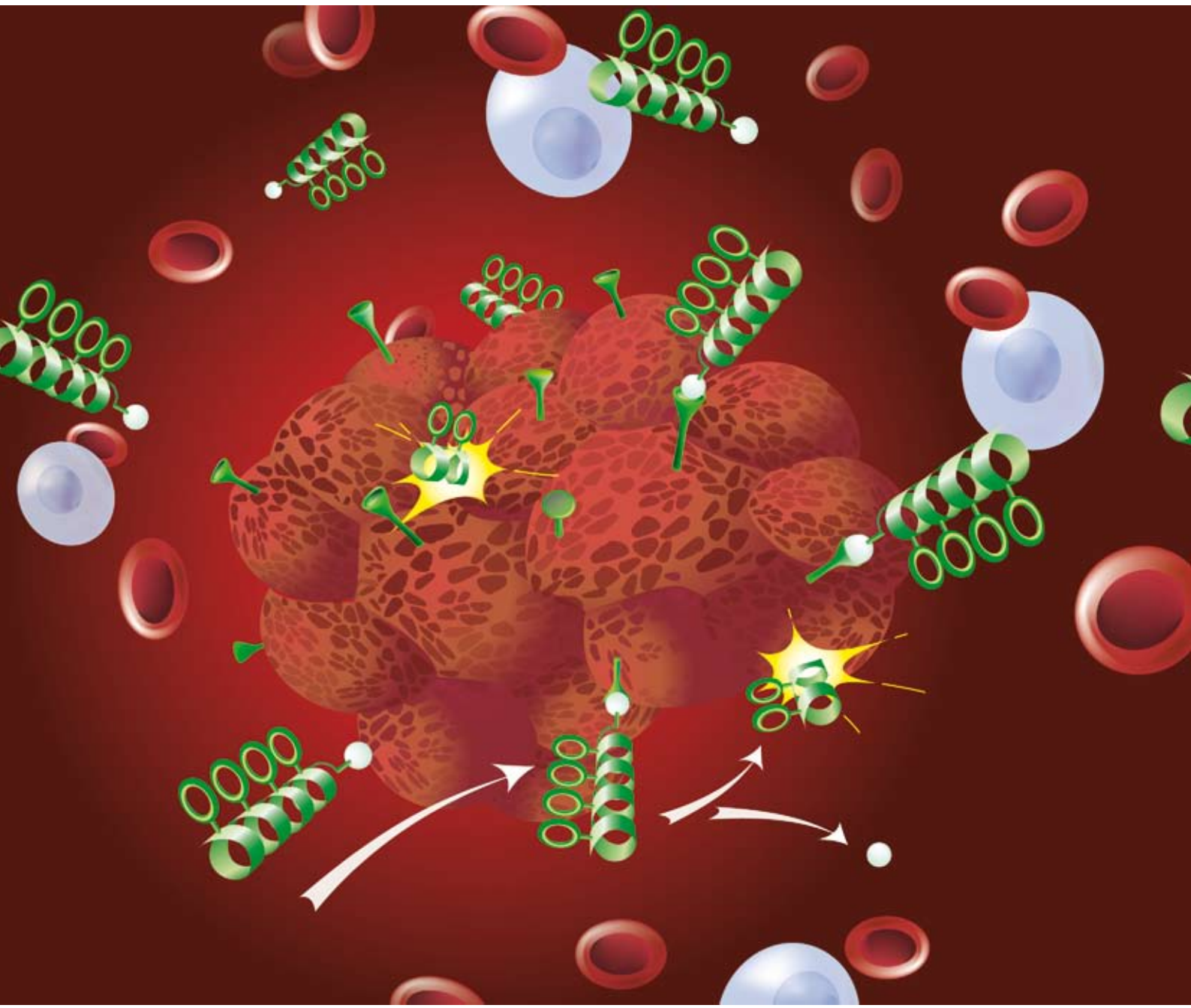
# ChemComm

Chemical Communications

www.rsc.org/chemcomm

Number 18 | 14 May 2008 | Pages 2061–2168



ISSN 1359-7345

RSC Publishing

**COMMUNICATION**

Normand Voyer *et al.*

Nanoscale tools to selectively destroy cancer cells

# Nanoscale tools to selectively destroy cancer cells†

**Pierre-Luc Boudreault, Mathieu Arseneault, François Otis and Normand Voyer***

*Received (in Cambridge, UK) 14th January 2008, Accepted 12th March 2008*
*First published as an Advance Article on the web 7th April 2008*
**DOI: 10.1039/b800528a**

**We present experimental data that demonstrate the potential of synthetic crown ether modified peptide nanostructures to act as selective and efficient chemotherapeutic agents that operate by attacking and destroying cell membranes.**

The term nanotechnology refers to the exploitation of nanometre dimension objects having unique properties due to their size. The present manuscript describes our first step towards exploiting designed peptide nanostructures to kill cancerous cells efficiently by destroying their membrane integrity. We present the development of novel nanochemotherapeutic agents that are activated selectively at the surface of certain cancer cells.

Recently, we reported a family of helical crown ether peptides of different lengths containing neutral polar benzo-21-crown-7 side-chains aligned on one face of the helix (Fig. 1).[1–5] It was observed that the 14-residue helical peptides having on average a length of 2.0 nm exerted a powerful membrane-disrupting effect leading to the leakage of large fluorescent dyes from synthetic vesicles and hemoglobin from erythrocytes.[1–3]

Biophysical studies on these peptide nanostructures showed that the peptide backbone adopts, in the presence of vesicles, the helical conformation necessary for the lytic activity. They also demonstrated that the polarity of the peptide termini impacts strongly on the lytic activity.