ORGANIC LETTERS

2008
Vol. 10, No. 1
5−8

A Luminescent Sensor for Tyrosine Phosphorylation

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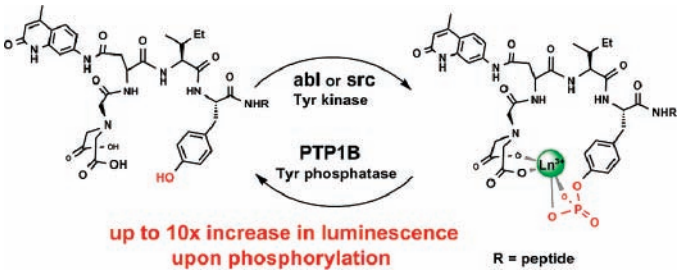
Received August 7, 2007

ABSTRACT



We have developed a luminogenic probe for tyrosine phosphorylation based on a short peptide sequence containing an iminodiacetate moiety near the site of phosphorylation. In response to kinase activity, the probe provides a strong luminescence enhancement, resulting from the increased ability of the probe to bind and sensitize Tb^{3+} and Eu^{3+} ions upon phosphorylation.

Phosphorylation and dephosphorylation of proteins and peptides are the principal chemical mechanisms of intracellular communication, and the protein kinases and phosphatases that control this chemistry have been implicated as key players in a number of important signal transduction pathways.[1] Not surprisingly, these enzymes have been targets of intense study, both for their therapeutic potential and their role in fundamental biochemical processes. Although a number of successful strategies for inhibition of both the substrate[2] and cofactor[3] binding sites of protein kinases have been developed, the design of optical reporters for measuring and imaging kinase activity has generally presented a more formidable challenge. On a supramolecular scale, protein phosphorylation has profound consequences for protein conformation and protein−protein interactions, from which it derives its powerful signaling ability. On a local chemical level, however, the event is relatively subtle with respect to the π-electronic changes typically exploited in standard approaches to optical switch development,[4] making it a relatively evasive property with regard to sensor design and requiring more creative reporting mechanisms to be invented.[5,6]

(1) A comprehensive issue on the role of protein phosphorylation in signaling: (a) *Chem. Rev.* **2001**, *101*(8). The chemical biology of signal transduction: (b) *Acc. Chem. Res.* **2003**, *36* (6).

(2) (a) Shen, K.; Keng, Y.-F.; Wu, L.; Guo, X.-L.; Lawrence, D. S.; Zhang, Z.-Y. *J. Biol. Chem.* **2001**, *276*, 47311−47319. (b) Yeh, R.-H.; Lee, T. R.; Lawrence, D. S. *J. Biol. Chem.* **2001**, *276*, 12235−12240. (c) Lee, J. H.; Nandy, S. K.; Lawrence, D. S. *J. Am. Chem. Soc.* **2004**, *126*, 3394−3395. (d) Hah, J.-M.; Sharma, V.; Li, H.; Lawrence, D. S. *J. Am. Chem. Soc.* **2006**, *128*, 5996−5997.

(3) Bridges, A. J. *Chem. Rev.* **2001**, *101*, 2541−2572.

(4) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515−1566.

