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Antimicrobial activity and molecular analysis of Azoderivatives of β-diketones

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Running title: Mode of action of novel Azoderivative leads.

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 analogues 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, C5, showed maximum inhibition of transcription mechanism, followed by C8 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.

Keywords: azoderivatives of β-diketones, antimicrobial activity, transcription, translation, oxidation, single cell.

Abbreviations used

ADB-Azoderivatives of βdiketones

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

1. Introduction

Azoderivatives of βdiketones (ADB), derived from corresponding βdiketones and aryldiazonium salt are of recent interest in research due to its interesting pharmacological activity(Feilmeier et al., 2000; M. Kandhavelu et al., 2012; Semd R 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(M. Kandhavelu et al., 2012), anti-tubercular and anticonvulsant activities (Feilmeier et al., 2000; Semd 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 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 analysing translational changes. *E.coli* K12, transformed with

pQE30 vector containing ROS sensor coding ro-iR mutant was used for investigatingredoxresponse.

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 30^0C, 250 rpm for 13 hrs in the presence of chloramphenicol and kanamycin, in LB media. A preculture was grown in LBbroth at37^0C, 250 rpm,with an initial cell density of $5*10^7$ cells/ml for 2 hrs with the induction of 100 ng/mlaTc at 75 min and 1mM 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 two hours 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 1^{st} 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 treated with MIC_{50} of the selected compounds (**5, 8,** and **11**) for 2 hrs with the addition of IPTG after 1 hr of treatment. As a positive control we used 50mg/mL concentration of rifampicin which as a transcriptional inhibitor. The tubes were then

centrifuged for concentrating the cells and microscopic slides were prepared on 1% agarose. The cells were observed under microscope, images were acquired and these images were used for data analysis to investigate the impact of drugs on transcriptional pathway.

2.3.2. *Microscopy and image analysis*

Cells were visualized in a Nikon Eclipse (TE2000-U, Nikon, Japan) inverted microscope with C1 confocal laser-scanning system. For image acquisition, we used Nikon EZ-C1 software. GFP fluorescence was monitored using an argon ion laser (Melles-Griot), at 488nm excitation and 514nm emission.

The images were then analysed for determining the mean mRNA count per cell using custom software written in MATLAB 2011b (MathWorks). Cells were detected from fluorescence images by a semi-automatic method. Fluorescent spots in the cells were automatically segmented using density estimation with a Gaussian kernel (Chen et al., 2008) and Otsu's thresholding (Xu et al., 2011). Finally, background-corrected spot intensities were calculated and summed to produce the total spot intensity in each cell.

2.3.3. *Translational activity*

E.coli K12 strain was transformed with YFP plasmid and the positive clones were screened and used for studying the effect of compounds on translation. The cells were overnight grown from frozen culture in LB medium with chloramphenicol (34µg/mL) at 30^0C, 250 rpm for 13 hrs. The preculture was prepared in LB for 2hrs at 37^0C, 250 rpm and incubated with MIC_{50} of selected compounds with the addition of IPTG after 1hr of treatment. Soon after IPTG induction, 200 µL each of treated and un-treated cells were transferred into 96-well plate. Known antimicrobial were used as a control for following cellular changes of the selected strains. All measurements were done in quintuplicate in 96-well plate (Greiner). Plates were incubated at room temperature and Fluorescence emission was monitored every hour from 0 to 4 hrs using fluroskan accent using Ex_{508nm}- Em_{538nm} pair. The test was performed in two biological repeats for improved accuracy of the results. One-way ANOVA test suggest that the data is significant at 95 % confidence level.

