenylalanine (2)

Following the procedure described in the literature[21] 33 g (0.20 mol) of (*S*)-phenylalanine (**1**) was dissolved in concd H_2SO_4 and reacted with concd HNO_3, yielding 27.46 g (65%) **2** as a white crystalline solid. TLC ($CHCl_3$/CH_3OH = 3:1): R_f = 0.07 and (10% w/v aq ammonium acetate/CH_3OH = 1:1): R_f = 0.83. UV/vis (MeOH): 210.0, 229.5, 261.5. IR (KBr): 3433 (br, OH), 1741 (C═O), 1514 (s, NO_2). ^{1}H NMR (400 MHz, CD_3OD): δ = 3.36 (dd, 1H, J = 15.3 and 7.2, CH_2), 3.46 (dd, 1H, J = 15.3 and 6.4, CH_2), 4.38 (dd, 1H, J = 6.4 and 7.2), 7.60 (d, 2H, ArH), 8.27 (d, 2H, ArH).

4.1.2. (*S*)-*p*-Nitrophenylalanine methyl ester hydrochloride (3)

Prepared as described in the literature[25] compound **2** (10.0 g, 47.6 mmol) was treated with CH_3OH, saturated with HCl (g), to give the compound **3** as a white solid (7.4 g, 60%). TLC ($CHCl_3$/CH_3OH = 4:1): R_f = 0.75 and (10% aqueous solution of ammonium acetate/CH_3OH = 1:1): R_f = 0.83. UV/vis (MeOH): 207.0, 228.5, 264.0. IR (KBr): 1745 (s, C═O). ^{1}H NMR (400 MHz, CD_3OD): δ = 3.27–3.42 (m, 2H), 3.83 (s, 3H), 4.41 (t, 1H), 7.52 (d, 2H, ArH), 8.23 (d, 2H, ArH). Elemental Anal. Calcd for $C_{10}H_{12}N_2O_4$·HCl: C, 46.08; H, 5.03; N, 10.75; Cl, 13.60. Found: C, 45.93; H, 4.82; N, 10.66; Cl, 13.33.

4.1.3. *N*-[(Methoxycarbonyl)methyl]-(*S*)-*p*-nitrophenylalanine methyl ester (4)

Prepared as described in the literature[26] 26.1 g (0.10 mol) **3** was reacted with methyl bromoacetate (45.9 g, 0.30 mol) and triethylamine under nitrogen yielding **4** as a pale yellow oil (24.9 g, 84%). TLC ($CHCl_3$/CH_3OH = 50:1): R_f = 0.53 and (10% aqueous solution of ammonium acetate/CH_3OH = 1:1): R_f = 0.85. UV/vis (MeOH): 207.0, 232.0, 270.5. IR (KBr): 3443 (m, NH), 1739 (s, C═O), 1519 (s, NO_2). ^{1}H NMR (400 MHz, CD_3OD): δ = 3.10 (dd, 1H, J = 14.8 and 6.9, ArCH_2CH), 3.14 (dd, 1H, J = 14.8 and 6.9, ArCH_2CH), 3.40 (d, 1H, J = 17.7, NHCH_2CO), 3.42 (d, 1H, J = 17.7, NHCH_2CO), 3.66 (s, 3H, CO_2CH_3), 3.70 (s, 3H, CO_2CH_3), 3.71 (t, 1H, J = 6.9, ArCH_2C*H*), 7.48 (d, 2H, ArH), 8.16 (d, 2H, ArH). ^{13}C NMR (100 MHz, CD_3OD): δ = 39.4 ($ArCH_2$), 49.1 ($HNCH_2$), 51.97 (CH_3), 52.11 (CH_3), 62.37 (CH), 124.09 (Ar), 131.25 (Ar), 146.46 (Ar), 147.99 (Ar), 173.35 (C═O), 174.61 (C═O). GC–MS (m/z): 237 (42), 177 (19), 160 (100), 100 (49), 72 (11), 59 (5); calcd for $C_{13}H_{16}N_2O_6$: 296.28.