(5) For small molecule/short peptide conjugates, see: (a) Bowman, B. F.; Peterson, J. A.; Stull, J. T. *J. Biol. Chem.* **1992**, *267*, 5346−5354. (b) Higashi, H.; Sato, K.; Ohtake, A.; Omori, A.; Yoshida, S.; Kudo, Y. *FEBS Lett.* **1997**, *414*, 55−60. (c) Chen, C.-A.; Yeh, R.-H.; Lawrence, D. S. *J. Am. Chem. Soc.* **2002**, *124*, 3840−3841. (d) Shults, M. D. and Imperiali, B. J. *Am. Chem. Soc.* **2003**, *125*, 14248−14249. (e) Chen, C.-A.; Yeh, R.-H.; Yan, X.; Lawrence, D. S. *Biochim. Biophys. Acta* **2004**, *1697*, 39−51. (f) Rothman, D. M.; Shults, M. D.; Imperiali, B. *TRENDS Cell Biol.* **2005**, *15*, 502−509. (g) Wang, Q.; Cahill, S. M.; Blumenstein, M.; Lawrence, D. S. *J. Am. Chem. Soc.* **2006**, *128*, 1808−1809. (h) Balakrishnan, S.; Zondlo, N. J. *J. Am. Chem. Soc.* **2006**, *128*, 5590−5591. (i) Wang, Q.; Dai, Z.; Cahill, S. M.; Blumenstein, M.; Lawrence, D. S. *J. Am. Chem. Soc.* **2006**, *128*, 14016−14017. (j) Tremblay, M. S.; Zhu, Q.; Martí, A. A.; Dyer, J.; Halim, M.; Jockusch, S.; Turro, N. J.; Sames, D. *Org. Lett.* **2006**, *8*, 2723−2726. (k) Choi, Y.; Ho, N.-H.; Tung, C.-H. *Angew. Chem., Int. Ed.* **2007**, *46*, 707−709. (l) Shults, M. D.; Janes, K. A.; Lauffenburger, D. A.; Imperiali, B. *Nature Methods* **2005**, *2*, 277−284.

(6) For protein-derived reporters, see: (a) Post, P. L.; Trybus, K. M.; Taylor, D. L. *J. Biol. Chem.* **1994**, *269*, 12880−12887. (b) Zhang, J.; Ma, Y.; Taylor, S. S.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14997−15002. (c) Ting, A. Y.; Kain, K. H.; Klemke, R. L.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 15003−15008. (d) Wang, Q.; Lawrence, D. S. *J. Am. Chem. Soc.* **2005**, *127*, 7684−7685.

10.1021/ol701920x CCC: $40.75
Published on Web 12/11/2007

Herein, we report the development of a tyrosine kinase reporter substrate that provides a significant increase in luminescence upon tyrosine phosphorylation (up to 10-fold). This probe enables a straightforward and practical assay for tyrosine phosphorylation where the optical readout can be programmed to appear at a variety of wavelengths ranging from ~490 to 700 nm depending on the choice of lanthanide metal.

As part of a program aimed at developing optical sensors for metabolic and signaling enzymes,[5j,7] we became interested in designing a reporting system for protein phosphorylation based on the luminescent lanthanide ions, whose sharp emission lines and long radiative lifetimes facilitate their spectral and temporal resolution from background fluorescence.[8] Since the luminescent lanthanides absorb light poorly ($\epsilon \sim 1\ M^{-1}\ cm^{-1}$), sensitization of the lanthanide excited-state via an organic photon antenna is required to achieve a useful luminescence intensity. We sought to exploit this fact, and the known ability of phosphates and phosphotyrosine[9] to bind trivalent lanthanides, to design a small peptide-based reporting domain whose ability to bind and sensitize a trivalent lanthanide ion would be dependent on the phosphorylation state of a proximal tyrosine residue. The presence of an appropriate sensitizing chromophore in the reporting domain would effectively gate the luminescence intensity as a function of peptide phosphorylation, allowing kinase and phosphatase activity to be measured.

Our general design was inspired by work done in the Lawrence group,[5c] which showed that a phosphorylated amino acid could serve as an iminodiacetate surrogate in ethylene glycol tetraacetate (EGTA)-type cooperative chelation of a divalent cation. Since the tricationic, oxophilic lanthanides can also be expected to bind with high affinity to such a motif,[8a] we envisioned a peptide containing an iminodiacetate moiety that would bind tightly to Tb^{3+} or Eu^{3+} and sensitize its luminescence as a function of nearby tyrosine phosphorylation (Figure 1). The intervening peptide sequence should contain an appropriate organic photon antenna, and the domain surrounding the key tyrosine residue should contain kinase recognition elements. Balakrishnan and Zondlo[5h] recently reported a strategy for monitoring serine phosphorylation that involves replacing a key glutamate residue in a Ca^{2+} (or Tb^{3+}) binding EF-hand domain with