2.3.4. *Cellular Oxidation*

Cellular oxidation was monitored using *E.coli* roGFP2-iR, a redox sensitive *E.coli* possessing a mutation on roGFP-sensor protein (Lohman and Remington, 2008). Overnight culture of the bacteria was grown in M63 medium at 30^0C, 250 rpm for 13 hrs. Pre-culture was prepared with a cell density of $5*10^7$ cells/mL, in M63 for 2hrs at 37^0C, 250 rpm with addition of IPTG after 1 hr for inducting the target protein. Culture was diluted to reach a cell density of $2*10^8$ cells/mL and treated with the MIC_{50} of selected drugs for 2 hrs. As a positive control we used of H_2O_2 which acts as an oxidizing agent. Then the culture was distributed into 1.5 ml centrifuge tubes and centrifuged at 8000rpm for 1 min to collect the pellet. These concentrated cells were suspended in ~50 µl medium and used for microscopy. The slides were prepared with 1% agarose and microscopy was conducted to analyse the ratio of fluorescence. The observed changes in ROS activity are statistically significant with 90 % confidence interval.

2.3.5. *Ratiometric analysis of single cell fluorescence*

Cells were observed under microscope using two different excitation wavelengths 405nm and 488nm both with an emission wavelength of 514nm. Image processing was conducted with MatLab as described in the transcription section and mean fluorescence intensity was determined for the total population, individually for both excitation wavelengths. A ratiometric analysis was conducted to determine oxidative changes in comparison to control. Ratiometric analysis of the fluorescence intensity was determined (Lohman and Remington, 2008) and was analysed using the equation $R=F_{405}/F_{488}$. This ratio is indicative of the oxidative changes, as the ratio is directly proportional to oxidation.

2.3.6. *Statistical analysis*

Spectrophotometric results for determination of MIC, was conducted in three biological repeats with two technical repeats. Tests of statistical significance were done using Sidak's multiple comparison tests in conjunction with ANOVA using Graphpad to determine the significance of the difference between untreated and treated samples as well as to confirm the significance in changes, when cells were incubated with different concentrations of the drug. For translation analysis, each time point represents a mean of 6 repeats unless otherwise stated. Both technical and biological repeats were maintained and the results were validated using Dunnett's multiple comparison tests in conjunction with ANOVA. In both cases data were validated against 95% confidence level.

3. Results and discussion

3.1.*Antibacterial activity*

Multidrug resistance of *E.coli* has been extensively studied by various groups (Manges et al., 2001; van der Donk et al., 2012). The *E.coli* strain considered for our study has resistance to chloramphenicol, kanamycin and spectinomycin. We were concerned to analyze the effect of a set of novel azoderivatives of β-diketones on *E.coli* cell growth using optical density measurements, by testing *E.coli* against different concentrations of the drugs. Cells in exponential growth phase (a cell density of $2*10^8$ cells/mL) were treated with dilutions of the drugs ranging from 1µg/mL to 1000 µg/mL. Minimum inhibitory concentration (MIC) of all compounds was determined by macro-dilution method. From dose-response curve generated from the OD_{600} values, MIC_{50} values were determined. The MIC_{50} data for the *E.coli* K12 is summarized in **Table 1**. Significant reduction of E.coli cell growth was observed for all the compounds analyzed. In *E.coli* there are several proteases targets ribosomal proteins in a stressful environment. Moreover, it has been shown that antibiotics can cause degradation of ribosomal RNA and proteins. Also it is known that degradation of ribosomal-RNAs is the major cause of cell death. Our results also show that the selected compounds affect translation process (discussed below), which possibly influence protein degradation, thus reducing the cell survival.

The compounds used in this study are 5-(2-(2-hydroxyphenyl)hydrazono)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione(**C5**), 4-hydroxy-5-(2-(2,4,6-trioxo-tetrahydro-pyrimidin-5(6H)-ylidene)hydrazinyl)benzene-1,3-disulfonic acid(**C6**), 5-(2-(2-hydroxy-4-nitrophenyl)hydrazono)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione(**C7**) and 5-chloro-3-(2-(4,4-

dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-2-hydroxybenzenesulfonic acid(**C8**). The antimicrobial activity of **5–8,** as well as known analogs, 5-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-4-hydroxybenzene-1,3-disulfonic acid(**C9**) and 2-(2-carboxyphenylhydrazo)malononitrile(**C11**) were taken for the study as they displayed better growth inhibition compared to others. Figure 1 shows dose-response curves of the selected compounds. Streptomycin was used as a standard while no drug treatment was considered as control. Compounds **5**, **8** and **11** shows relatively higher inhibitory effects on *E.coli* cell growth at low concentrations whereas **6** and **9** had inhibitory activity at higher concentrations. MIC_{50} of **5**, **8** and **11** determined from the dose-response curve were identified at 0.79 mg/mL, 0.42 mg/mL, and 0.62 mg/mL respectively.