[1,3] We sought to exploit the unusual membrane-lytic ability of our crown-modified peptide nanostructures for developing efficient nanoscale chemotherapeutics. Our approach consists of preparing prodrug analogs of **1** that are inactive when given systemically, but convert *in vivo* into their active form by desired metabolic processes. Some anticancer prodrugs have been designed for selective activation by a specific enzyme secreted in proximity to cancer cells.[6–9] However, the results described herein constitute to our knowledge the first description of artificial membrane disrupting nanostructures designed to be activated by a specific proteolytic process.

To design activable nanochemotherapeutics, the core 14-mer **1** was linked through its *N*- or *C*-termini to negatively charged dipeptide chains. The peptide linkage between the cytolytic peptide agent and the dipeptide chain could be specifically cleaved by the proteolytic activity of prostate-specific membrane antigen (PSMA), a type II membrane protein over-expressed selectively in LNCaP prostate cancer cell line.[9–12]

Indeed, this enzyme has an extracellular catalytic activity able to hydrolyze progressively terminal γ-linked glutamate residues.[9–14] The high level of over-expression of PSMA exhibited by prostate cancer cells and its unique exopeptidase activity make it a very interesting model for developing prodrug activation processes and selective anticancer therapy.[10,13,14]

For the study, we prepared procytolytic peptide nanostructures **4–8** consisting of a 14-mer (**1**) bearing four crown ethers and modified at the *N*- or *C*-termini by two glutamic acid and/or aspartic acid residues linked by their α, β, or γ carboxyl group (Fig. 2). They were synthesized by a combination of solution and solid-phase synthesis, using the *tert*-butyloxycarbonyl/benzyl protection strategy. Target compounds **1–8** were purified by high performance liquid chromatography (HPLC), and characterized by circular dichroism and mass spectrometry. The synthesis and characterization are described elsewhere.[1]