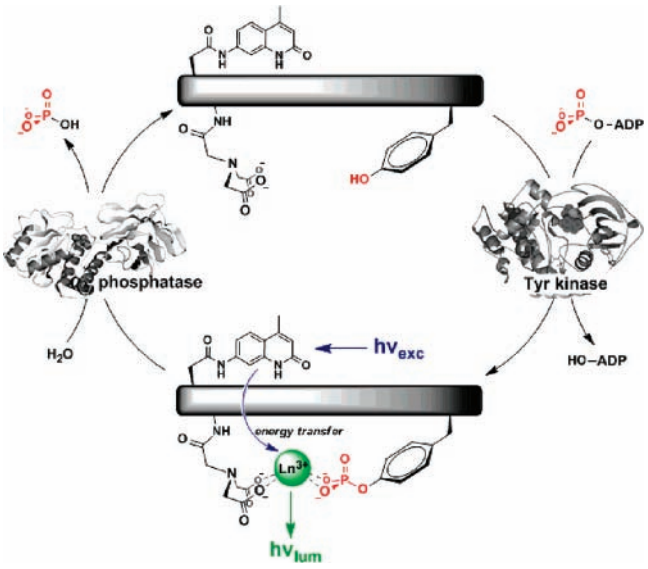
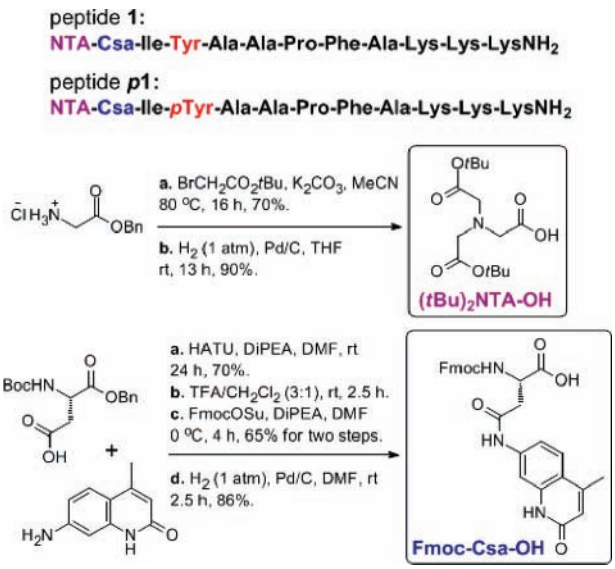


Figure 1. Design of a tyrosine kinase/phosphatase sensor that binds to Ln^{3+} (Ln = Tb or Eu) and sensitizes its luminescence as a function of phosphorylation state.

Ser/*p*Ser; the resulting domain binds and sensitizes Tb^{3+} as a function of the Ser phosphorylation state, since only *p*Ser is an adequate steric and electronic Glu mimic.

With these design features in mind, a pair of Tyr/*p*Tyr-containing peptides were prepared by standard Fmoc-based solid-phase peptide synthesis (**1** and ***p*1**, Scheme 1). Custom

Scheme 1



building blocks **(*t*-Bu)$_2$NTA-OH** and **Fmoc-Csa-OH** (NTA = nitrilotriacetic acid; Csa = carbostyril aspartamide) were readily synthesized from commercially available amino acid derivatives and facilitated the introduction of the desired iminodiacetate and organic photon antenna components,

(7) (a) Chen, G.; Yee, D. J.; Gubernator, N. G.; Sames, D. *J. Am. Chem. Soc.* **2005**, *127*, 4544−4545. (b) Froemming, M. K.; Sames, D. *Angew. Chem., Int. Ed.* **2006**, *45*, 637−642. (c) Yee, D. J.; Balsanek, V.; Bauman, D. R.; Penning, T. M.; Sames, D. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13304−13309. (d) Tremblay, M. S.; Halim, M.; Sames, D. *J. Am. Chem. Soc.* **2007**, *129*, 7570−7577. (e) Halim, M.; Tremblay, M. S.; Jockusch, S.; Turro, N. J.; Sames, D. *J. Am. Chem. Soc.* **2007**, *129*, 7704−7705.