Previous studies from our group have demonstrated the potential of **5** and **11** against gram positive bacteria comparable to naficillin and chloramphemicol (M. Kandhavelu et al., 2012). In the present study, streptomycin, a drug with known MIC, used as a standard, showed a greater inhibitory effect when compared to the new compounds and MIC_{50} value was interpreted as 50µg/ml. Thus it is clear from the studies that the compounds are more effective in gram positive bacteria, whereas high concentrations are needed to have an inhibitory action on gram negative bacteria. The results validated using ANOVA in conjunction with Sidak's multiple comparison test with 95% confidence level were found to be significant.

3.2. *Transcriptional activity*

The effect of compounds on gene expression was analyzed by quantifying the average number of mRNAs produced per cell(M. Kandhavelu et al., 2012). The fluorescent cells observed under the microscope were processed and analyzed using a semi-automated cell segmentation program in MATLAB. Transcriptional response to the drug treatment was analyzed by comparing the mean mRNA count per cell, between the treated and untreated cells. All cells produced bright green fluorescent spots against the cell background, as expected, as an outcome of RNA-MS2-GFP molecules (Figure 2a). If the transcription pathway has been repressed by the drug, the intensity of the spot is expected to be lower than control due to the production of low number of fluorescent mRNAs. To extract the mRNA numbers from the spot we used image processing method as described in the methods section (Figure 2b). Indeed, comparison of the average RNA production per cell demonstrated a reduction in gene expression for treated cells when compared to the control. The drugs were dissolved in DMSO; therefore we also analyzed the effect of DMSO which displayed a slight decline in the transcription level as opposed to untreated cells (Figure 2c). Drug **8** exhibited maximum transcriptional reduction of32.3% in comparison to the control, while **5** and **11** displayed the lower transcriptional impact among the set, 23% and 4% respectively. Figure 2c displays the effect of standard drug relative to its control. The standard drug streptomycin showed 29.7% of transcriptional activity reduction. Compared to the positive control rifampicin, compounds **5** and **8** are showing higher transcription inhibition response, 10.5% and 20.5% respectively. In case of compound **11**, transcription inhibition is not greater than rifampicin, yet it has inhibitory effects compared to the untreated cells. The results were validated using Dunnett's multiple comparison test against the control and determined to be significant at the 5% level of significance.

3.3. *Translational response*

The cells were treated with the $MIC_{50}$for the compounds and streptomycin as a standard. Translational responses of the drugs were analyzed using a fluorescence plate reader. Experiments were done in six replicas and readings were taken every 15 minutes for 4 hours starting immediately after the induction. Compared to controls, the treated cells showed decreased fluorescence in relation to their translational activity (Figure 3a, 3b, 3c). Interestingly, **8** which showed lowest effect on transcription was found to have maximum effect on translation. Indeed, **8** displays 8.5 times reduction when compared to control, followed by **5** and **11** reducing translation 4.5 and 2.2 folds, respectively. Streptomycin at MIC_{50} didn't had significant inhibition on translation probably due to the presence of multidrug resistant character of the strain, still showed reduction in the translation level. All the conditions shows increase in fluorescence production over the time, in a decelerating mode. Data was validated using Sidak's multiple comparison test from which all the compounds were found to be significantly different from the control (P value <0.05 for all compounds).