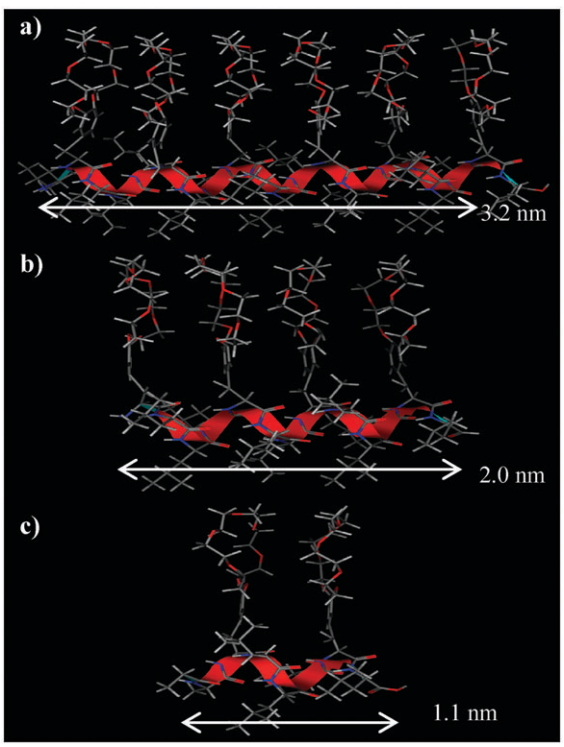
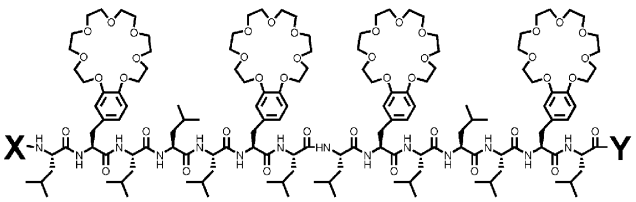



**Fig. 1** Average dimensions of (a) 21-, (b) 14- and (c) 7-mer peptide nanostructures under an α-helical conformation.

*Département de chimie and Centre de recherche sur la fonction, la structure et l'ingénierie des protéines, Faculté des sciences et génie, Université Laval, Québec, QC, Canada G1K 7P4. E-mail: normand.voyer@chm.ulaval.ca*

† Electronic supplementary information (ESI) available: Biological activity assays, flow cytometry, HeLa cell peptide uptake assays. See DOI: 10.1039/b800528a




| Entry | Compound | X | Y | $GI_{50}$ (μM)[a] | |
|---|---|---|---|---|---|
| | | | | LNCaP | PC-3 |
| **1** | $^{+}NH_3$-14-mer-OH | $H_2$ | -OH | >50 | >50 |
| **2** | Boc-14-mer-OMe | Boc- | -OMe | 43 | >50 |
| **3** | Boc-14-mer-OH | Boc- | -OH | 62 | 84 |
| **4** | γ-Glu-γ-Glu-14-mer-OH | γ-Glu- γ-Glu- | -OH | 19 | >100 |
| **5** | Glu-Glu-14-mer-OH | Glu-Glu- | -OH | >100 | >100 |
| **6** | Glu-Asp-14-mer-OH | Glu-Asp- | -OH | >50 | >50 |
| **7** | Boc-14-mer-γ-Glu-Glu-OH | Boc- | -γ-Glu-Glu-OH | 82 | >100 |
| **8** | Boc-14-mer-Glu-Glu-OH | Boc- | -Glu-Glu-OH | >50 | >50 |

[a]It should be noted that DMSO (the vehicle) has no observable effects.

**Fig. 2** $GI_{50}$ values from cell-growth inhibition assays of crown peptide nanostructures (average of triplicate runs, see supplementary information†).

The ability of peptide nanostructures **1–8** to inhibit cell proliferation was evaluated using two different cell lines: PSMA-positive LNCaP cells and PSMA-negative PC-3 cells.[13] The results are summarized in Fig. 2.

