Chinese Chemical Letters 31 (2020) 2945–2949

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Communication

Sensing cytochrome P450 1A1 activity by a resorufin-based isoform-specific fluorescent probe

Qiang Jin[a], Hongying Ma[a], Lei Feng[b], Ping Wang[a], Rongjing He[a], Jing Ning[b], Ling Yang[a], Guangbo Ge[a,*]

[a] Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
[b] College of Pharmacy, Dalian Medical University, Dalian 116044, China

ARTICLE INFO

Article history:
Received 11 May 2020
Received in revised form 17 May 2020
Accepted 26 May 2020
Available online 27 May 2020

Keywords:
Cytochrome P450 1A1
Fluorogenic sensor
Activity sensing
Resorufin
Isoform-specificity
Bioimaging

ABSTRACT

Cytochrome P450 1A1 (CYP1A1), a heme-containing monooxygenase, is of particular importance for human health because of its vital roles in the metabolic activation of pro-carcinogenic compounds to the carcinogens. Deciphering the relevance of CYP1A1 to human diseases and screening of CYP1A1 modulators require reliable tool(s) for probing this key enzyme in complex biological matrices. Herein, a practical and ultrasensitive fluorescence-based assay for real-time sensing CYP1A1 activities in biological systems has been developed, *via* designing an isoform-specific fluorogenic sensor for CYP1A1 (**CHPO**). The newly developed fluorogenic substrate for CYP1A1 has been carefully investigated in terms of specificity, sensitivity, precision, quantitative linear range and the anti-interference ability. The excellent selectivity, strong anti-interference ability and fast response kinetics, making the practicability of **CHPO**-based CYP1A1 activity assay is better than that of most reported CYP1A1 activity assays. Furthermore, **CHPO** has been successfully used for imaging CYP1A1 activities in living cells and human tissues, as well as for high-throughput screening of CYP1A1 inhibitors using tissue preparations as enzyme sources. Collectively, this study provided a practical fluorogenic sensor for real-time sensing CYP1A1 in complex biological systems, which would strongly facilitate the investigations on the relevance of CYP1A1 to human diseases and promote high-throughput screening of CYP1A1 modulators for biomedical applications.

The cytochromes P450 enzymes (CYPs) are a superfamily of hemoprotein monooxygenases that catalyze oxygen insertion into a vast array of substrates, including lipids, steroids, drugs and environmental toxins [1,2]. Over the past few decades, CYP-associated studies have been further extended from the xenobiotic metabolism field to almost all sub-fields in biology and life science, particularly in the subfield of human health and disease [3,4]. Cytochrome P450 1A (CYP1) subfamily plays critical roles in the metabolic activation of a great variety of procarcinogenic compounds to endotoxic intermediates or ultimate carcinogens, and thus has attracted increasing attention in the fields of toxicology and oncology [5]. In the human body, the CYP1A subfamily comprises of two members, including CYP1A1 and CYP1A2. CYP1A2 is mainly distributed in the human liver (~13%–15%), while CYP1A1 is mainly distributed in extrahepatic tissues [6,7].

Generally, CYP1A1 levels in lung and other extrahepatic tissues are extremely low, but this monooxygenase can be greatly induced by a variety of xenobiotics, *via* bioactivation of the aryl hydrocarbon nuclear receptor [8,9]. It has been reported that nicotine and benzo[α]pyrene can strongly induce human CYP1A1, which make the levels of CYP1A1 in the lung cells of smokers are much higher than that in nonsmokers [10,11]. Epidemiological studies have validated that the abnormal high expression of CYP1A1 is tightly linked to the pathogenesis of lung cancer [12,13]. Consistently, CYP1A1 inhibitors can be used to prevent the occurrence and development of lung cancer and other types of cancer [14–16]. In addition, recent studies have found that CYP1A1 plays crucial roles in the development of other diseases, such as nonalcoholic fatty liver disease (NAFLD) [17].