4.1.4. 3-(*S*)-(4-Nitrobenzyl)-2,6-dioxo-1,4,7,10-tetraazacyclododecane (5)

Following the procedure described in the literature[26] a solution of compound **4** (26.75 g, 0.09 mol) and dry diethylenetriamine (9.29 g, 0.09 mol) in absolute CH_3OH was refluxed for 11 days under nitrogen yielding **5** as a pale yellow solid (4.73 g, 16%). TLC (10% w/v aqueous ammonium acetate/CH_3OH = 1:1): R_f = 0.60. UV/vis (MeOH): 233.0, 272.0. IR (KBr): 3270 (s, NH), 1655 (C═O), 1512 (NO_2). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.72 (N*H*CH_2), 2.49–3.34 (m, 13 H, ArCH_2, ArCH_2C*H*, NCH_2), 4.03 (N*H*CH_2), 7.49 (d, 2H, ArH), 7.74 (N*H*CO), 7.78 (N*H*CO), 8.14 (d, 2H, ArH). ^{13}CNMR (100 MHz, DMSO-d_6): δ = 37.14 (CH_2), 37.26 (CH_2), 38.45 (CH_2), 45.01 (CH_2), 45.35 (CH_2), 51.95 (CH_2), 64.60 (CH), 123.23 (Ar), 130.50 (Ar), 146.23 (Ar), 147.12 (Ar), 171.72 (C═O), 172.84 (C═O). Elemental Anal. Calcd for $C_{15}H_{21}N_5O_4$: C, 53.72; H, 6.31; N, 20.88. Found: C, 53.16; H, 6.19; N, 20.38.

4.1.5. 2-(*S*)-(4-Nitrobenzyl)-1,4,7,10-tetraazacyclododecane trihydrochloride (6)

Prepared as described in the literature.[26,27]

4.1.5.1. Caution: explosion hazard. All glass wares were dried in an oven. In a three necked 1-L flask a suspension of compound **5** (3.0 g, 9 mmol) in 300 mL of dry THF under nitrogen, 200 mL of 1 mol/L BH_3/THF solution was added drop wise at 0 °C during 3 h. This reaction mixture was stirred for 1 h at 0 °C. Temperature was increased gradually and the mixture refluxed for 24 h. After that 90 mL water was added slowly during 1 h to destroy excess BH_3 and thereafter the mixture was cooled to 0 °C and concd HCl was added slowly during 1 h. The resulting mixture was refluxed for 14 h. Two thirds of the solvent was removed in vacuo and the resulting precipitate was filtered off and recrystallised from absolute CH_3OH and dried in vacuum. Compound **6** was obtained as white solid (1.09 g, 28%). TLC (CH_3OH/10% aq ammonium acetate w/v = 1:1): R_f = 0.18 and ($CHCl_3$/MeOH/25% aq NH_3 = 100:30:6): R_f = 0.11 as free base. UV/vis (MeOH): 205.0, 233.0, 271.0. IR (KBr): 3446 (w, br, NH), 1515 (s, NO_2). ^{1}H NMR (400 MHz, D_2O): δ = 2.4–3.25 (m, 16 H, NCH_2, $ArCH_2$), 3.4 (m, 1H, NCHC), 7.49 (d, 2H, ArH), 8.14 (d, 2H, ArH). ^{13}C NMR (100 MHz, D_2O): δ = 39.34 ($ArCH_2$), 43.22 (CH_2), 45.00 (CH_2), 45.52 (CH_2), 46.48 (CH_2), 46.77 (CH_2), 47.05 (CH_2), 50.13 (CH_2), 56.55 (CH), 126.65 (Ar), 132.90 (Ar), 147.39 (Ar), 149.32 (Ar). Elemental Anal. Calcd for $C_{15}H_{25}N_5O_2$·3HCl·H_2O: C, 41.44; H, 6.95; N, 16.11; Cl, 24.46. Found: C, 41.92; H, 6.80; N, 16.01; Cl, 24.25.

