Graphical Abstract

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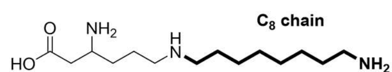
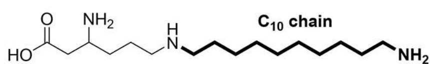
De-novo designed β-lysine derivatives can both augment and diminish the proliferation rates of *E. coli* through the action of Elongation Factor P

Ciara M. McDonnell[a], Magda Ghanim[b], J. Mike Southern[a]*, Vincent P. Kelly[b]* and Stephen J. Connon[a]*





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De-novo designed β-lysine derivatives can both augment and diminish the proliferation rates of *E. coli* through the action of Elongation Factor P

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ABSTRACT

An investigation into the effect of modified β-lysines on the growth rates of eubacterial cells is reported. It is shown that the effects observed are due to the post translational modification of Elongation Factor P (EFP) with these compounds catalysed by PoxA. PoxA was found to be remarkably promiscuous, which allowed the activity of a wide range of exogenous β-lysines to be examined. Two chain-elongated β-lysine derivatives which differ in aminoalkyl chain length by only 2 carbon units exhibited opposing biological activities – one promoting growth and the other retarding it. Both compounds were shown to operate through modification of EFP.

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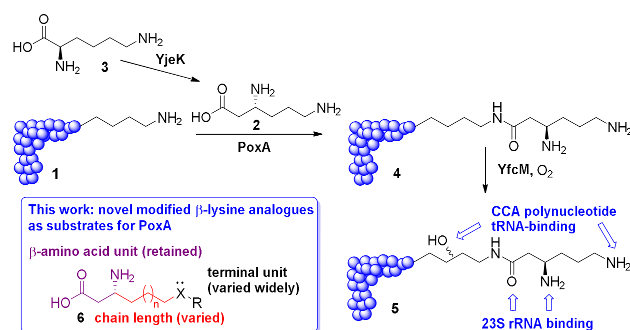
The looming crisis posed by the slow pace of development of new antibacterial chemotherapeutic agents relative to the emergence of drug-resistance (to almost every class of antibacterial drug)[1-4] makes the search for new antibacterial targets a critical strand of research in contemporary medicinal chemistry: no new classes of anti-eubacterial drug have emerged in the last 40 years.[5]

One of the time-honoured antibacterial drug design strategies is to exploit differences between eukaryotic and eubacterial translation - with subsequent disruption of protein synthesis -[5-8] which has led to the emergence of (*inter alia*) a diverse array of eubacterial ribosome modifications, proteins which protect the ribosome and mistranslation strategies which bring about resistance.[9-11]

We are interested in the concept of substrate-based drug design, that is, using existing enzymatic apparatus to deliver drugs which can covalently modify a specific target and modulate its activity, rather than agonism, antagonism or inhibition of targets. This has the potential advantage of tremendous target specificity - at the cost of requiring the design of a molecule that causes phenotypical effects very distinct from the natural substrate for the enzyme (which it must out-compete), without inhibiting the enzyme itself. We have successfully applied this approach to the treatment of a murine model of Multiple Sclerosis[12,13] and wished to evaluate its potential in the exploitation of elongation factor P (EFP) as a potentially novel target in antibacterial chemotherapy.



Scheme 1. Post-translational modification of EFP and the generic structure of the novel artificial PoxA substrates **6**.

