Accepted Manuscript

Modification of existing antibiotics in the form of precursor prodrugs that can be subsequently activated by nitroreductases of the target pathogen

Ayhan Çelik, Gülden Yetiş, Mehmet Ay, Tuğba Güngör

PII: S0960-894X(16)30692-8
DOI: http://dx.doi.org/10.1016/j.bmcl.2016.06.081
Reference: BMCL 24034

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 9 April 2016
Revised Date: 25 June 2016
Accepted Date: 27 June 2016

Please cite this article as: Çelik, A., Yetiş, G., Ay, M., Güngör, T., Modification of existing antibiotics in the form of precursor prodrugs that can be subsequently activated by nitroreductases of the target pathogen, *Bioorganic & Medicinal Chemistry Letters* (2016), doi: http://dx.doi.org/10.1016/j.bmcl.2016.06.081

Modification of existing antibiotics in the form of precursor prodrugs that can be subsequently activated by nitroreductases of the target pathogen

Ayhan Çelik,[a *] Gülden Yetiş[a] Mehmet Ay[b] and Tuğba Güngör[b]

[a]*Department of Chemistry, Gebze Technical University Cayirova Campus, Gebze-Kocaeli, 41400, Turkey*

[b]*Department of Chemistry, Canakkale 18 Mart University, Çanakkale, Turkey*

* Corresponding author: E-mail:ayhancelik@gtu.edu.tr (Ayhan Çelik); Tel: +90 2626053087, Fax: +90 262 605 3005

Abstract- The use of existing antibiotics in the form of prodrug followed by activation using enzymes of pathogenic origin could be a useful approach for antimicrobial therapy. To investigate this idea, a common antibiotic, sulfamethoxazole has been redesigned in the form of a prodrug by simple functional group replacement. Upon reductive activation by a type I nitroreductase from a pathogen, the drug displayed enhanced antimicrobial capacity. This strategy could improve the efficacy and selectively of antibiotics and reduce the incidence of resistance.

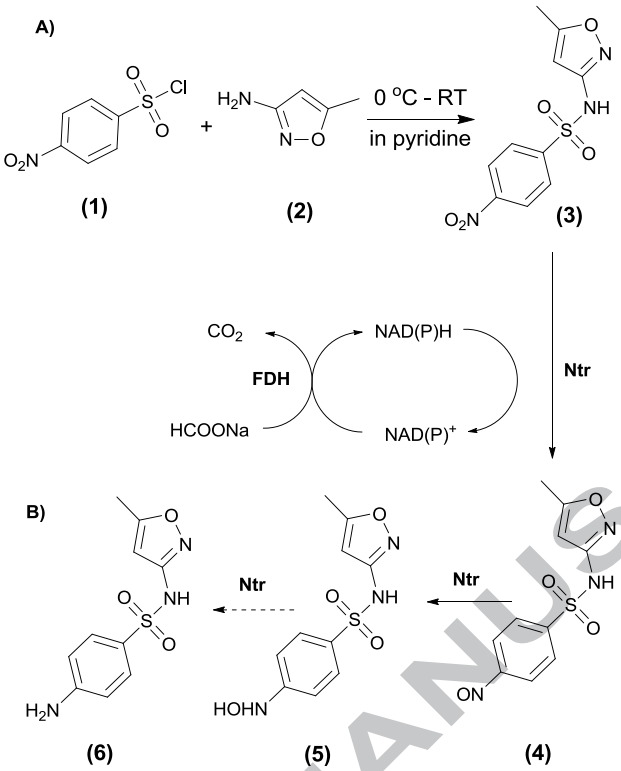
Keywords: nitroreductase, prodrugs, sulfamethoxazole

Since its original development in 1958, the prodrug strategy has been extensively utilized to improve the physicochemical and pharmacokinetic properties of drugs in order to lessen their adverse effects. In fact, approximately one third of drugs currently on the market are in the form of prodrugs with improved ADMET properties.[1] Moreover, this strategy has evolved into several novel applications that have attracted increasing attention over the past 20 years and has recently entered clinical trials for cancer therapy.[2] The prodrug concept is based on the conversion of an inactive, or less active, derivative of a drug (prodrug) into an active drug (or its metabolites), which can then stimulate its desired pharmacological effect in the body. This conversion can either be chemically or enzymatically stimulated. Among the enzymes that are traditionally used for activation are unspecific esterases,[3] alkaline phosphateses,[4] peptidases[5] and specific cytochrome P450s.[6,7]