4.1.6. 2-(*S*)-(*p*-Nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid tetrabenzyl ester (7)

Prepared according to the literature.[13,28] Free base of 2-(*p*-nitrobenzyl)-1,4,7,10-tetraazacyclododecane (4-NO_2-Bn-cyclen) was prepared by dissolving the HCl salt (**6**, 434.8 mg, 1 mmol) in 2 mL of H_2O. The pH was raised to about 13 by addition of solid NaOH and the free base was extracted with $CHCl_3$ (3 × 25 mL). After drying the $CHCl_3$ solution with K_2CO_3, the solvent was removed and the residue was dried in vacuo. The free base was dissolved in 10 mL of anhydrous MeCN, benzyl bromoacetate (2.29 g, 10 mmol) and anhydrous Na_2CO_3 (1.06 g, 10 mmol) were added. The mixture was stirred at 70 °C under argon for 96 h. The reaction mixture was filtered to remove excess Na_2CO_3 and the solvent was evaporated. The resulting yellow oil was purified by flash chromatography using first $CHCl_3$ and finally $CHCl_3$/MeOH (15:4) as eluent to give **7** (766 mg, 85%). **7** was used for the next step without further purification. TLC ($CHCl_3$/CH_3OH = 15:2): R_f = 0.46. UV/vis (MeOH): 206.5, 283.0. IR (KBr): 1732 (s, C=O), 1515 (s, NO_2). ^{1}H NMR (400 MHz, CD_3OD): δ = 2.10–3.80 (m, 25H, $ArCH_2C$, NCH_2, NCH), 5.00–5.40 (m, 8H, $ArCH_2O$), 7.20–7.40 (m, 20H, ArH), 7.48 (d, 2H, ArH), 8.05 (d, 2H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 44.05–67.13 ($ArCH_2$, NCH_2, CH), 123.64–135.20 (Ar), 146.33 (Ar), 146.64 (Ar), 147.75 (Ar), 173.45 (C=O), 173.81 (C=O), 173.91 (C=O), 173.99 (C=O).

4.1.7. 2-(*S*)-(*p*-Aminobenzyl)-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (8)

Following the procedure described in the literature[29] **7** (1.58 g, 1.75 mmol) was hydrogenated to compound **8** (802.5 mg, 90%) white crystal. TLC (10% w/v aqueous ammonium acetate/CH_3OH = 1:1): R_f = 0.44. UV/vis (MeOH): 204.0, 242.0. IR (KBr): 3430 (s, br, NH_2), 1700 (s, C=O). ^{1}H NMR (400 MHz, D_2O): δ = 2.5–4.0 (m, 25H, NCH, $ArCH_2$, NCH_2, NCH_2CO), 7.2–7.4 (m, 4H, ArH). ^{13}C NMR (100 MHz, D_2O): δ = 34.30–63.66 ($ArCH_2$, NCH_2, NCH_2CO, NCH), 126.45 (Ar), 132.36 (Ar), 133.84 (Ar), 140.67 (Ar), 173.48 (C=O). MS (*m*/*z*): $[M+H]^+$: 510.1, $[M+Na]^+$: 532.1, $[M+NaCl+H]^+$: 568.0, $[M+NaCl+Na]^+$: 590.0, $[M-H]^-$: 508.7, $[M+Na-2H]^-$: 530.1, $[M+NaCl-H]^-$: 566.0, $[M+NaCl+Na-2H]^-$: 588.2; calcd for $C_{23}H_{35}N_5O_8$: 509.25.

4.1.8. [Gd-(*p*-NH_2-Bn-DOTA)] (9)

Prepared according to the literature.[30] $GdCl_3 \cdot 6H_2O$ (74.34 mg, 0.2 mmol) was added to a stirred solution of compound **8** (101.91 mg, 0.2 mmol) in 100 mM aqueous Na_2CO_3 solution (pH ~8). This mixture was stirred at 80 °C for 12 h. The solvent was evaporated, yielding **9**. TLC (MeCN/H_2O = 10:3): R_f = 0.22 and (MeOH/10% aq w/v CH_3COONH_4 solution = 1:1): R_f = 0.51. UV/vis (H_2O): 217.0, 236.0. IR (KBr): 3416 (s, br, NH_2), 1604 (s, C=O). MS (*m*/*z*): $[M+2Na]^+$: 708.9, $[M+NaCl+2Na]^+$: 766.9, $[M-H]^-$: 661.3; calcd for $C_{23}H_{31}GdN_5O_8^-$: 662.77.