(8) For a general review, see: (a) Parker, D.; Dickins, R. S; Puschmann, H.; Crossland, C.; Howard, J. A. K. *Chem. Rev.* **2002**, *102*, 1977−2010. (b) For a review of lanthanide-based switches, see: Gunnlaugsson, T.; Leonard, J. P. *Chem. Commun.* **2005**, 3114−3131. For recent examples of biologically relevant switches, see: (c) Lee, K.; Dzubeck, V.; Latshaw, L.; Schneider, J. P. *J. Am. Chem. Soc.* **2004**, *126*, 13616−13617. (d) Terai, T.; Kikuchi, K.; Iwasawa, S.-y.; Kawabe, T.; Hirata, Y.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 6938−6946.

(9) (a) dos Santos, C. M. G.; Fernández, P. B.; Plush, S. E.; Leonard, J. P.; Gunnlaugsson, T. *Chem. Commun.* **2007**, 3389−3391. (b) Atkinson, P.; Bretonniere, Y.; Parker, D.; Muller, G. *Helv. Chim. Acta* **2005**, *88*, 391−405. (c) Ringer, D. P.; Etheredge, J. L.; Dalrymple, B. L.; Niedbalski, J. S. *Biochem. Biophys. Res. Commun.* **1990**, *168*, 267−273. (d) Galvan, B.; Christopoulos, T. K. *Clin. Biochem.* **1996**, *29*, 125−131.

respectively (Scheme 1). The carbostyril 124 chromophore in **Csa** provides highly efficient sensitization of Tb^{3+} luminescence and also sensitizes Eu^{3+}, allowing the readout of this system to be extended to ~700 nm.[10] The sequence C-terminal to the Tyr residue contains recognition elements common to both the src and abl protein tyrosine kinases (PTKs).

In the presence of Tb^{3+} or Eu^{3+}, peptide **1** exhibits negligible emission, while its phosphorylated counterpart ***p*1** sensitizes both Tb^{3+} and Eu^{3+} luminescence (Figure 2a,b),

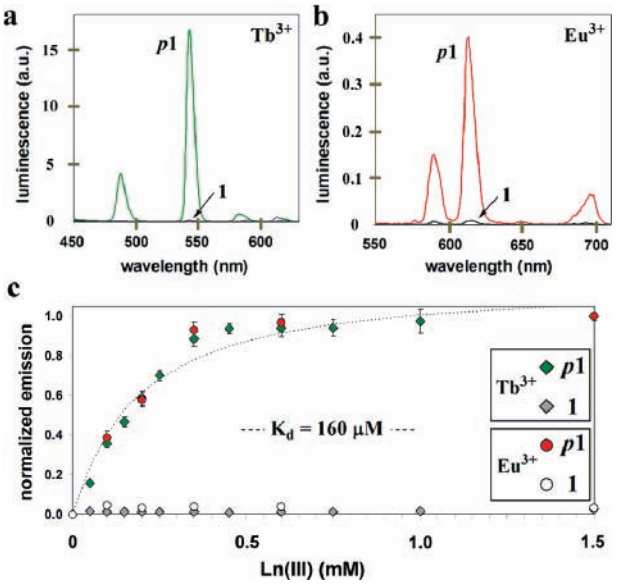


Figure 2. Emission spectra of **1** and ***p*1** in the presence of Tb^{3+} (a, gray and green lines, respectively) and Eu^{3+} (b, gray and red lines, respectively). Titration of **1**/***p*1** with Tb^{3+} (gray and green diamonds, respectively) and Eu^{3+} (white and red circles, respectively) (c) Conditions: 50 μM peptide, 1500 μM $LnCl_3$ [Ln = Tb (a) or Eu (b)] or 0−1500 μM $LnCl_3$ (c), 10 mM HEPES, pH 7.4; excitation, 340 nm; emission, 530−560 nm (Tb^{3+}) or 580−625 nm (Eu^{3+}). Error bars are derived from standard deviations between four independent trials.

constituting an excellent phosphorylation-responsive luminescent switch. The peptides were titrated with Tb^{3+} and Eu^{3+}, and as expected, the phosphorylated peptide ***p*1** exhibited a dramatic increase in Tb^{3+} and Eu^{3+} luminescence, which could be correlated to an apparent dissociation constant (K_d) of 160 μM (Figure 2c).[11] The nonphosphorylated peptide **1** showed no significant luminescence even after addition of 30 equiv of Tb^{3+}. While ***p*1**[Tb^{3+}] is more than an order of magnitude brighter than ***p*1**[Eu^{3+}] (compare parts a and b of Figure 2), the latter is still desirable for its red emission band centered at 696 nm.