Nitroreductases (NTRs) are a relatively new class of enzyme in the area of prodrug activation.[8,9] Unlike traditional enzymes, which activate mostly carrier-linked prodrugs (drug with promoiety), NTRs are involved in the activation of bioprecursor prodrugs, which are transformed by direct reduction or reduction followed by elimination to the active agent.[10] The best known example is the combination of NfsB from *E. coli* with CB1954, which are currently in clinical trials (phase I/II) for patients with late stage tumors.[11,12,13,14] Given their versatility, we decided to explore the potential use of NTR enzymes for antibiotic activation. The work comprises the synthesis of novel 'Pre-antibiotics' of known antibiotics and their reductive activation by a NTRs followed by metabolite analysis and characterization. By adopting this strategy, it might then be possible to re-design existing drugs as precursor prodrugs that could be activated by bacterial NTRs from pathogens of interest for enhanced efficacy and possibly selective antimicrobial activity. As prodrug activating enzymes, NTRs from pathogenic sources have a number of advantages. Specifically, (i) the absence, or low catalytic activities, of mammalian equivalent enzymes to the bacterial NTRs, and (ii) differences in substrate specificity between the bacterial NTRs and the corresponding mammalian enzymes. These properties help prevent nonspecific prodrug activation at sites other than the pathogen itself.

In order to test the principle, there are two main requirements: (i) a NTR from a pathogen, and (ii) a drug molecule that is used for the treatment of diseases caused by the pathogen of interest. Therefore, we have focused on Urinary Tract Infections (UTI) and sulphonamide-based antibiotics that have been used to treat UTIs. Based on whole genome sequence data, a novel NTR from *Staphylococcus saprophyticus*, has been cloned and the

recombinant enzyme purified in an active form (named SsapNtrB).[15] *S. saprophyticus* is an uropathogenic Staphylococcus usually isolated from young female patients presenting with uncomplicated UTI.[16] Sulfamethoxazole (SMX) is a sulphonamide bacteriostatic antibiotic, which is used to treat UTI and RTI (Respiratory Tract Infections). The antibiotic is usually given in combination with trimethoprim (in a 5:1 ratio in co-trimoxazole) to combat infections caused by most Gram-positive and many Gram-negative organisms including Streptococcus, *Staphylococcus aureus* (including MRSA), *Escherichia coli*, *Haemophilus influenza*, and oral anaerobes.[17] SMX is on the World Health Organization's List of Essential Medicines-2015, as one of the most important medications required in a basic healthcare system (http://www.who.int/medicines).[18] The prodrug form of sulfamethoxazole, namely N-(5-methylisoxazol-3-yl)-4-nitrobenzenesulfonamide (**3**), was synthesized following a literature procedure [19] using 4-nitrobenzenesulfonyl chloride (**1**) and 3-amino-5-methylisoxazol (**2**) (Scheme 1A) in 53% yield. Detailed synthetic steps and characterisation of 3 has been described in the ESI†. Briefly, 4-nitrobenzenesulfonyl chloride was added to a solution of 3-amino-5-methylisoxazol in pyridine at 0˚C. After stirring at this temperature for few hours, the mixture was allowed to warm to room temperature and stirred for a further 24 h. The solution was then poured into cold water. The resulting yellow precipitate was filtered, washed several times with water and dried at room temperature. The crude product was purified by crystallization with hexane/ethyl acetate.



Scheme 1. (A) Synthesis of the prodrug of sulfamethoxazole (NO2-SMX, **3**) and (B) its possible reduction products (NO-SMX, **4**; N4-OH-SMX, **5** and SMX, **6**) catalysed by a nitroreductase enzyme (SsapNtrB) together with a cofactor regeneration system (middle).