4.2. Synthesis of mifepristone derivatives by attaching linkers

4.2.1. Desmethylmifepristone (DMM) [17β-hydroxy-17α-propynyl-11β-(4-*N*-methylaminophenyl)-estra-4,9-dien-3-one (11)

Following the procedure described in the literature[1,31] 500 mg (1.16 mmol) of mifepristone (**10**) was converted to 135 mg of compound **11** (28%) as a fluffy pale yellow solid. Purification: HPLC: column 1, acetonitrile: H_2O, (84:16, vol/vol); flow: 23 mL/min; t_R = 3.4 min. Adduct **10** was recovered (130 mg, 26%), t_R = 4.3 min. Analytical HPLC: column 2; acetonitrile: H_2O (90:10, vol/vol); flow: 1 mL/min): t_R = 4.822. UV/vis: (MeOH): 209.0, 250.0, 303.5. TLC (cyclohexane/EtOAc = 4:6): R_f = 0.51 and (CH_2Cl_2/MeOH, 9:1): R_f = 0.71. UV/vis (MeOH): 209.0, 250.0, 303.5. IR (KBr): 3387 [m (br), N–H, O–H], 2929 (s, C–H), 2870 (m, C–H), 2250 (w, C≡C), 1654 [s (br),C=O], 1615 (s, C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.50 (s, 3H, C-18 H), 1.40 (m, 1H, C-15 H), 1.42 (m, 1H, C-7 H), 1.68 (m, 2H, C-14 H, C-15 H), 1.84 (s, 3H, C-21 H), 1.89 (td, *J* = 11.4, *J* = 3.2, 1H, C-16 H), 1.98 (m, 1H, C-7 H), 2.16 (m, 1H, C-16 H), 2.21 (m, 1H, C-12 H), 2.29 (m, 4H, C-1 H, 2 × C-2 H, C-12 H), 2.42 (dd, *J* = 10.4, *J* = 0.8, 1H, C-8 H), 2.52 (m, 2H, C-6 H), 2.73 (m, 1H, C-1 H), 2.86 (s, 3H, NCH_3), 4.31 (d, *J* = 6.4, 1H, C-11 H), 5.52 (s, 1H, C-4 H), 6.61 (m, 2H, C-3′/5′H), 6.97 (m, 2H, C-2′/6′H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 3.7 (q, C-21), 13.6 (q, C-18), 23.2 (t, C-15), 25.6 (t, C-1), 27.2 (t, C-7), 31.0 (t, C-6), 36.7 (t, C-2), 38.8 (t, C-12, C-16), 39.0 (d, C-8), 39.4 (d, C-11), 40.5 (q, NCH_3), 46.7 (s, C-13), 49.7 (d, C-14), 79.9 (s, C-17), 82.0 (s, C-20), 82.4 (s, C-19), 112.7 (d, C-3′, C-5′), 122.5 (d, C-4), 127.3 (d, C-2′, C-6′), 128.8 (s, C-10), 132.0 (s, C-1′), 146.8 (s, C-9), 148.4 (s, C-4′), 156.9 (s, C-5), 199.5 (s, C-3). MS: (*m*/*z*): 415 (M^+, 100), 397 (7), 331 (11), 266 (71), 213 (23), 120 (47), 107 (76), 91 (54); calcd for $C_{28}H_{33}NO_2$: 415.25.