We next turned our attention to monitoring the enzyme-catalyzed interconversion of **1** and ***p*1**. After incubation of **1** with src PTK and ATP for 20 h, a ~10-fold increase in Tb^{3+} luminescence was observed that was dependent on the presence of src PTK (Figure 3a). Detailed analysis of the

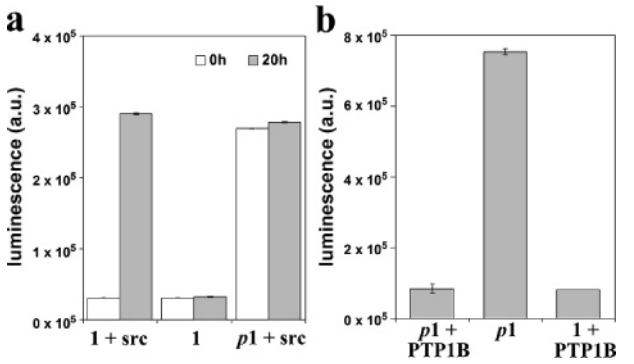


Figure 3. Phosphorylation of **1** catalyzed by Src PTK (a) and dephosphorylation of ***p*1** catalyzed by PTP1B (b). Conditions: (a) 250 μM peptide, 1.8 μg/mL src PTK, 1 mM ATP, 50 mM Tris·HCl, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% Brij 35, pH 7.5, 30 °C, 20 h; then diluted 10× with 10 mM HEPES (pH 7.4) containing $TbCl_3$ (750 μM); (b) 250 μM peptide, 1.0 μg/mL PTP1B, 5 mM HEPES, 100 mM NaCl, 5 mM DTT, pH 7.4, 37 °C, 1.5 h; then diluted with $TbCl_3$ as described for (a). Excitation at 340 nm; luminescence is the integrated emission intensity from 530−560 nm region. Error bars are standard deviations from three runs.

phosphorylation rate afforded the kinetic parameters for probe **1** (K_m = 56 ± 13 μM; V_{max} = 0.69 ± 0.09 μmol min^{-1} mg^{-1}), which are comparable to those obtained with the optimized peptide (K_m = 33 μM; V_{max} = 0.8 μmol min^{-1} mg^{-1}).[12] This data shows that the nonphysiological amino acid derivatives present in peptide **1** are well tolerated by the Src PTK. Hydrolysis of ***p*1** by protein tyrosine phosphatase 1B (PTP1B) also proceeded smoothly, giving a 9-fold decrease in luminescence intensity in the presence of the enzyme after 1.5 h (Figure 3b).[13]

Since the src and abl PTK families phosphorylate similar consensus peptide sequences, we also examined the reaction of **1** with abl PTK. Indeed, a time−course assay revealed an increase in luminescence that corresponded to phosphorylation of **1** as confirmed by HPLC and MALDI-MS analysis (Figure 4a, gray diamonds), and the kinetic parameters were obtained (K_m = 26 ± 6 μM; V_{max} = 0.8 ± 1 pmol min^{-1} unit^{-1}). Although the K_m value for probe **1** is several fold higher than the published value for an optimized peptide (K_m = 4 μM, EAIYAAPFAKKK),[12] the catalytic activity is similar as judged by comparison to the number provided for the optimized peptide by the commercial

(10) Li, M.; Selvin, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 8132−8138.

(11) A nonlinear least squares regression analysis was performed using Microsoft Excel. See: Long, J. R.; Drago, R. S. *J. Chem. Educ.* **1982**, *59*, 1037−1039.

(12) Songyang, Z.; Carraway, K. L.; Eck, M. J.; Harrison, S. C.; Feldman, R. A.; Mohammadi, M.; Schlessinger, J.; Hubbard, S. R.; Smith, D. P.; Eng, C.; Lorenzo, M. J.; Ponder, B. A. J.; Mayer, B. J.; Cantley, L. C. *Nature* **1995**, *373*, 536−539.