3.4. Effect on Cellular Oxidation

Cellular oxidation was determined with the aid of a redox sensor fluorescent protein which exhibits two excitation peaks corresponding to the neutral and anionic form of the chromophore (Lohman and Remington, 2008). Oxidation causes an increase in the neutral chromophore at the cost of anionic chromophore. This ratiometric behavior of roGFP protein permits the monitoring of cellular oxidation. Fluorescence was observed at 408 nm and 488 nm. Fluorescence spectra showed two distinct emission maxima in both excitation wavelengths, corresponding to oxidized and reduced form of the protein. Figure 4a and 4b show the changes in fluorescence intensity exhibited by the sensor protein at two excitation wavelengths. Ratiometric fluorescence emission of the treatments and control is shown in the Figure4c. All the compounds exhibited an increase in oxidation upon treatment, **8** was found to have the highest increment of oxidative effect at MIC_{50} (1.84folds), followed by **5** (1.35 folds) and **11** (1.06 folds). Figure 4c shows that compared to the positive control, H_2O_2, MIC50 the selected drugs showed lower oxidative effect as expected.

4. Conclusion

The objective of the current study was to screen and analyze the antimicrobial activity of a set of novel azoderivatives of β-diketones. This study revealed that all the compounds considered have an antibacterial effect on *E.coli* cell growth. Among the compounds considered in the study, compound **8** has the highest antibacterial effect. It appears to have a strong negative impact on transcription, translation, and promote cellular oxidation. Also, the result shows that the **5** and **11** exhibited lower effect on transcription, translation and oxidation. The obtained results demonstrate that the compounds derived from β-diketones exhibited antimicrobial activity. The antibacterial activity of these compounds has been already verified and reported by our group in Gram-positive as well as in Gram-negative bacteria, using a whole cell bacterial luminescence biosensor method, and was found to be effective against *Staphylococcus aureus* and *Staphylococcus epidermidis*. Compound **5**effectively inhibits the growth of *Staphylococcus aureus* at the MIC_{50} of 10µg/ml. Compound **11** showed potent activity against *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* at MIC_{50} of 10µg/ml and 17µg/ml, respectively. Conclusively, we suggest the role of azoderivatives of β-diketones as a potent class of

antimicrobial agents and our studies also indicates **11** to be a lead compound against both Gram-positive and Gram-negative multidrug resistant strains.

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Transparency declaration

None to declare

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Figures caption:

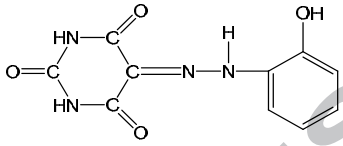
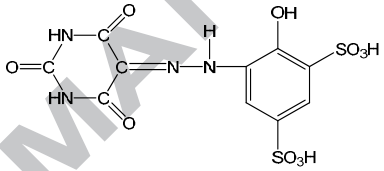
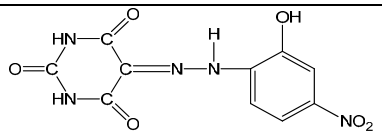
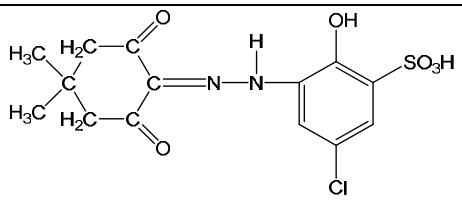
Figure 1: The graph represents the dose-response curve exhibited by *E.coli*. OD_{600} values are plotted against various concentrations of compounds (µg/ml), after 2 hours of treatment. Each data point represents an average of three replicas. Error bars represent SEM.

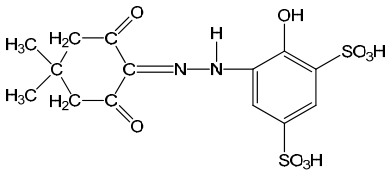
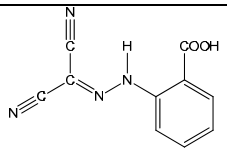
Figure 2: (a) Unprocessed images of cells displaying tagged RNA molecules as bright fluorescent spots as detected by fluorescent microscope (image converted in grey scale). (b) Segmented images with detected cells and spots. (c) Transcriptional response to various compounds compared to the effect of solvent (DMSO) which serves as a control. Histogram represents the mean mRNA produced per cell (in %maximum) after 2 hrs of treatment with MIC_{50} of compounds. Error bars represent SEM.