4.2.2. 3-*N*-Methyl-*N*-[4-(11β,17β)-17-hydroxy-3-oxo-17α-(1-propynyl)estra-4,9-dien-11-yl]-phenylaminopropionic acid (12)

Prepared according to the literature.[31,32] 3-Bromopropionic acid (367 mg, 2.4 mmol) was dissolved in 4 mL of water and the pH of the solution was brought to 7.7 by the addition of $NaHCO_3$. Compound **11** (200 mg, 0.48 mmol) was dissolved in 4 mL EtOH and was added to the previous aqueous mixture, the pH was kept at 9 during the reaction by the addition of $NaHCO_3$. This mixture was stirred for 20 h at 70 °C, cooled to room temperature and pH was adjusted to 6 by adding HCl/EtOH. Solvent was evaporated and the product was extracted with ethyl acetate three times and the extract was dried over sodium sulfate. After evaporation of the solvent the product (**12**) was purified by HPLC column 1 (flow rate 23 mL/min, λ = 254 nm, acetonitrile/water 6:4, retention time for = 5.22 min). After lyophilisation, **12** was isolated as yellow solid (120 mg, 51%). TLC (ethyl acetate): R_f = 0.16, (chloroform/MeOH/25% aqueous ammonia solution = 100:30:6): R_f = 0.41. Analytical HPLC (column 2; acetonitrile/H_2O (90:10, vol/vol); flow: 1 mL/min): t_R = 4.161 min. UV/vis (MeOH): 207.5, 263.5, 303.0. UV/vis (CH_2Cl_2): 300.0. IR (KBr): 3424 (m, br, O–H), 2939 (s, C–H), 2870 (m, C-H), 2242 (w, C≡C), 1728 (s, C=O), 1656 (s, C=O), 1615(s, C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.53 (s, 3H, C-18 H), 1.35 (m, 1H, C-15 H), 1.46 (m, 1H, C-7 H), 1.72 (m, 1H, C-14 H), 1.73 (m, 1H, C-15 H), 1.89 (s, 3H, CH_3, C-21 H), 1.94 (t, 1H, C-16 H), 2.02 (m, 1H, C-7 H), 2.22 (m, 1H, C-16 H), 2.25 (m, 1H, C-12 H), 2.32 (m, 1H, C-1 H), 2.35 (m, 1H, C-12 H), 2.37 (m, 1H, C-2 H), 2.44 (m, 1H, C-2 H), 2.46 (m, 1H, C-8 H), 2.60 (m, 4H, C-6 H, C-2″H), 2.75 (m, 1H, C-1 H), 2.93 (s, 3H, $-NCH_3$), 3.63 (m, 2H, C-1″ H), 4.36 (d, *J* = 6.6, 1H, 11-H), 5.78(s, 1H, C-4 H), 6.81 (d, *J* = 7.2, 2H, C-3′/5′H), 7.06 (d, 2H, C-2′/6′H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 3.84 (q, C-21), 13.81 (q, C-18), 23.34 (t, C-15), 25.81 (t, C-1), 27.33 (t, C-7), 31.14 (t, C-6), 31.22 (t, C-2″), 36.76 (t, C-2), 38.90 (t, 2C, C-12, C-16), 39.18 (d, C-8), 39.45(q, NCH_3), 39.69 (d, C-11), 46.88 (s, C-13), 49.66 (t, C-1″), 49.78 (d, C-14), 80.18 (s, C-17), 82.30 (s, C-19), 82.54 (s, C-20), 114.59 (d, C-3′, C-5′), 122.74 (d, C-4), 127.95 (d, C-2′, C-6′), 129.25 (s, C-10), 135.10 (s, C-1′), 145.60 (s, C-4′), 146.66 (s, C-9), 157.28 (s, C-5), 175.85 (s, C-3″), 200.01 (s, C-3). MS (*m*/*z*): $[M+Na]^+$ 510.4; calcd for $C_{31}H_{37}NO_4$: 487.27.