Direct reductive activation of **3** (NO_2-SMX) catalysed by *Ssap*NtrB in the presence of NADH or NADPH (or with a cofactor regeneration system) *in vitro*, results in potentially three metabolites: a nitroso (NO-SMX, **4**), a N^4-hydroxylamino (N-OH-SMX, **5**) and an amine derivative (SMX, **6**) of the prodrug (Scheme 1B) depending on the relative stability of the metabolites. It should be noted that the amine derivative (SMX) is the actual antibiotic, SMX. Preliminary activity experiments with a fixed substrate concentration showed that the purified enzyme has potential for prodrug activation. The rate of depletion of NADH was observed by monitoring the absorbance at 340 nm. (Fig. 1A). Therefore, the reductive metabolism of 3 by SsapNtrB was investigated in more detail. HPLC analysis together with LC-MS/MS analysis revealed that the dominant metabolite of **3** is the N^4-hydroxy-sulfamethoxazole, together with a small quantity of **4**. (Fig. 1B).

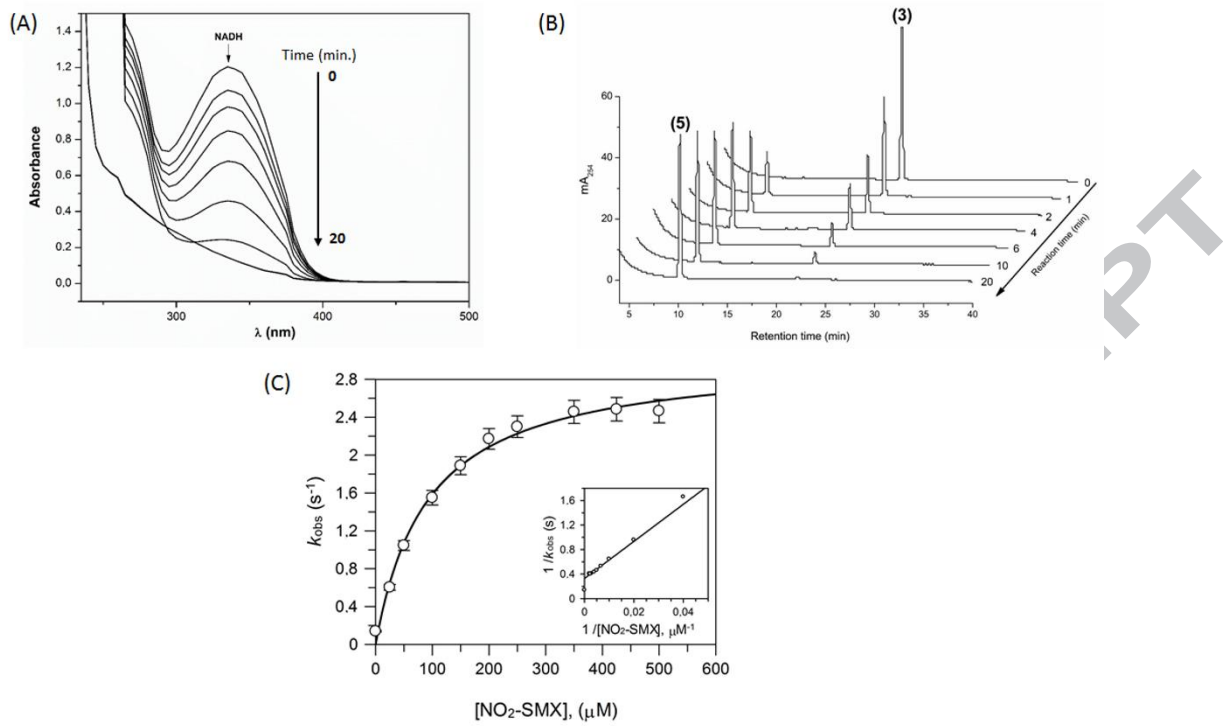


Figure 1. (A) Time dependent Uv-vis absorption spectra of the reaction in which the prodrug (NO2-SMX, **3**) reacted with SsapNtrB in the presence of NADH. The consumption of the cofactor is indicated by an arrow at 340 nm. (B) Corresponding HPLC analysis of the metabolite(s) of the prodrug. (C) Steady state kinetic data of SsapNtrB catalysed reduction of **3** by NADH (Insert: Lineweaver–Burk plot).