EFP (**1**, Scheme 1) was first thought to primarily serve to facilitate the formation of the first peptide bond during protein synthesis,[14,15] however its main function is now understood to be to rescue ribosomes from stalled states during the synthesis of highly challenging[16] (from a kinetic standpoint) polyproline-

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containing peptides.[17,18,19,20,21] Depending on the sequence being translated (and the identity of the amino-acid being added next to the nascent polyproline-polypeptide, in particular Pro-Pro-X triplets[22]), the location of the polyproline-related codons on the mRNA and the level of translation of the gene itself, this pausing can lead to ribosome queuing and deleterious effects on translation in Δ*efp E. coli* strains.[23]

EFP is a tRNA-mimicking, 3-domain L-shaped translation elongation factor[24] which undergoes two unusual post-translational modifications (Scheme 1) in relevant eubacteria such as *E. coli* and *S. enterica*. First, Lys34 is ligated with (*R*)-β-lysine[25,26] (**2** – itself derived from the isomerisation of (*S*)-lysine (**3**) under the influence of YjeK[27]) catalysed by PoxA (also known as YjeA) to give **4**. This then undergoes hydroxylation at the Lys34 residue mediated by YfcM[28] to yield the final enzyme form **5**. It is noteworthy that alternative post-translational modifications of either a lysine or arginine residue in eubacteria are known, in addition to similar yet distinct processes in the eukaryotic and archaeal EFP analogues (named eIF5A, and aIF5A respectively).[21]

Stalling is alleviated through EFP binding in between the ribosomal P and E sites when the E site is vacant, where EFP recognises and aids in rigidifying P-site $tRNA^{Pro}$. Specifically, it has been recently shown that the long chain modification occupies a crevice pocket adjacent to the $tRNA^{pro}$ 3'-CCA sequence (just short of the location of the peptide-transfer centre[29]), where it forces the polyproline end of the nascent peptide chain to change to a conformation compatible with the exit tunnel and more conducive to peptide bond formation.[17, 30, 31]

The elongation factor was initially thought to be essential for eubacterial growth,[32] however current thinking regarding its essentiality is more nuanced, evolving, complex and organism-dependent.[21] EFP does not appear to be universally essential, yet its absence is often associated (including in pathogens such as *Salmonella enterica*) with slower growth/growth defects,[33,34,35] and susceptibility to antibiotics[36] - especially in phases of more rapid eubacterial proliferation where translational demand is high.[37] In some organisms, including the increasingly problematic human pathogen *Mycobacterium tuberculosis,* EFP is reported to be essential.[21, 38]

Given the ribosome stall-alleviating role of EFP (*inter alia*[39-43]) and since it has been shown that *i*) EFP binds during most (if not all) elongation cycles[44] (and not just during polyproline-associated stalling events), *ii*) the Lys34 hydroxyl unit (not necessary for function[17,18,28]) and the amino group from the ligated β-lysine residue form hydrogen bonds with the ribosyl-backbone of the CCA P-site tRNA polynucleotide, while the β-lysine carbonyl- and amine moieties bind to the conserved nucleotide A2439 of the 23S rRNA[31] and *iii*) the β-amino acid modification protrudes close in space to the peptide transfer centre;[29,31] we postulated that if PoxA could decorate EFP with β-amino acids other than **2**, that it would offer an opportunity to influence eubacterial translation very close to the peptide-forming event either in dramatic or subtle ways, depending on the substrates incorporated into EFP and the downstream phenotypic effects of same.

Until very recently, almost nothing was known about the promiscuity of PoxA. In early 2021, Lassak *et al.* reported the post translational modification of EFP with 8 non-canonical β-amino acid substrates.[45] It was found that these were accepted by the enzyme (notably, all possessed the same or similar chain lengths to **2**), yet **2** proved superior to all in rescuing polyproline peptide-synthesising ribosomes from stalled states. This prompted us to report the results of a study involving the exposure of eubacteria (both wild-type and mutants) to a wide range of β-amino acids of diverse chain length and functionality (**6**, Scheme 1), and the effect of same on both eubacterial growth and antibiotic sensitivity.