Consistent with our working model and previous results, the most lytic compound (**3**) inhibited cell growth of both cell lines without selectivity. Peptide nanostructure **3** is quite active with $GI_{50}$ of 62 and 84 μM for LNCaP and PC-3 cells respectively, and almost complete inhibition at 100 μM. On the other hand, no inhibition was observed in the case of $^{+}NH_3$-14-mer-OH **1** at tested concentrations. This could be due to the lower solubility of this zwitterionic peptide. Independently of the cell lines used, no inhibition occurs with *N*-terminal modified 14-mers **5** and **6** which have acidic dipeptides linked with the usual α linkage. However, modified 14-mer **4** with two γ-linked glutamate residues at the *N*-terminal position showed a significant selectivity toward LNCaP cells with a $GI_{50}$ of 19 μM. Contrarily, PC-3 cells not over-expressing PSMA were not affected by the presence of **4** within the concentration range used in the cell assay. Hence, it can be proposed that **4** exerts its cytotoxicity towards LNCaP cells by having its propeptide cleaved by the PSMA over-expressed at the surface of those cells.

To verify the toxicity against normal human cells, we performed a hemolysis assay[15] (Table 1). At 1 μM, no peptide nanostructures caused hemolysis. At 6 μM, the 14-mer modified at the *C*-terminal with a glutamic and/or aspartic acid chain (**7** and **8**) caused a significant hemoglobin leakage. Interestingly, no leakage occurs when using 14-mers modified at their *N*-terminal at every concentration studied. These observations support the proposal that the cytotoxicity of the peptide nanostructures under investigation can be modulated by modifying *N*-terminal groups. The absence of hemolysis activity of **4–6** is probably due to a decrease in the overall hydrophobicity of the peptide nanostructures resulting from addition of an aspartic acid and/or glutamic acid chain at the *N*-terminal. This is supported by recent studies finding that an increase of hydrophobicity leads to an increase in cytolytic activity toward eukaryotic cells.[15] Previous results showed that the inhibition of hemolytic activity of **4–6** is not due to an important disruption of their active helical conformation.[1]

**Table 1** Hemoglobin leakage induced by peptide nanostructures **3–8**

| Entry | Compound | Hemolysis (%) | | |
|---|---|---|---|---|
| | | 1 μM | 6 μM | 10 μM |
| — | DMSO[a] | 0 | 0 | 0 |
| **3** | Boc-14-mer-OH | −2 | <1 | 12 |
| **4** | γ-Glu-γ-Glu-14-mer-OH | −1 | <1 | 2 |
| **5** | Glu-Glu-14-mer-OH | 1 | <1 | <1 |
| **6** | Glu-Asp-14-mer-OH | −1 | <1 | <1 |
| **7** | Boc-14-mer-γ-Glu-Glu-OH | 0 | 15 | 45 |
| **8** | Boc-14-mer-β-Asp-Glu-OH | 3 | 13 | 23 |

[a] Used as solvent for all peptide solutions.

In order to determine how cancer cells died in the presence of **4**, apoptosis and necrosis assays have been performed (Fig. 3).[16] Apoptosis, or programmed cell death, is a process

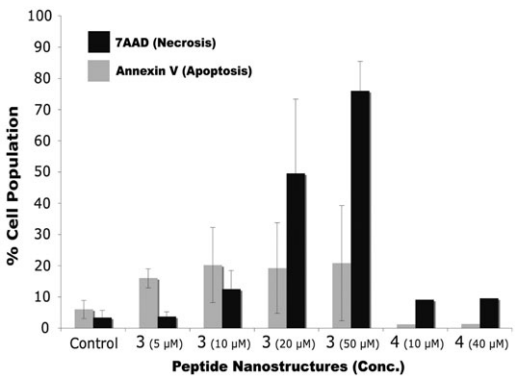



**Fig. 3** Results for PC-3 cells death mechanism (apoptosis or necrosis) when incubated with peptide nanostructures **3–4** (2 h in 5 nM Tes, 100 mM NaCl, pH 7.4).

