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Antimicrobial activity and molecular analysis of azoderivatives of β-diketones

Anisha Viswanathan [a], Adrien Sala [a], Olli Yli-Harja [a,b], Meenakshisundaram Kandhavelu [a,*]

[a] Molecular Signaling Lab, Computational Systems Biology Research Group, Department of Signal Processing, Tampere University of Technology, P.O. Box 553, 33101 Tampere, Finland
[b] Institute for Systems Biology, 1441N 34th Street, Seattle, WA 98103-8904, USA

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ABSTRACT

The emergence and increase in the number of multidrug resistant microorganisms have highly increased the need of therapeutic trials, necessitating a deep exploration on novel antimicrobial response tactics. This study is intended to screen and analyze the activity of a novel set of azoderivatives of β-diketones and their known analogs for antimicrobial properties. The compounds were analyzed to determine their minimum inhibitory concentration. Hit compounds 5-(2-(2-hydroxyphenyl)hydrazono)pyrimidine-2,4,6(1H,3H,5H)-trione (C5), 5-chloro-3-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-2-hydroxybenzenesulfonic acid (C8), 2-(2-carboxyphenylhydrazo)malononitrile (C11) were then considered in evaluating their effect on transcription, translation and cellular oxidation impact. All three compounds were found to have in vitro inhibitory action on *E. coli* cell growth. The study also revealed that those compounds have a notable impact on cellular activities. It is determined that the newly synthesized azoderivative of barbituric acid (C8) have maximum growth inhibitory activity among the three compounds considered, characterized by a MIC50 of 0.42 mg/ml. The MS2 reporter system was used to detect the transcriptional response of the bacteria to the treatment with the selected drugs. All three compounds are found to down regulate the transcriptional pathway. The novel compound, C8, showed maximum inhibition of transcription mechanism, followed by C5 and C11. The effect of the compounds on translation was analyzed using a Yellow Fluorescent protein reporter system. All the compounds displayed reductive impact on translation of which C8 was found to the best, exhibiting 8.5-fold repression followed by C5 and C11, respectively. Fluctuations of the Reactive Oxygen Species (ROS) concentrations were investigated upon incubation in hit compounds using ROS sensor protein. All the three compounds were found to contribute to oxidative pathway. C8 is found to have the best oxidative effect than C5 and C11. All experiments were repeated at least twice, the results being verified to be significant using statistical analysis.

1. Introduction

Azoderivatives of β-diketones (ADB), derived from corresponding β-diketones and aryldiazonium salt are of recent interest in research due to its interesting pharmacological activity (Feilmeier et al., 2000; Kandhavelu et al., 2012c; Semde et al., 1998). Derivatives of barbituric acid, called barbiturates, are produced by alkylating diethyl malonate, followed by reaction with urea. Although barbituric acid itself is pharmacologically inactive, barbiturates are very active drugs, generally behave as depressants of central nervous system, and have been shown to have antifungal (Kidwai et al., 2000), antibacterial (Kandhavelu et al., 2012a,b,c), anti-tubercular and anticonvulsant activities (Feilmeier et al., 2000; Semde et al., 1998). The significance of ADB as bistate molecular switches (Kopylovich et al., 2011) and regulators of ionophore selectivity (Kopylovich et al., 2003) due to their tautomeric balances have been reported. The role of ADBs as antimicrobial agents, their efficiency in multidrug resistant organisms and their modes of action leading to cell death, still remains unexplored.

The diversity of drug resistance mechanisms among microorganisms, strategies to tackle them and the alternatives to treat microbial infections are actively discussing topics in the context of the limited success of therapeutic trials. Antimicrobial resistance

Abbreviations: ADB, azoderivatives of β-diketones; DMSO, dimethyl sulfoxide; aTc, anhydrotetracycline; MIC, minimum inhibitory concentration; IPTG, isopropyl β-D-1-thiogalactopyranoside; SEM, standard error of mean; ANOVA, analysis of variance; mg, milligram; ml, milliliter; ng, nanogram.

* Corresponding author. Tel.: +358 417488772.
E-mail address: meenakshisundaram.kandhavelu@tut.fi (M. Kandhavelu).