The investigation began with the synthesis of β-lysine (**2**) as its dihydrochloride salt, together with chain extended and contracted analogues **7**-**11** (Figure 1A). All compounds were synthesised as racemates. We considered the use of *E. coli* K12 strains from the Keio collection advantageous due to the availability of various mutants implicated in the EF-P pathway: including Δ*efp,* Δ*yjeK* and Δ*poxA* variants. Access to these mutants would allow the examination of the effect of candidate molecules on each step of the post-translational modification of EFP. Lysogeny broth was selected as the medium for monitoring the growth of eubacteria. Wild-type and mutant strains in the exponential growth phase were cultivated at 37 °C in 96-well microplates; each well containing a different candidate molecule. This experiment allowed for the quantification of *E.coli* growth at t = 240 min in the presence of **2** and **7-11** for the wild-type strain and Δ*yjeK* strain, respectively. The control, in this case, was the eubacterial cells growing in the absence of supplements. Visible growth was measured by turbidity (optical density at 600 nm) following incubation. It was found that none of the compounds had an appreciable effect on the wild type cells (data not shown). In contrast, Δ*yjeK E. coli* cells deficient in β-lysine (**2**) provided intriguing results (Figure 1B): as expected, the shorter chained β-amino acids **7** and **8** had no appreciable influence on eubacterial growth, however the chain-extended homologue **9** mediated a significant, and unexpected, reduction in doubling time. Further elongation of the β-amino acid residue (*i.e.* **10** and **11**) proved ineffectual.

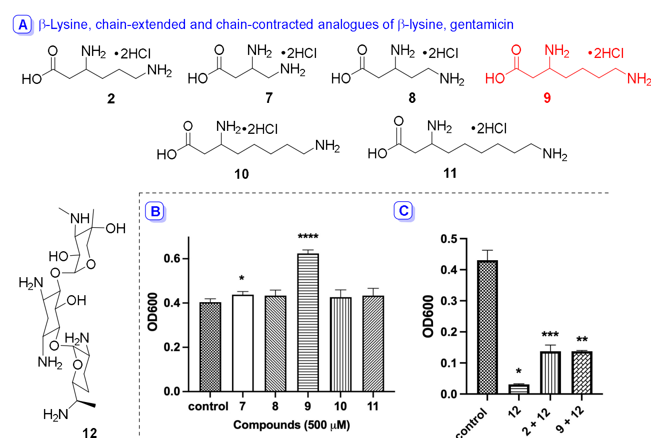

Figure 1. A: Structures of β-lysine dihydrochloride (**2 •2HCl**), simple chain-extended/chain-contracted analogues **7-11** and gentamicin (**12**). **B:** Differences in doubling times of a culture of Δ*yjeK E. coli* cells discernible by OD_{600} in the presence of compounds **7-10** at 240 min. Data are means ± SD (n = 3). **C:** Restoration of growth by **9** following translational arrest in the presence of gentamicin (**12**) represented as OD_{600} of Δ*yjeK* cells at 240 min. Data are means ± SD (n = 3). Unpaired t-test with Welch's correction vs. control was performed, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, not significant values not indicated.

The aminoglycoside gentamicin (**12**) inhibits eubacterial growth by binding to the 30S subunit of the ribosome.[46] It has been demonstrated that strains of *S. enterica* which lacked the genes

encoding *PoxA* and *YjeK* (*i.e.* WN353 Δ*poxA* and WN354 Δ*yjeK* respectively) were more sensitive to the action of **12**.[47] In contrast, the growth of the wild-type strain was shown not impacted by the presence of **12** - implicating the EFP pathway in the resistance mechanism. Additionally, it was found that supplementation of the eubacterial culture with **2** could restore growth of the Δ*yjeK* mutants growing in the presence of **12**.[47] It was envisaged that executing a similar experiment involving **9** in *E. coli* could be used to demonstrate the activity of this compound in the target pathway. Thus, a screening assay was carried out to investigate the impact of supplementation with **9** on the sensitivity of *E. coli* Δ*yjeK* cells to the aminoglycoside **12** (Figure 1C). Exposure of the Δ*yjeK* cells to the experimentally determined (see ESI for details) MIC of **12** (6 μg/mL) led to a dramatic reduction in growth as expected, however an identical experiment in the presence of **9** led to a partial restoration of growth of a similar magnitude to that observed using the natural PoxA substrate **2**. These data strongly indicate that *i*) unnatural β-lysine analogues can potentially serve as PoxA substrates and *ii*) that EF-P is post translationally modified by the chain-extended homologue **9** in *E. coli* and is able to perform a similar function (with similar competency) to wild-type variants decorated with the natural amino acid derivative **2**. A subsequent repeat of this experiment involving **7**, **8**, **10** and **11** resulted in no restoration of growth (data not shown).