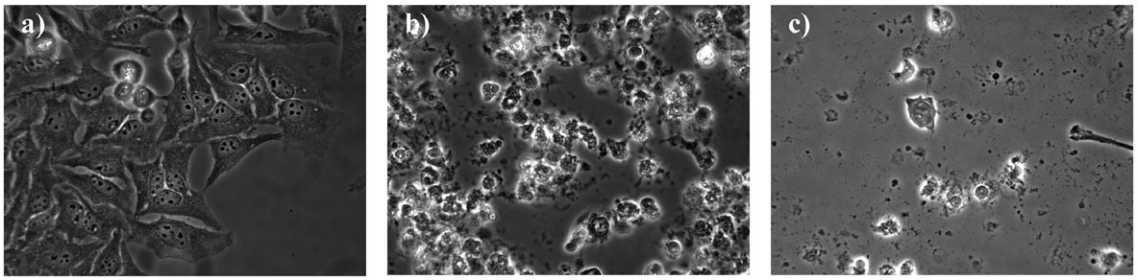



**Fig. 4** Phase contrast microscopy images of HeLa cells incubated with (a) 0 μM, (b) 100 μM of Boc-14-Mer-OH **3** for 48 h and (c) same as in (b) but illustrating the intact cell nucleus (see text for details).

in which cells actively participate in their destruction by triggering such processes as DNA fragmentation and protein mislocalization. Necrosis, or nonprogrammed cell death, is a process of cell death which is characterized by interorganelle swelling, depletion of ATP stores, and loss of plasma membrane integrity. In the present study, the fraction of apoptotic cells was determined by measuring the amount of phosphatidylserine (PS) binding protein Annexin V. Necrotic cells were determined by staining with 7-amino actinomycin D (7AAD), a membrane-impermeable dye that binds to DNA.

Compound **4** was added to PC-3 cells and, consistent with cell-growth inhibition assays, there was no significant fraction of apoptotic and necrotic cells ($<5\%$). Results also show that **3** induced a large amount of cell necrosis ($>70\%$ at 50 μM) which suggests that the crown peptide nanostructure acts as a membrane-disrupting agent. On the basis of the results with PC-3 cells and **3**, it is reasonable to assume that **4** uses a similar mode of action with LNCaP cells.[17]

To gather further evidence on the mechanism our nanostructures use on cell membranes, we performed additional experiments on HeLa cancer cells (PSMA-negative). We aimed to observe the action of **3** on the plasma membrane using microscopy.[18] Cancer cells were cultivated and then incubated with **3** for 48 h. The cells were then washed, fixed and mounted on glass slides in order to observe them by phase contrast microscopy. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) prior to mounting in order to assess the position of each cell. Fig. 4a shows cancer cells with no added peptide nanostructures. It should be noted that the solvent used (DMSO) has no observable effects. Fig. 4b illustrates the same cells with 100 μM of **3**; cell death was nearly complete. Indeed, the cell membranes were clearly destroyed. Interestingly, 4b and 4c were taken on the same glass slide and both show important membrane damage. In 4c, though, the nuclei are not as damaged as in 4b, suggesting that the nanostructure starts by disrupting the external membrane before the nucleus membrane. We also performed the same experiment with 50 μM of **3** and observed both altered and healthy cells, as expected since the concentration is near the $GI_{50}$ for Boc-14-mer-OH **3**.

In conclusion, we have described the first example of designed peptide nanostructures having the ability to attack and selectively destroy cancerous cells by puncturing their membranes after selective enzymatic activation. The overall activity profile of peptide nanostructures reported herein constitutes the first step towards efficient nanochemotherapeutics exploiting the unusual bioactivity of synthetic membrane active compounds of nanometre dimensions. Studies are currently being pursued to determine in more detail the mechanism of action of this new class of antitumor agents and to improve their potency.

The authors thank NSERC, FQRNT, the Université Laval and Gertrude Fiset (illustration).