Deciphering the key roles of CYP1A1 in human diseases or xenobiotic metabolism, as well as screening of CYP1A1 modulators, require reliable and practical tool(s) for accurately sensing this key enzyme in complex biological systems. Over the past two decades, optical probes have been widely used for sensing a great variety of biomolecules, owing to their inherent advantages

* Corresponding author.
E-mail address: geguangbo@dicp.ac.cn (G. Ge).

https://doi.org/10.1016/j.cclet.2020.05.038
1001-8417/

including ultra-high sensitivity, noninvasiveness, easy-to-use, high spatiotemporal resolution and suitable for high-throughput detection [18–25]. Although several fluorogenic substrates or sensors for CYP1A enzymes have been reported [26–28], the isoform-specific fluorogenic sensor for highly sensitive sensing of CYP1A1 activities in complex biological systems have been rarely reported. In addition, the previously reported fluorogenic substrates for CYP1A1 have some limitations, such as poor sensitivity, poor anti-interference ability and narrow linear response ranges [29,30]. Thus, it is urgent and necessary to develop more practical and accurate assays for highly selective and ultra-sensitive probing CYP1A1 activities in complex biological matrix.

In this study, to get an ideal substrate-type fluorogenic sensor for CYP1A1, resorufin was selected as the basic fluorophore, owing to its good physical-chemical and fluorescence properties, including good water solubility, good cell-permeability and easy fluorescence quenching *via* 7-hydroxy substitution [31,32]. Hence, a series of *O*-alkylated resorufin derivatives were purposely designed and then evaluated their potential as good substrates for CYP1A1 through reaction phenotyping-based experimental screening. Following phenotypic screening, we found that all synthesized *O*-alkylated derivatives could be rapidly dealkylated by CYP1A, while chloroethyl derivative (7-(2-chloroethoxy)-3*H*-phenoxazin-3-one, **CHPO**) displayed the best combination of isoform-specificity (Fig. 1a and Fig. S1 in Supporting information), high sensitivity and good reactivity towards CYP1A1 over other human CYPs. Meanwhile, molecular docking simulation was carried out to provide a deeper insight into CYP1A1 mediated CHPO dechloroethylation. As shown in Figs. 1b and c, CHPO created strong interactions with CYP1A1 *via* van der Waals forces and π-π stacking interactions, while the distance between the *O*-demethylation site of CHPO and heme of CYP1A1 was 4.2 Å. These findings suggested that CHPO is a good substrate for CYP1A1, which inspired us to fully characterize the performance and the applicability of **CHPO** for sensing CYP1A1 activities in complex systems, as well as for high-throughput screening of CYP1A1 inhibitors in complex biological samples. The synthetic procedure of **CHPO**, as well as structural characterization by HRMS, ^{1}H NMR, and ^{13}C NMR are described in Scheme S1 and Figs. S11-S13 (Supporting information).

With this probe in hand, we firstly investigated the sensing ability of CHPO to CYP1A1 under physiological conditions (pH 7.4 at 37 °C). Fortunately, CHPO was very stable in phosphate buffer (100 mmol/L, a simulated physiological pH 7.4) without any metabolites or degradation products detected after 30 min incubation at 37 °C, but it could be rapidly converted to a single metabolite by CYP1A1 (Fig. S2 in Supporting information). The newly developed fluorogenic sensor CHPO displayed an absorption band at 460 nm, while a new absorption band centered at 560 nm emerged upon addition of CYP1A1 (Fig. S3a in Supporting information). Such changes in absorption bring the solution from colorless to yellow, which could be clearly distinguished by "naked eyes". Correspondingly, upon addition of CYP1A1, a strong red fluorescence emission was recorded at 590 nm (Fig. S3b in Supporting information). The time course of the fluorescence spectra changes following co-incubation of CHPO (5 μmol/L) with CYP1A1 were carefully investigated and depicted in Fig. 2a, upon addition of CYP1A1, the fluorescence intensities were linearly related to the incubation times of up to 30 min (Fig. 2b). The effects of pH values on the fluorescent intensities of CHPO and metabolite resorufin were also performed. As shown in Fig. S4 (Supporting information), the fluorescence intensity of resorusfin was stable over the pH range of 7–11. These results demonstrated that CHPO could serve as a practical long-wavelength "turn-on" fluorogenic sensor for sensing the real activities of CYP1A1 under physiological conditions.