Metabolite **4** is relatively unstable under the reaction conditions used in this study. The formation of **4** was only observed using direct mass spectrometric analysis using ESI-MS in negative mode with molecular ion $[M-H]^-$ at m/z=266 (with fragmentations, m/z=170, 122 and 92) indicating a molecular mass of 267 Da (Fig. S4, ESI†). The main metabolite of **3**, however, was the hydroxylamine species (**5**) with ESI-MS in negative mode and molecular ion $[M-H]^-$ at m/z = 268 (with fragmentations, m/z= 251, 155, 107) indicating a molecular mass of 269 Da (Fig. S5, ESI†). The amine derivative, **6** has not been observed *in vitro* under the experimental setup described here. A cofactor regeneration system for NAD(P)H was utilized using NAD- or NADP-dependent formate dehydrogenases in the presence of auxiliary substrate sodium formate (FDHs)[20,21] to ensure the complete conversion of **3** without cofactor limitation and forcing the reaction to go to completion. Steady state kinetics of the reduction of **3** obeyed Michaelis-Menten kinetics and a typical plot of rate versus substrate concentration is shown in Fig. 1C. Steady state parameters based on NADH consumption, k_{cat} and K_M were 2.95 ± 0.11 s^{-1} and 92.61±7.25 µM, respectively. At a fixed substrate concentration, NADH usage vs product formation (done by HPLC) was estimated to be 2.5 to

1 (NADH used: product formed). Given that N^4-hydroxylamino (N^4-OH-SMX, 5) is the major product and requires at least 4 electrons for its formation, the results are as expected.

To demonstrate the antimicrobial ability of the prodrug upon reduction, antimicrobial susceptibility assays were conducted. For quantifying the ability of prodrugs of SMX (**3**) and its N^4-hydroxy-SMX derivative (**5**) by comparison to the unmodified antibiotic itself, we conducted both disk diffusion[22] and liquid assays[23] for *S. saprophyticus*. The results are shown in Figure 2A and 2B, respectively. In the agar diffusion assay, zones of inhibition with a radius of 10-12 mm and 16-18 mm were observed for entries **5** and **6**, respectively. By contrast, no zone of inhibition was observed for the prodrug (**3**) along with the control experiments, in which co-solvent (DMSO) alone was applied. This is rather surprising, but not totally unexpected. Results are interpreted from this type of assay based on the assumption that the antibiotic diffuses freely in the solid nutrient medium. However, sometimes this assumption is incorrect. In such cases, significant deviation from the expected behaviour is observed for the analysis of diffusion in solid agar. A number of antibiotics fall into this category, in particular those with hydrophobic and amphipathic characteristics. Given that the logD values at pH 6.8 for entries **3**, **5** and **6** are 0.81, 0.53 and 0.24, respectively,[24] it is clear that the prodrug (**3**) has a low diffusion rate on the plate assay. It is known[25] that certain antibiotics can be problematic to test with the disk diffusion method because of the specific physiochemical properties of the molecules. This is particularly the case for antibiotics with a hydrophobic and amphipathic nature (*e.g.* subtilin). However, improved membrane permeability of the prodrug was demonstrated in the liquid assay (Fig. 2B). Our results clearly indicated that the prodrug (**3**) has enhanced inhibitory effects over that of SMX in liquid medium. More details regarding minimum inhibitory concentration (MIC) values for entries **3**, **5** and **6** and common antibiotics (nitrofurazone, nitrofurantoin) for *S. saprophyticus* ATCC 15305 were given in Table S1 (ESI†).