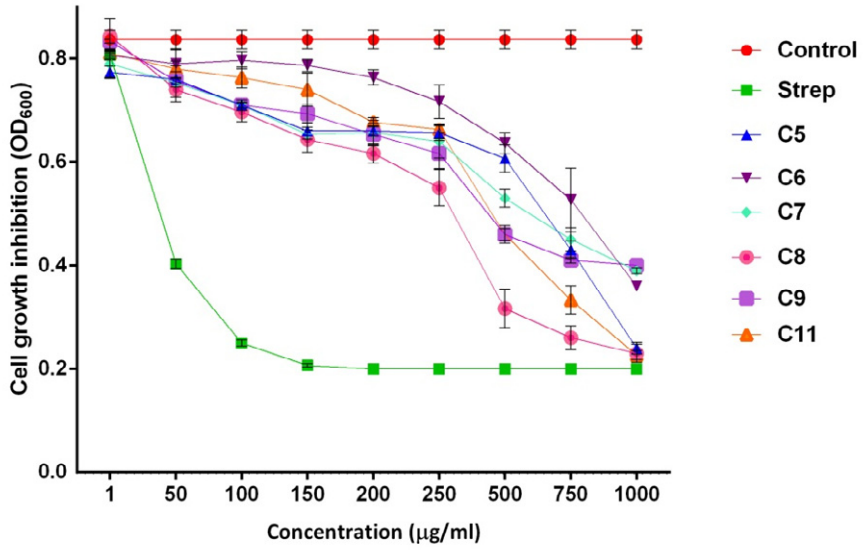
Figure 3: (a) Kinetic analysis of fluorescence reading for 4 hrs, from 1st hr of treatment with MIC_{50} of hit compounds. Each data point indicates an average of six replicates. Error bars represent SEM. (b) Relative expression of fluorescence after 2 hrs of treatment, displayed in % maximum. Effect of the standard drug on translation compared to control (H_2O), displayed in % maximum.

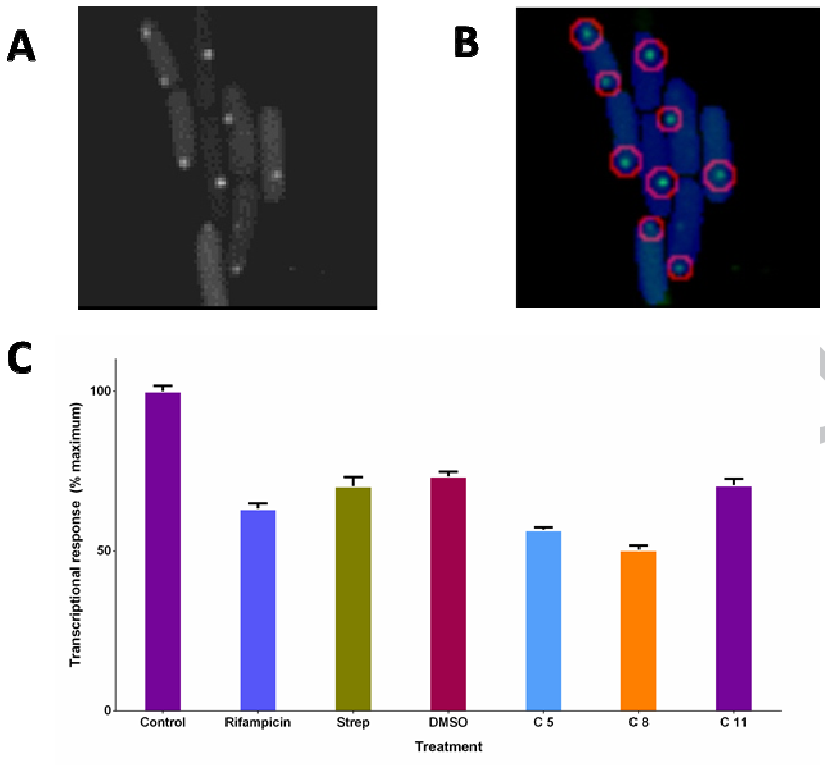
Figure 4: (a) The unprocessed monochromic image of treated cells observed at Ex_{408} nm. Grey scaled image. (b) Unprocessed monochromic image of treated cells observed at Ex_{488} nm. Cells were treated with MIC_{50} of the selected compounds for 2 hrs and analyzed for ratiometric values at the two excitation wavelengths. Treated cells were then compared against the control to determine the oxidation level. Grey scaled image. (c) The histogram represents the mean ratiometric fluorescence values of test compounds from two independent tests, on a logarithmic scale. Error bar represents SEM. (d) The histogram represents the mean of ratiometric fluorescence values of standard drug and control at two excitation wavelengths from two independent tests, on a logarithmic scale. Error bar represents SEM.