## Notes and references

1 P. L. Boudreault and N. Voyer, *Org. Biomol. Chem.*, 2007, **5**, 1459.
2 E. Biron, F. Otis, J. C. Meillon, M. Robitaille, J. Lamothe, P. Van Hove, M. E. Cormier and N. Voyer, *Bioorg. Med. Chem.*, 2004, **12**, 1279.
3 Y. R. Vandenburg, B. D. Smith, E. Biron and N. Voyer, *Chem. Commun.*, 2002, 1694.
4 F. Otis, N. Voyer, A. Polidori and B. Pucci, *New J. Chem.*, 2006, **30**, 185.
5 For other elegant examples of peptide nanostructures see: (*a*) A. L. Sisson, M. R. Shah, S. Bhosale and S. Matile, *Chem. Soc. Rev.*, 2006, **35**, 1269; (*b*) S. Fernandez-Lopez, H. S. Kim, E. C. Choi, M. Delgado, J. R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D. A. Weinberger, K. M. Wilcoxen and M. R. Ghadiri, *Nature*, 2001, **412**, 452; (*c*) N. Sakai, J. Mareda and S. Matile, *Mol. BioSyst.*, 2007, **3**, 658; (*d*) J. T. Fletcher, J. A. Finlay, M. E. Callow, J. A. Callow and M. R. Ghadiri, *Chem.–Eur. J.*, 2007, **13**, 4008.
6 K. D. Bagshawe, C. J. Springer, F. Searle, P. Antoniw, S. K. Sharma, R. G. Melton and R. F. Sherwood, *Br. J. Cancer*, 1988, **58**, 700.
7 K. D. Bagshawe, S. K. Sharma, C. J. Springer, P. Antoniw, G. T. Rogers, P. J. Burke, R. Melton and R. Sherwood, *Antibody, Immunoconjugates, Radiopharm.*, 1991, **4**, 915.
8 S. K. Sharma, K. D. Bagshawe, P. J. Burke, J. A. Boden, G. T. Rogers, C. J. Springer, R. G. Melton and R. F. Sherwood, *Cancer*, 1994, **73**, 1114.
9 A. Mhaka, S. Janssen, A. Gady, M. Rosen and S. Denmeade, *Eur. J. Cancer*, 2002, **38**, S36.
10 A. Mhaka, A. M. Gady, D. M. Rosen, K. M. Lo, S. D. Gillies and S. R. Denmeade, *Cancer Biol. Ther.*, 2004, **3**, 551.
11 J. T. Pinto, B. P. Suffoletto, T. M. Berzin, C. H. Qiao, S. L. Lin, W. P. Tong, F. May, B. Mukherjee and W. D. W. Heston, *Clin. Cancer Res.*, 1996, **2**, 1445.
12 J. Pinto, B. Suffoletto, T. Berzin, C. Qiao, S. Lin, W. Tong and W. Heston, *FASEB J.*, 1996, **10**, 2862.
13 P. Warren, L. J. Li, W. Song, E. Holle, Y. Z. Wei, T. Wagner and X. Z. Yu, *Cancer Res.*, 2001, **61**, 6783.
14 S. R. Denmeade, A. Nagy, J. Gao, H. Lilja, A. V. Schally and J. T. Isaacs, *Cancer Res.*, 1998, **58**, 2537.
15 S. Castano, I. Cornut, K. Buttner, J. L. Dasseux and J. Dufourcq, *Biochim. Biophys. Acta*, 1999, **1416**, 161.
16 H. J. Hiddinga and N. L. Eberhardt, *Am. J. Pathol.*, 1999, **154**, 1077.
17 Due to technical limitations, it was not possible to perform the same experiment with LNCaP cells.
18 X. Y. Wang, X. F. Bao, M. McFarland-Mancini, I. Isaacsohn, A. F. Drew and D. B. Smithrud, *J. Am. Chem. Soc.*, 2007, **129**, 7284.