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depends on various factors leading to adaptive and protective mechanism such as altering gene expression patterns and cell physiology so as to combat stress. The resistance could be direct or indirect ways such as growth cessation (Miller et al., 2004), inducing changes to antimicrobial targets (Gunn, 2001), alterations to membrane barrier functions (Delcour, n.d.), promotion of resistant growth modes such as biofilms (Landini, 2009) and favorable mutations (Shee et al., 2011). Indeed, classification of antibiotics is mainly based on their biological impact and is categorized as bactericidal and bacteriostatic. Recent studies focus on a third-category drugs that induce endogenous reactive oxygen species (Dwyer et al., 2012; Foti et al., 2012). A deep exploration of novel antibacterial response strategies is crucial to combat antimicrobial resistance in the current situation.

A recent report from our group describing the antibacterial activities of ADBs in gram positive bacteria- *S. aureus*, *S. epidermidis*, and *P. aeruginosa*- mentioned the potential of ADBs as antibacterial agents. The present study, an extension of our previous work, focuses on the antibacterial activities of novel ADBs on *E. coli*. We considered 13 new ADBs for which we analyzed their antibacterial potential, involvement in cellular oxidation and the interruption of cellular activities such as transcription and translation. Involvement of Reactive Oxygen Species (ROS) in various cell death pathways have been discussed by Scott and Brent (Dixon and Stockwell, 2013), hence the necessity to investigate the redox fluctuation of the cell in response to drug treatments.

In highly varied biological systems, the sensibility and versatility of fluorescent protein reporter method used in our study are comparable to the likes of fluorescence microscopy (Gogoi et al., 2006; Webb et al., 2001; Wymelenberg et al., 1997), flowcytometry (Dhandayuthapani et al., 1995) and fluorimetry (Feilmeier et al., 2000) making it a reliable method for antibacterial activity analysis. Exploration of drug effects on cellular events is significant, as they are involved in various pathways and biological systems are highly varied. We considered green and yellow fluorescent protein reporters for detecting the effect of potential compounds for transcriptional and translational activity at the single cell level. A redox sensitive Green Fluorescent Protein (GFP) biosensor (Lohman and Remington, 2008) was used for studying ROS level fluctuation considering the fact that ROS are involved in signaling various cell death pathways(Dixon and Stockwell, 2013). The results of our study have been discussed in the following sections.

2. Material and methods

2.1. Bacterial strains and chemicals

2.1.1. Chemicals

The components of Lysogeny Broth (**LB**) broth are: Tryptone medium (Fluka, #BCBJ2249V), Yeast extract (LabM, UK, #MC001). For selective growth, chloramphenicol (Sigma–Aldrich, USA, #100M0061V), kanamycin (Sigma–Aldrich, USA, #SLBB0945V), ampicillin (Sigma–Aldrich, USA, #BCBF0407V), streptomycin (Sigma–Aldrich, USA, #081M13803V) are used. Isopropyl β-D-1-thiogalactopyranoside (Sigma–Aldrich, USA, #092M4001V) and aTc (Sigma–Aldrich, USA, #A1200000) were used for induction of promoter of plasmid and target proteins. Components of M63 media are $(NH4)_2SO_4$(Sigma–Aldrich, USA, #SLBB3959V), KH_2PO_4 (Sigma–Aldrich, USA, #120M0157V), $FeSO_4$ (Sigma–Aldrich, USA, #041M1753V), Glycerol (Sigma–Aldrich, USA, #STBB9416V), MEM aminoacids (Sigma–Aldrich, USA, #RNB**8**084), $MgSO_4{\cdot}7H2O$ (Sigma–Aldrich, USA, #MKBJ2382V). For transcription and ROS positive controls we used (Sigma–Aldrich, USA, #0001438603) and H_2O_2(Sigma–Aldrich, USA, #SZBB3540V).