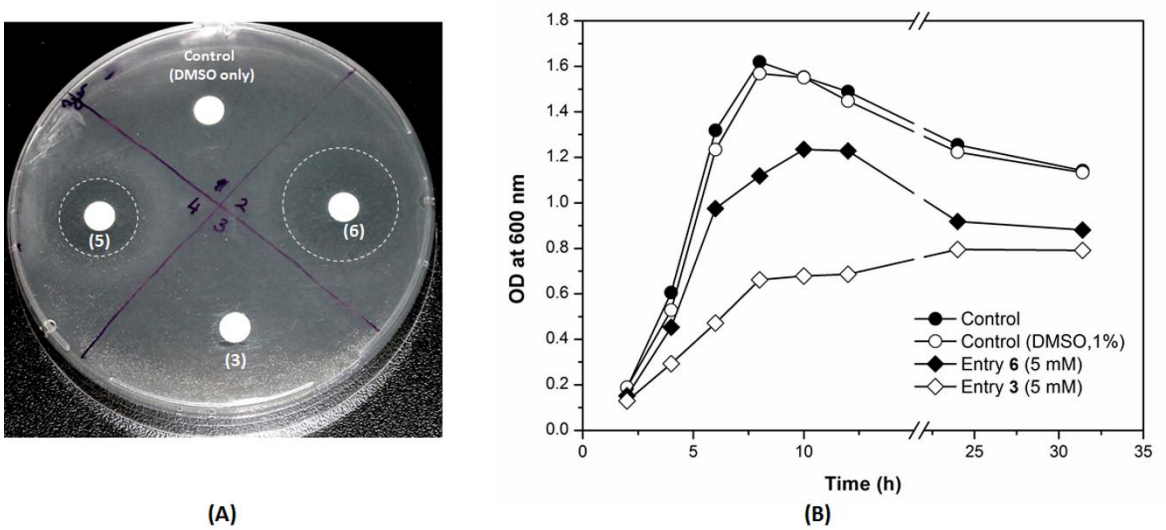


Figure 2. (A) Inhibitory activity of entries of **3**, **5** and **6** against *S. saprophyticus* as determined by the disk diffusion susceptibility test. (B) Inhibitory activity of entries of **3** and **6** against *S. saprophyticus* as determined growth curves in liquid assay. SMX, NO_2-SMX (**3**) and the enzymatic reduction metabolite (**5**) were prepared in DMSO, which was used for all stock solutions and final concentration was 1%.

In conclusion, we have demonstrated that the use of existing FDA approved antibiotics in the form of pre-antibiotics could be a useful strategy for combating certain infectious diseases caused by bacterial pathogens. This was demonstrated using the example of the sulphonamide-based antibiotic of sulfamethoxazole (SMX). The prodrug of SMS (NO_2-SMX, **3**) was synthesized and characterized. The reductive activation of **3** catalyzed by *Ssap*NtrB from the pathogen *S. saprophyticus* yielded N^4-hydroxy-SMX (**5**) as the major metabolite. The metabolite showed comparable antimicrobial activity with that of the antibiotic (SMX) in the agar diffusion assay, whereas the prodrug showed no clear zone on the plate assay, possibly due to its low solubility. In the liquid assay, however, the prodrug showed superior antimicrobial activity over that of SMX, probably due to its increased membrane permeability and hence absorption followed by reduction *via* pathogen specific stimuli (*e.g.* nitroreductases). Moreover, combating pathogens by exploiting their own enzymes may provide a useful alternative strategy not only for using lower amounts of drug with improved selectively but also with the potential of limiting the development of antibiotic resistance. SMX is metabolized in human by acetylation to generate N-acetyl and N^4-acetyl derivatives, which limits its availability for antimicrobial activity.[26,27] Moreover, increasing the plasma concentration of SMX to >400 mg/L leads to toxic adverse effects. The prodrug strategy, which masks the biological activity of an active compound before it reaches its target, may

overcome these limitations. In addition, our strategy could improve antibiotic specificity for the target pathogen and help optimize its pharmacokinetics. *In silico* analysis of the toxic potential of entries **3-6** showed that the differences are insignificant (Table S2, ESI†). These findings indicate that converting an amine to a nitro, nitroso and hydroxylamine moiety does not create any undesired properties. In addition, our strategy is generic and can be used to improve many antibiotics presently on the market.

Acknowledgements

This work was supported by The Scientific and Technological Research Council of Turkey (TUBITAK, Grant No.110T754&113Z706). We would like to thank Dr. Gareth A. Roberts (University of Edinburgh, UK) for his critical reading of this manuscript and his comments.

References

1 Rautio, J.; Kumpulainen, H.; Heimbach, T.; Oliyai, R.; Oh, D.; Jarvinen, T.; Savolainen, J. *Nat Rev Drug Discov* **2008**, *7*, 255.

2 Williams, E. M.; Little, R. F.; Mowday, A. M.; Rich, M. H.; Chan-Hyams, J. V.; Copp, J. N.; Smaill, J. B.; Patterson, A. V.; Ackerley, D. F. *Biochem J* **2015**, *471*, 131.