(13) Other NTA−Csa−peptide conjugates were investigated and also exhibited a marked increase in luminescence upon phosphorylation; however, the shape of the titration curve differed and the amount of excess metal added needs to be optimized for each sequence.

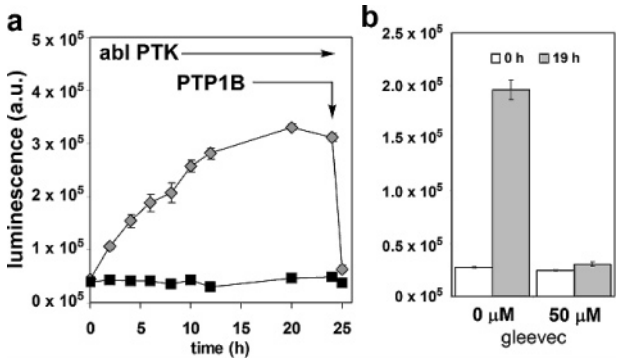


Figure 4. Time-course assay of the phosphorylation of **1** by abl kinase, followed by in situ dephosphorylation of ***p*1** by PTP1B (gray diamonds): **1** without abl (black squares) (a) Inhibition of phosphorylation of **1** by abl kinase with Gleevec (b). Conditions: 250 μM peptide, 350 units/mL abl PTK, 1 mM ATP, 50 mM Tris·HCl, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% Brij 35, pH 7.5, 30 °C, 0−24 h; at 24 h, 2.5 μg PTP1B added; aliquots removed and diluted 10× with 10 mM HEPES (pH 7.4) containing $TbCl_3$ (1.25 mM). Excitation: 340 nm, emission: 530−560 nm (luminescence was measured as the integrated emission intensity over this region). Error bars are derived from standard deviations between three independent trials.

vendor.[14] When the reaction was judged complete by comparison to a positive control, PTP1B was added to the assay mixture and a sharp decrease in luminescence intensity was observed that corresponded to complete hydrolysis of ***p*1** as judged by comparison to the negative control (Figure 4a, black squares). This experiment demonstrates that reversible protein phosphorylation can be probed in situ by the luminescent switching pair **1**/***p*1** in an operationally straightforward procedure that involves simply diluting an aliquot of the enzyme assay in buffer containing Tb^{3+} or Eu^{3+}.

(14) Src PTK was obtained from New England Biolabs.

Kinase inhibition can be readily evaluated using the reporter system. This was demonstrated by addition of imatinib mesylate (Gleevec), a known inhibitor of the abl kinase family and a successful drug for the treatment of chronic myelogenous leukemia,[15] which completely inhibited the phosphorylation reaction (no luminescence enhancement, Figure 4b).

In summary, we have developed a luminescent sensor for tyrosine kinase and phosphatase activity based on a peptide whose ability to bind and sensitize Tb^{3+} and Eu^{3+} is enhanced upon phosphorylation. Detection of src and abl kinase activity, as well as PTP1B-catalyzed phosphotyrosine hydrolysis, is facilitated by a dramatic 8−10-fold change in luminescence intensity that accompanies the interconversion of **1** and ***p*1**. Additionally, the luminescent output can be programmed to appear at a variety of wavelengths depending on the choice of lanthanide ion. The Eu^{3+} complex of ***p*1** has the longest wavelength emission of any phosphorylation sensor yet reported. The kinetic parameters show that probe **1** is comparable to the optimized peptide substrates or peptide reporters developed by others, and thus, the assay format described herein is suitable for in vitro evaluation of inhibitors and competitive substrates. The long radiative lifetimes of Tb^{3+} and Eu^{3+} will enable highly sensitive, background-free detection of kinase activity using time-resolved emission measurement.[8d]

Acknowledgment. We gratefully acknowledge financial support from the G. Harold & Leila Y. Mathers Charitable Foundation.

Supporting Information Available: Detailed synthetic procedures, spectral and photophysical characterization, and assay protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

OL701920X

(15) Wolf, D.; Tilg, H.; Rumpold, H.; Gastl, G.; Wolf, A. M. *Curr. Cancer Drug Targets* **2007**, *7*, 251−258.