Specificity is one of the most important features of a fluorogenic sensor for sensing or imaging the target enzyme(s) in living systems. In this study, a panel of P450 isoforms and endogenous molecules were used to evaluate possible interferences on CHPO. To our delight, only CYP1A1 caused remarkable changes in fluorescence response, whereas no obvious changes in fluorescence detection were observed upon addition of other CYP isoforms (Fig. 2c) and common metal ions and amino acids

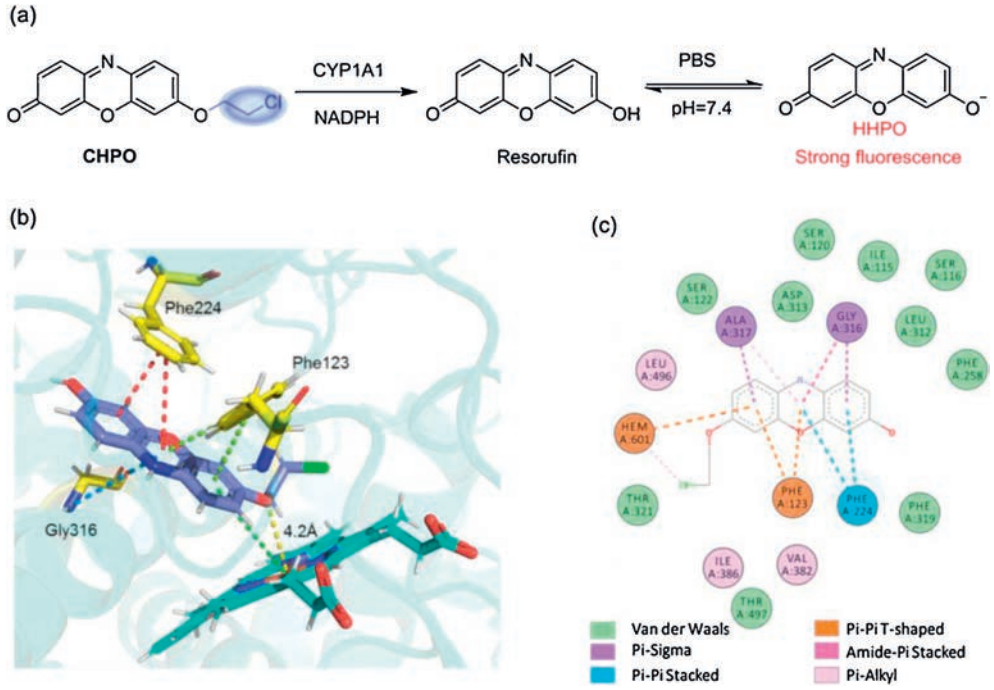

Fig. 1. (a) Structure of **CHPO** and its fluorescence response toward CYP1A1. (b) Predicted binding pose of **CHPO** within the catalytic cavity of CYP1A1. (c) The 2D representation of interactions between **CHPO** and the key residues in the catalytic cavity of CYP1A1.