4.2.3. 6-*N*-Methyl-*N*-[4′-[17β-hydroxy-3-oxo-17α-(1-propynyl)-estra-4,9-dien-11β-yl]-phenylaminohexanoicacid (13)

Following the procedure described for the synthesis of **12**, reaction of 6-bromohexanoic acid (468 mg, 2.4 mmol) and compound **11** (200 mg, 0.48 mmol) gave after purifying by flash-chromatography with EtOAc and MeOH and preparative HPLC (column 2; acetonitrile/H_2O (56:44, vol/vol), flow: 23 mL/min; t_R = 8 min 30 s) 100 mg (39%) of compound **13** as a fluffy yellow solid. Analytical HPLC (column 3; acetonitrile/H_2O (90:10, vol/vol); flow:

1 mL/min): t_R = 4.668 min. TLC (chloroform/MeOH/25% aqueous ammonia solution = 100:30:6): R_f = 0.47 and (EtOAc): R_f = 0.36. UV/vis (MeOH): 207.0, 262.5, 303.0. IR (KBr): 3420 (m, br, OH), 2937 (s, CH), 2866 (m, CH), 2242 (w, C≡C), 1730 (s, C=O), 1659 (s, C=O), 1612 (s, C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.55 (s, 3H, C-18 H), 1.36 (m, 1H, C-15 H), 1.38 (m, 2H, C-3″ H), 1.46 (m, 1H, C-7 H), 1.56 (m, 2H, C-2″ H), 1.68 (m, 2H, C-4″ H), 1.72 (m, 1H, C-14 H), 1.73 (m, 1H, C-15 H), 1.89 (s, 3H, CH_3, C-21 H), 1.94 (t, 1H, C-16 H), 2.02 (m, 1H, C-7 H), 2.22 (m, 1H, C-16 H), 2.28 (t, J = 7.0, 2H, C-5″ H), 2.32 (m, 1H, C-1 H), 2.35 (m, 1H, C-12 H), 2.40 (m, 1H, C-2 H), 2.44 (m, 1H, C-2 H), 2.46 (m, 1H, C-8 H), 2.57 (m, 2H, C-6 H), 2.73 (m, 1H, C-1 H), 2.88 (s, 3H, -NCH_3), 3.26 (t, J = 8.0, 2H, C-1″ H), 4.34 (d, J = 6.8, 1H, 11α-H), 5.76 (s, 1H, C-4 H), 6.59 (m, 2H, C-3′/5′ H), 6.98 (m, 2H, C-2′/6′ H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 3.80 (q, C-21), 13.68 (q, C-18), 23.27 (t, C-15), 24.67 (t, C-4″), 25.80 (t, C-1), 26.39 (t, C-2″), 26.66 (t, C-3″), 27.32 (t, C-7), 31.09 (t, C-6), 34.34 (t, C-5″), 36.85 (t, C-2), 38.19 (q, NCH_3), 38.74 (t, C-12), 38.83 (t, C-15), 39.07 (d, C-8), 39.46 (d, C-11), 46.82 (s, C-13), 49.83 (d, C-14), 52.71 (t, C-1″), 80.13 (s, C-17), 82.35 (s, C-20), 82.37 (s, C-19), 112.20 (d, C-3′, C-5′), 122.62 (d, C-4), 127.54 (d, C-2′, C-6′), 128.98 (s, C-10), 131.27 (s, C-1′), 146.86 (s, C-9), 147.24 (s, C-4′), 156.97 (s, C-5), 173.60 (s, C-6″), 199.64 (s, C-3). MS (*m/z*): $[M+Na]^+$ 552, $[M]^-$ 529; calcd for $C_{34}H_{43}NO_4$: 529.32.

4.3. Synthesis of desired conjugates

4.3.1. 3-*N*-Methyl-*N*-[4′-[17β-hydroxy-3-oxo-17α-(1-propynyl)-estra-4,9-dien-11β-yl]-phenyl-amino-propanoyl-[Gd-(*p*-NH_2-Bn-DOTA)] conjugate (16)