3 Redinbo, M. R.; Bencharit, S.; Potter, P. M. *Biochem Soc T* **2003**, *31*, 620.

4 Huttunen, K. M.; Raunio, H.; Rautio, J. *Pharmacol Rev* **2011**, *63*, 750.

5 Zhang, J.; Kale, V.; Chen, M. *AAPS J* **2015**, *17*, 102.

6 Huttunen, K. M.; Mahonen, N.; Leppanen, J.; Vepsalainen, J.; Juvonen, R. O.; Raunio, H.; Kumpulainen, H.; Jarvinen, T.; Rautio, J. *Pharm Res* **2007**, *24*, 679.

7 Ortiz de Montellano, P. R. *Future Med Chem* **2013**, *5*, 213.

8 Denny, W. A. *Curr Pharm Design* **2002**, *8*, 1349.

9 Prosser, G. A.; Copp, J. N.; Syddall, S. P.; Williams, E. M.; Smaill, J. B.; Wilson, W. R.; Patterson, A. V.; Ackerley, D. F. *Biochem Pharmacol* **2010**, *79*, 678.

10 Hu, L. In *Prodrug Approaches to Drug deivery*; John Wiley & Sons, Nerw Jersey, 2005. pp 125.

11 James, N. D.; Patel, P.; Mautner, V.; Young, J. G.; Hull, D.; Searle, P.; Leung, H. Y.; Ellis, J.; Wallace, M.; Young, L. S. *J Clin Oncol* **2004**, *22*, 399s.

12 Rooseboom, M.; Commandeur, J. N.; Vermeulen, N. P. *Pharmacol Rev* **2004**, *56*, 53.

13 Searle, P. F.; Salman, A.; Viney, R.; Patel, P.; Onion, D.; James, N. D.; Porfiri, E.; Mautner, V. *Hum Gene Ther* **2010**, *21*, 522.

14 Patel, P.; Young, J. G.; Mautner, V.; Ashdown, D.; Bonney, S.; Pineda, R. G.; Collins, S. I.; Searle, P. F.; Hull, D.; Peers, E.; Chester, J.; Wallace, D. M.; Doherty, A.; Leung, H.; Young, L. S.; James, N. D. *Mol Ther* **2009**, *17*, 1292.

15 Celik, A.; Yetis, G. *Bioorgan Med Chem* **2012**, *20*, 3540.

16 Kuroda, M.; Yamashita, A.; Hirakawa, H.; Kumano, M.; Morikawa, K.; Higashide, M.; Maruyama, A.; Inose, Y.; Matoba, K.; Toh, H.; Kuhara, S.; Hattori, M.; Ohta, T. *P Natl Acad Sci USA* **2005**, *102*, 13272.

17 Goodyer, L. *Int J Nurs Stud* **2000**, *37*, 555.

18 Organization, W. H. In *WHO Model List of Essential Medicines* 2015. pp 13.

19 Dea-Ayuela, M. A.; Castillo, E.; Gonzalez-Alvarez, M.; Vega, C.; Rolon, M.; Bolas-Fernandez, F.; Borras, J.; Gonzalez-Rosende, M. E. *Bioorgan Med Chem* **2009**, *17*, 7449.

20 Davis, B. G.; Celik, A.; Davies, G. J.; Ruane, K. M. In *NOVEL ENZYME*, United States, 2010.

21 Demir, A. S.; Talpur, F. N.; Sopaci, S. B.; Kohring, G. W.; Celik, A. *J Biotechnol* **2011**, *152*, 176.

22 Institute, C. a. L. S. In *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement*; Clinical and Laboratory Standards Institute, USA, 2014. pp 68.

23 Sakinc, T.; Michalski, N.; Kleine, B.; Gatermann, S. G. *FEMS microbiology letters* **2009**, *299*, 60.

24 Majewsky, M.; Wagner, D.; Delay, M.; Brase, S.; Yargeau, V.; Horn, H. *Chem Res Toxicol* **2014**, *27*, 1821.

25 Bonev, B.; Hooper, J.; Parisot, J. *J Antimicrob Chemother* **2008**, *61*, 1295.

26 Sim, E.; Abuhammad, A.; Ryan, A. *Br J Pharmacol* **2014**, *171*, 2705.

27 Cribb, A. E.; Nakamura, H.; Grant, D. M.; Miller, M. A.; Spielberg, S. P. *Biochem Pharmacol* **1993**, *45*, 1277.

Graphical abstract