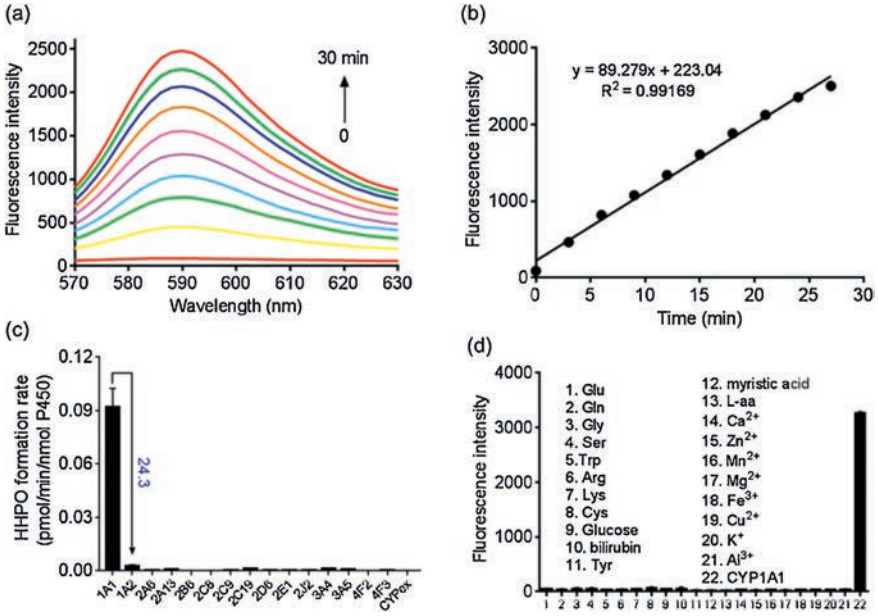

Fig. 2. (a) Time-course of the fluorescence response of **CHPO** (1 μmol/L) in the presence of CYP1A1 (2 nmol/L). (b) The linear relationship between the fluorescence intensity of HHPO and incubation time. λ_{ex} = 540 nm. Fluorescence changes of **CHPO** (5 μmol/L) upon addition of different (c) human P450 isoforms and (d) various analytes in aqueous solution.

(Fig. 2d and Fig. S5 in Supporting information). Based on these results, we can say that probe CHPO displayed excellent selectivity for CYP1A1 over other biologically relevant species and could be of value for highly selective sensing of CYP1A1 in complex biological samples.

To further confirm that the fluorescence changes in complex biological systems were CYP1A1-dependent, a series of selective inhibitors of human CYP isoforms were used to inhibit CHPO *O*-demethylation in human liver preparations. As shown in Fig. S6 (Supporting information), the formation of resorufin could be potently inhibited by ABT (a broad CYP inhibitor) and furafylline (a selective and potent inhibitor of CYP1A). By contrast, inhibitors of other CYP isoforms displayed minor effects on this (CYP1A mediated) biotransformation. These results indicated that the CHPO *O*-demethylation was selectively catalyzed by CYP1A in human tissues. Next, the enzymatic kinetics of CHPO *O*-dechloroethylation in different enzyme sources including human liver microsomes (HLM), CYP1A1 and CYP1A2 was well characterized under physiological conditions (pH 7.4 at 37 °C). As shown in Fig. S7 (Supporting information), CYP1A1-mediated CHPO *O*-dechloroethylation in both HLM and recombinant CYP1A isoforms followed Michaelis-Menten kinetics, as evidenced by the corresponding Eadie-Hofstee plots. Compared with CYP1A2, both CYP1A1 and HLM catalyzed CHPO *O*-dechloroethylation in a more efficient way, displaying very high affinity (K_m < 1 μmol/L) and good reactivity in CHPO *O*-dechloroethylation (Table S1 in Supporting information). As a result, the metabolic clearance of CHPO *O*-dechloroethylation in CYP1A1 is more than 100-fold of that in CYP1A2. These findings suggested that CYP1A2 is hardly catalyzed CHPO *O*-dechloroethylation, while CHPO can act as a highly specific probe for CYP1A1 for quantitative detection of CYP1A1 in complex biological systems.

The detection sensitivity of CHPO for sensing CYP1A1 was also investigated. As shown in Fig. S8 (Supporting information), an excellent linear correlation (R = 0.99) between fluorescence response and CYP1A1 concentration was present within the range of 0.15 nmol/L to 0.9 nmol/L, with the limit of detection as low as 0.073 nmol/L. Such high sensitivity can be attributed to the very low background fluorescence signal around 590 nm of metabolite resorufin.