2.1.2. Bacterial strains and plasmids

E. coli K12 DH5α pro was used for drug screening transcription, translation and oxidation studies. For detecting transcriptional interference of drugs, *E. coli* K12 containing two constructs: (i) PROTET-K133 carrying $P_{LtetO-1}$-MS2d-GFP, and (ii) a pIG-BAC (P_{lac}-mRFP1-MS2-96bs) vector, carrying a 96 binding site array under the control of $P_{lac}$was used (Golding and Cox, 2004). *E. coli* K12 strain with a plasmid PAK400c carrying P_{lac}-YFP gene coding for yellow fluorescent protein was used for analyzing translational changes. *E. coli* K12, transformed with pQE30 vector containing ROS sensor coding ro-iR mutant was used for investigating redox response.

2.1.3. Synthesis of drugs used

For the present study we considered a set of novel azo derivatives of β-diketones: (*E*)-3-(2-(1-ethoxy-1,3-dioxobutan-2-ylidene)hydrazinyl)-2-hydroxy-5-nitrobenzenesulfonic acid (**1**), 2-(2-(2-hydroxy-4-nitrophenyl)hydrazono)-2H-indene-1,3-dione (**2**), (Z)-5-chloro-2-hydroxy-3-(2-(4,4,4-trifluoro-1,3-dioxo-1-(thiophen-2-yl)butan-2-ylidene)hydrazinyl)benzenesulfonic acid (**3**), 5-chloro-2-hydroxy-3-(2-(2,4,6-trioxo-tetrahydropyrimidin-5(6H)-ylidene)hydrazinyl)benzenesulfonic acid (**4**), 5-(2-(2-hydroxyphenyl)hydrazono)pyrimidine-2,4,6(1H,3H,5H)-trione (**5**), 4-hydroxy-5-(2-(2,4,6-trioxo-tetrahydro-pyrimidin-5(6H)-ylidene)hydrazinyl)benzene-1,3-disulfonic acid (**6**), 5-(2-(2-hydroxy-4-nitrophenyl)hydrazono)pyrimidine-2,4,6(1H,3H,5H)-trione (**7**) and 5-chloro-3-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-2-hydroxybenzenesulfonic acid (**8**) as well as known analogs 5-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-4-hydroxybenzene-1,3-disulfonic acid (**9**), 2-(2-sulfophenylhydrazo)malononitrile (**10**), 2-(2-carboxyphenylhydrazo)malononitrile (**11**), 2-(2-(2,4-dioxopentan-3-ylidene)hydrazinyl)phenylarsonic acid (**12**) and 5-(2-(2,4-dioxopentan-3-ylidene)hydrazinyl)-2,3-dihydrophthalazine-1,4-dione (**13**) were studied. The new compounds **C1–8** (scheme of synthesis has been previously described and the known analogs **C9**–**C13** were synthesized via the Japp–Klingemann (Frank and Phillips, 1949) reaction between the respective aromatic diazonium salt and methylene active compounds in a water solution containing sodium acetate or sodium hydroxide.

2.2. Antibacterial activity assay

E. coli K12 was used for determining the MIC values of the selected compounds. From a frozen culture, an overnight culture of the bacteria was grown at 30 °C, 250 rpm for 13 h in the presence of chloramphenicol and kanamycin, in LB media. A preculture was grown in LB broth at 37 °C, 250 rpm, with an initial cell density of $5 * 10^7$ cells/ml for 2 h with the induction of 100 ng/ml aTc at 75 min and 1 mM IPTG at 90 min. The culture was then diluted in LB to obtain a cell density of $2 * 10^8$ cells/ml. The culture was then treated with 1 μg/ml, 50 μg/ml, 100 μg/ml, 150 μg/ml, 200 μg/ml, 250 μg/ml, 500 μg/ml, 750 μg/ml and 1000 μg/ml of the compounds for 2 h and the OD_{600} values were measured to determine the MIC_{50} of the compounds. All the compounds displaying better MIC was independently tested thrice using the same method as described above.

2.3. Cellular dynamics response to the compounds

2.3.1. Transcriptional activity

The bacteria were grown in LB media supplemented with the appropriate antibiotics as follows: 34 μg/ml of chloramphenicol, 50 μg/ml of kanamycin; induced with 100 ng/ml aTc at 1st hr of the pre-culture. The cells were then diluted to reach a cell density of $2 * 10^8$ cells/ml, redistributed in 1.5 ml centrifuge tubes, and