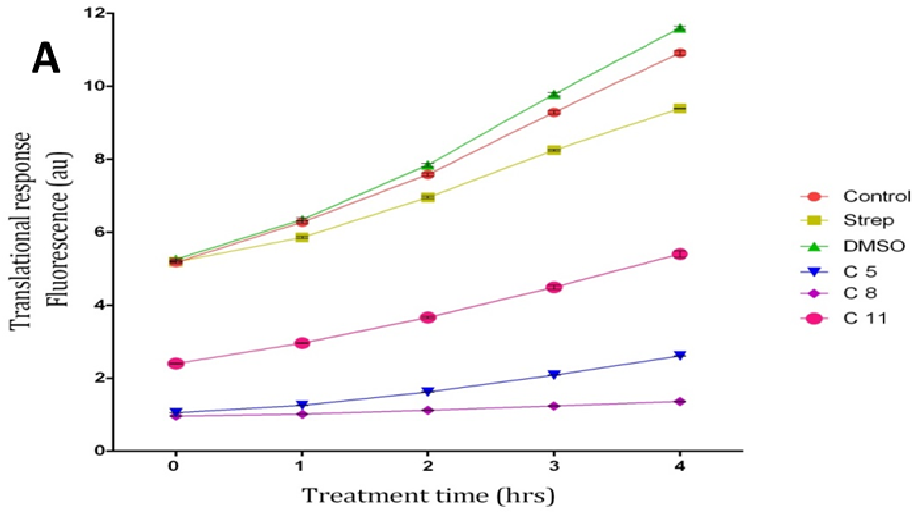
Table 1. Chemical structure and MIC values of compounds exhibiting better inhibitory action

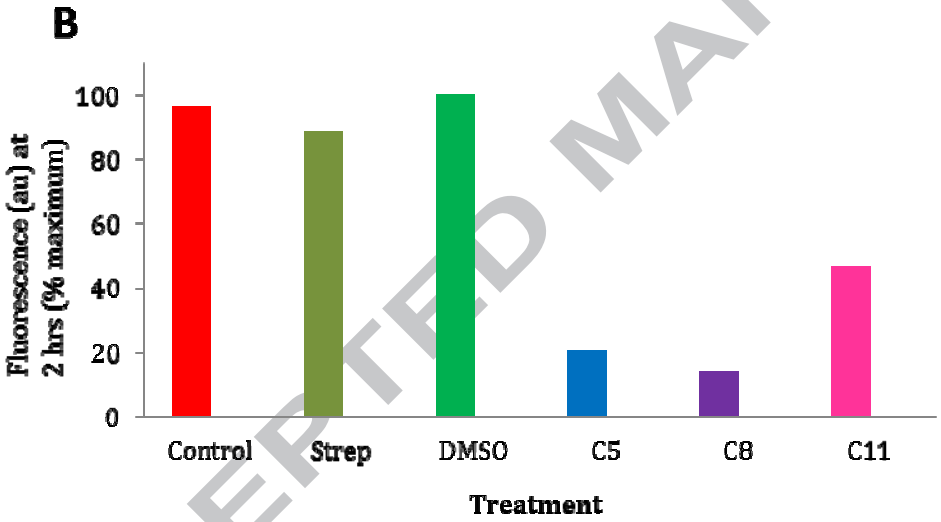
No:	Compound Name	Structure, Mw, Solubilisation	MIC_{50} (mg/mL) *E.coli*
5	5-(2-(2-hydroxyphenyl)hydrazono) pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione	 MW = 248, solublein DMSO	0.79
6	4-hydroxy-5-(2-(2,4,6-trioxo-tetrahydro-pyrimidin-5(6H)-ylidene)hydrazinyl) benzene-1,3-disulfonic acid	 MW = 408, soluble in H_2O and DMSO	0.93
7	5-(2-(2-hydroxy-4-nitrophenyl)hydrazono) pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione	 MW = 293, solublein DMSO	0.95
8	5-chloro-3-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-2-hydroxybenzenesulfonic acid	 MW = 375, soluble in H_2O and DMSO	0.42