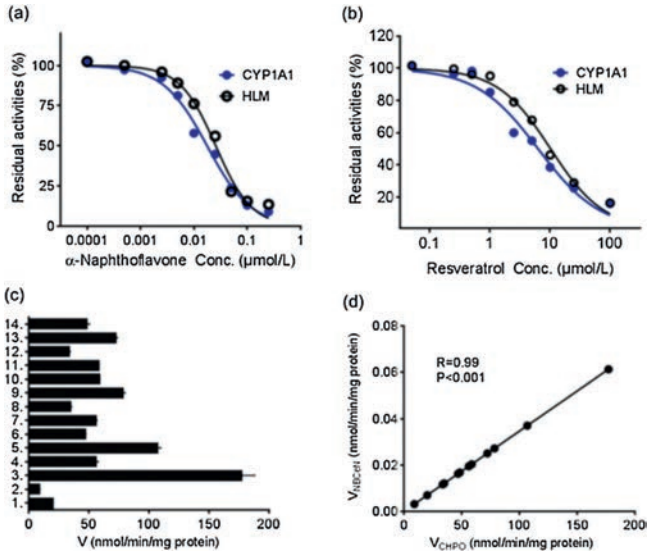

Fig. 3. Dose-inhibition curves of α-naphthoflavone (a) and resveratrol (b) on **CHPO** *O*-demethylation in HLM and CYP1A1, respectively. (c) CYP1A1 activities in a panel of fourteen individual HLMs using **CHPO** as the probe substrate. (d) Correlation analysis between the *O*-dechloroethylation rates of **CHPO** and that of **NBCeN** in a panel of individual HLM samples (n = 14).

Encouraged by the above findings, we next investigated the utility of CHPO for screening of CYP1A1 inhibitors by using human tissue preparations as enzyme sources. As shown in Figs. 3a and b, both the inhibition curves and the IC_{50} values of two known CYP1A inhibitors (α-naphthoflavone, resveratrol) towards CHPO-*O*-dechloroethylation in HLM were similar to those in CYP1A1 (Table S2 in Supporting information). These findings further confirmed that CHPO could be used as a highly selective fluorescent probe of CYP1A1, for high throughput screening of CYP1A1 modulators by using tissue preparations as enzyme sources. The applications of CHPO for measuring the real activities of CYP1A1 in human biological samples were then investigated. As depicted in Fig. 3c,

CHPO was used to determinate CYP1A1 activities in a panel of 14 HLMs. About 6-fold difference in CYP1A1 activity was observed among individuals, which was in accordance with previous literature reported variability in CYP1A1 activity assayed by other non-optical CYP1A1 substrates [33,34]. Moreover, a strong correlation with a high coefficient parameter ($R^2 = 0.96$, $P < 0.0001$) was gained between CHPO *O*-dechloroethylation rates and the *O*-dechloroethylation rates of NBCeN (a reported available probe of CYP1A1) (Fig. 3d). These findings strongly suggested that CHPO could be employed to measure the real activities of CYP1A1 in real samples, and the quantification was highly reliable.

To validate the practicality of the probe for sensing CYP1A1 activity in living cells, cell cytotoxicity assays were conducted. MTT assays showed that cell viability for cells was more than 80% upon addition of CHPO (5 μmol/L, about 20-fold of the K_m value) for 48 h (Fig. S9 in Supporting information), suggesting the low toxicity of the probe. In this case, confocal fluorescence imaging of CYP1A1 with CHPO (5 μmol/L) in living cells was then carried out (Fig. 4). As expected, after treated cells with CHPO for 90 min, brightly red fluorescence signals inside cells were observed (Figs. 4b and d). In sharp contrast, the fluorescence intensity was decreased significantly when the cells were pre-treated with resveratrol (a specific CYP1A1 inhibitor) for 30 min (Figs. 4c and e). This finding clearly demonstrated that the occurrence of fluorescence in red channel was dependent on the *O*-dechloroethylation of CHPO by intracellular CYP1A1. Meanwhile, the practicability of CHPO for sensing CYP1A1 in deep tissues was also investigated. Following co-incubation of CHPO with mouse lung slice in PBS (pH 7.4 at 37 °C) for 60 min, strong red fluorescence could be observed in the cytoplasm of mouse lung cells when excited at 540 nm (Fig. S10 in Supporting information). All these results revealed that CHPO could be used for fluorescence imaging of the real activities of CYP1A1 in living matrices, also suggested that CHPO was cell membrane permeable and capable of *in-situ* sensing endogenous CYP1A1 in living cells and tissues.