Extremely dried **9** (27 mg, 0.04 mmol) was dissolved in 8 mL of dry DMF in a 10 mL flask under N_2 using a sonicator (3–4 min). In another flask compound **12** (19.5 mg, 0.04 mmol) was dissolved in 0.8 mL of dry THF with 14 μL of triethylamine by stirring 5 min in an N_2 atmosphere. Isobutyl chloroformate (5.76 μL, 0.044 mmol) was added and this mixture was stirred about 2 min under N_2 atmosphere. The solution of **9** was then added and the reaction mixture was stirred for 5 h under N_2 atmosphere at room temperature. Solvent was evaporated and the residue was purified by silica gel column chromatography using MeCN/H_2O (20:7) as eluent yielding **16** after evaporation as a pale yellow solid. $C_{54}H_{67}GdN_6O_{11}$; (MW: 1133.40). TLC (MeCN/H_2O = 20:7): R_f = 0.39 and (MeOH: 10% aq w/v CH_3COONH_4 solution = 1:1): R_f = 0.58. UV/vis (DMSO): 253.0, 265.0. IR(KBr): 3421 (s, br, NH), 1609 (s, C=O). MS (*m/z*): $[M]^-$: 1132.2.

4.3.2. 6-*N*-methyl-*N*-[4′-[17β-hydroxy-3-oxo-17α-(1-propynyl)-estra-4,9-dien-11β-yl]-phenyl-amino-hexanoyl-[Gd-(*p*-NH_2-Bn-DOTA)] conjugate (17)

Following the procedure described for the synthesis of conjugate **16**. Product **17** was synthesised by the coupling reaction between **9** and **13** and purified by silica gel column chromatography using MeCN/H_2O (10:3) yielding **17** as a pale yellow solid. $C_{57}H_{73}GdN_6O_{11}$; (MW: 1175.49) calcd C, 58.24; H, 6.26; N, 7.15; found +1/3 H_2O: C, 57.92; H, 6.10; N, 7.08. TLC (MeCN/H_2O = 10:3): R_f = 0.32 and (MeOH: 10% aq w/v CH_3COONH_4 solution = 1:1): R_f = 0.52. UV/vis (DMSO): 252.0, 264.0, 281.5, 303.0. IR (KBr): 3422 (s, br, NH), 1605 (s, C=O). MS (*m/z*): $[M+2Na+H]^{2+}$: 610.6, $[M+Na+H]^+$: 1198.2, $[M]^-$: 1174.2.

4.4. Alkaline phosphatase assay

T47-D cells were co-incubated with increasing concentrations of either compound **10** (reference antagonist) or test compounds **11**, **12, 13**, **16** or **17** (10^{-11}–10^{-7} mol/L) and progesterone (10^{-9} mol/L). The alkaline phosphatase assay was done as reported previously[1,37,38] with some minor modifications. Cells were washed with 0.9% saline and stored at −80 °C for at least 30 min after 48 h incubation. After defrosting cells were incubated with 50-μL *p*-nitrophenyl phosphate (5 mM, Fluka) dissolved in aqueous diethanolamine (1 M, supplemented with 0.5 mM magnesium chloride and 20 μM zinc sulfate and adjusted to pH 9.8) for 2–5 h in the dark at room temperature. Absorbance of *p*-nitrophenolate was measured in a Lucy 1 multimode plate reader (anthos labtec instruments) at 405 nm (vs 690 nm as reference). For each compound, at least four independent experiments (with six data points each) were carried out. After background subtraction (absorbance of cells without hormonal treatment), absorbance was normalised to the absorbance resulting from the progesterone incubation. Median (central tendency) ± median absolute deviation (variability) was calculated. IC_{50} values were determined from dose–response curves and are given in nmol/L.

4.5. Determination of Gd concentration with ICPMS (inductively coupled plasma mass spectrometry)

Lysed cell pellets were quantitatively transferred into 15 mL polyethylene using 20% (v/v) nitric acid. The Gd-concentration was directly determined in these solutions with an Agilent 7500ce inductively coupled plasma mass spectrometer (Agilent, Waldbronn, Germany) at *m/z* 157. The calibration standards were prepared from a 10.0 ± 0.5 μg Gd/mL stock standard solution (P/N 4400-010031, CPI International, Amsterdam, Netherlands) in a concentration range of 1.0–100 ng Gd/L. Throughout the measurements Indium at *m/z* 115 was used as internal standard (P/N 4400-1000241, CPI International, Amsterdam, Netherlands) to compensate for instrumental drifts.

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