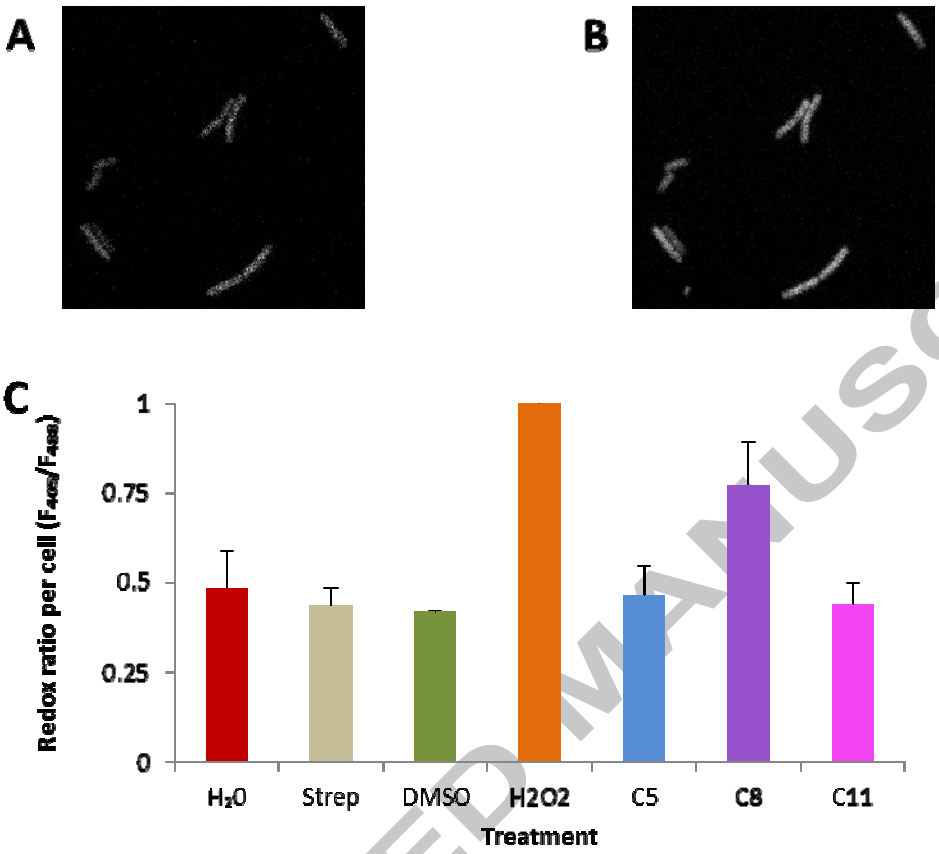
9	5-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-4-hydroxybenzene-1,3-disulfonic acid	 MW = 420, soluble in H_2O and DMSO	1.00
11	2-(2-carboxyphenylhydrazo) malononitrile	 MW = 214, soluble in H_2O and DMSO	0.62











Graphical abstract