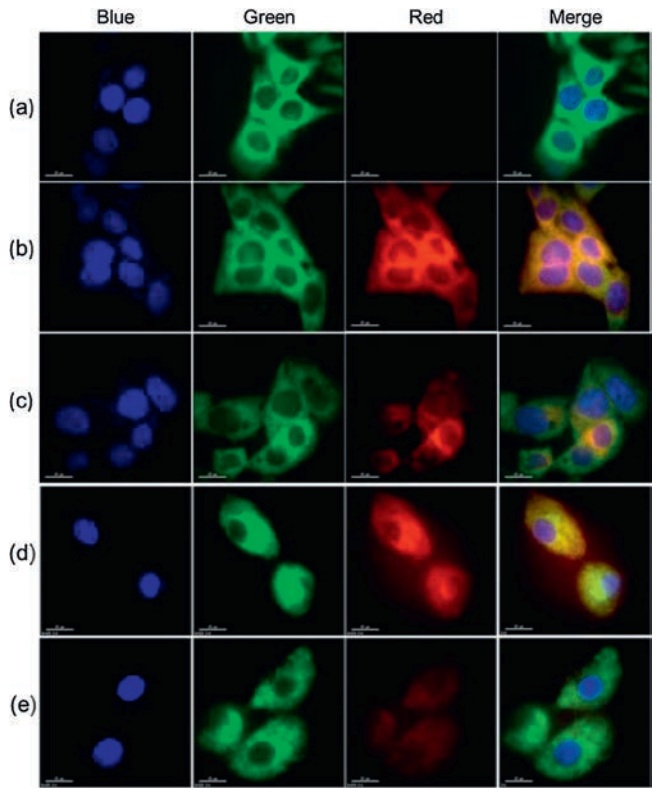

Fig. 4. Confocal fluorescence imaging of CYP1A1 in living HepG2 (a-c) and A549 cells (d-e). (a) The HepG2 cells treated with Hoechst 33342 (blue channel), ER tracker (green channel) for 20 min, (b) HepG2 and (d) A549 cells treated with CHPO (red channel) and two fluorescent dyes with clear subcellular localization (Hoechst 33342 and ER tracker); (c) HepG2 and (e) A549 cells pre-treated with a CYP1A inhibitor (resveratrol, 100 μmol/L) for 60 min and then incubated with CHPO in the presence of Hoechst 33342 and ER tracker.

In summary, on the basis of the substrate preference and catalytic properties of CYP1A1, a new resorufin derived fluorogenic sensor (CHPO) has been developed and characterized as a highly selective and isoform-specific fluorogenic sensor for CYP1A1. Our results demonstrated that CYP1A1 mediated CHPO *O*-dechloroethylation displayed excellent isoform specificity, good reactivity and high sensitivity. CHPO has been applied for sensing CYP1A1 activities in a panel of human biological samples, as well as for high throughput screening of CYP1A1 inhibitors by using tissue preparations as enzyme sources. Furthermore, CHPO was a cell membrane permeable agent, which has been confirmed by cellular imaging of endogenous CYP1A1 in living cells and tissue slice. Collectively, our findings suggested that CHPO was a practical optical substrate for sensing CYP1A1 activities in real samples, while this fluorogenic sensor provided a promising tool for exploring the biological roles of CYP1A1 in human diseases or xenobiotic metabolism.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 81922070, 81973286, 81803489, 81773687 and 81703604), the National Key Research and Development Program of China (Nos. 2016YFC1303900, 2017YFC1700200 and 2017YFC1702000), the Three-year Action Plan of Shanghai TCM Development (No. ZY-(2018-2020)-CCCX-5001), Drug Innovation Major Project (No. 2018ZX09731016), Program of Shanghai Academic/ Technology Research Leader (No. 18XD1403600), and Shuguang Program (No. 18SG40) supported by Shanghai Education Development Foundation and Shanghai Municipal Education Commission.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cclet.2020.05.038.

References

[1] U.M. Zanger, M. Schwab, Pharmacol. Ther. 138 (2013) 103–141.
[2] P. Manikandan, S. Nagini, Curr. Drug Targets 19 (2018) 38–54.
[3] W. Wang, K.Z. Sanidad, G. Zhang, Adv. Exp. Med. Biol. 1161 (2019) 115–123.
[4] L.N. Sausville, S.M. Williams, A. Pozzi, Pharmacol. Ther. 196 (2019) 183–194.
[5] N.J. Gooderham, S. Murray, A.M. Lynch, et al., Drug Metab. Dispos. 29 (2001) 529–534.
[6] D. Lang, M. Radtke, M. Bairlein, Chem. Res. Toxicol. 32 (2019) 1115–1122.
[7] H. Bulus, S. Oguztuzun, G. Guler Simsek, et al., Biotech. Histochem. 94 (2019) 1–9.
[8] O. Hankinson, Annu. Rev. Pharmacol. Toxicol. 35 (1995) 307–340.
[9] C. Knebel, T. Heise, U.M. Zanger, et al., Food Chem. Toxicol. 123 (2019) 481–491.
[10] J.C. Willey, E.L. Coy, M.W. Frampton, et al., Am. J. Respir. Cell Mol. Biol. 17 (1997) 114–124.
[11] J.H. Kim, M.E. Sherman, F.C. Curriero, et al., Toxicol. Appl. Pharmacol. 199 (2004) 210–219.
[12] R.E. Go, K.A. Hwang, K.C. Choi, J. Steroid Biochem. Mol. Biol. 147 (2015) 24–30.
[13] I. Spyrou, S. Sifakis, A. Ploumidis, et al., Tumor Biol. 35 (2014) 9549–9556.
[14] V.P. Androutsopoulos, A.M. Tsatsakis, D.A. Spandidos, BMC Cancer 9 (2009) 187.
[15] G.T. Diaz-Gerevini, G. Repossi, A. Dain, et al., Nutrition 32 (2016) 174–178.

[16] N.J. Horley, K.J.M. Beresford, S. Kaduskar, et al., Bioorg. Med. Chem. Lett. 27 (2017) 5409–5414.
[17] B. Huang, J. Bao, Y.R. Cao, et al., Biochem. (Mosc) 83 (2018) 595–602.
[18] Y. Chen, T. Wei, Z. Zhang, et al., Chin. Chem. Lett. 28 (2017) 1957–1960.
[19] L. Ding, Z. Tian, J. Hou, et al., Chin. Chem. Lett. 30 (2019) 558–562.
[20] Q. Jin, L. Feng, D.D. Wang, et al., ACS Appl. Mater. Interfaces 7 (2015) 28474–28481.
[21] Q. Jin, L. Feng, D.D. Wang, et al., Biosens. Bioelectron. 83 (2016) 193–199.
[22] J. Chan, S.C. Dodani, C.J. Chang, Nat. Chem. 4 (2012) 973–984.
[23] D. Chen, W.J. Qin, H.X. Fang, et al., Chin. Chem. Lett. 30 (2019) 1738–1744.
[24] Z.Z. Shao, C. Zhang, X.H. Zhu, et al., Chin. Chem. Lett. 30 (2019) 2169–2172.
[25] Z.J. Zhang, F. Wang, X.Q. Chen, Chin. Chem. Lett. 30 (2019) 1745–1757.
[26] Z.R. Dai, G.B. Ge, L. Feng, et al., J. Am. Chem. Soc. 137 (2015) 14488–14495.
[27] X. Zhang, Y. Zhou, X. Gu, et al., Talanta 186 (2018) 413–420.
[28] C. Zhang, A.M. Ren, J.F. Guo, et al., Phys. Chem. Chem. Phys. 20 (2018) 13290–13305.
[29] Z.R. Dai, L. Feng, Q. Jin, et al., Chem. Sci. 8 (2017) 2795–2803.
[30] J. Ning, Z. Tian, B. Wang, et al., Mater. Chem. Front. 2 (2018) 2013–2020.
[31] H. Li, W. Liu, F. Zhang, et al., Anal. Chem. 90 (2018) 855–858.
[32] X. Wu, X. Li, H. Li, W. Shi, H. Ma, Chem. Commun. (Camb.) 53 (2017) 2443–2446.
[33] H. Schweikl, J.A. Taylor, S. Kitareewan, et al., Pharmacogenetics 3 (1993) 239–249.
[34] X. Ding, L.S. Kaminsky, Annu. Rev. Pharmacol. Toxicol. 43 (2003) 149–173.