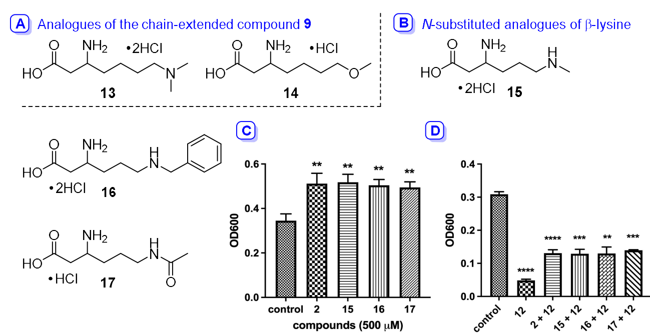

Figure 2. A: Structures of **13-14**; the inactive terminally-modified analogues of **9**. **B:** Structures of *N*-methyl β-lysine (**15**), *N*-benzyl β-lysine (**16**) and *N*-acetyl β-lysine (**17**). **C:** Differences in doubling times of a culture of Δ*yjeK E. coli* cells discernible by OD_{600} in the presence of compounds **15-17** at 240 min. Data are means ± SD (n = 3). **D:** Restoration of growth by **15-17** following translational arrest in the presence of 6 μg/mL gentamicin (**12**) represented as OD_{600} of Δ*yjeK* cells at 240 min. Data are means ± SD (n = 3). Unpaired t-test with Welch's correction vs. control was performed, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Given the ability of the chain-extended homo-β-lysine **9** to serve as a functional PoxA substrate, the contribution from the terminal primary amino substituent was investigated through the synthesis and evaluation of the tertiary amine **13** and the corresponding methyl ether **14** (Figure 2A). Both compounds had no effect on eubacterial growth (data not shown). Returning to the β-lysine core structure, secondary amines **15-16** and amide **17** were prepared to probe the role of hydrogen-bond donation/basicity at the amino acid residue (Figure 2B). While it was found that secondary amines were well tolerated and could increase the rate of proliferation of Δ*yjeK* cells with comparable activity to the natural substrate **2**, it was surprising to find that basicity is not a requirement for activity: the presence of a hydrogen bond donating unit at the chain terminus is sufficient to ensure activity (Figure 2C). All three compounds restored eubacterial growth in Δ*yjeK* cells (Figure 2D) in the presence of **12** to the same extent as β-lysine (**2**). It is noteworthy that **15-17** exhibited no influence on the growth of wild type *E. coli* cells.

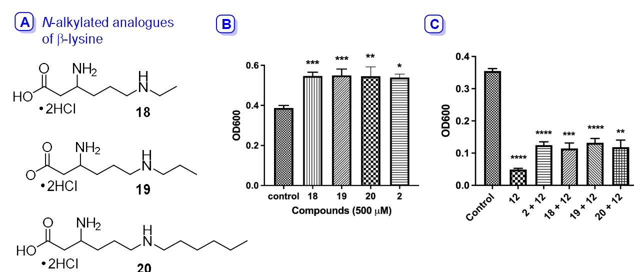

Figure 3. A: Structures of *N*-alkylated analogues of β-lysine **18-20**. **B:** Differences in doubling times of a culture of Δ*yjeK E. coli* cells discernible by OD_{600} in the presence of compounds **18-20** at 240 min. Data are means ± SD (n = 3). **C:** Restoration of growth by **18-20** following translational arrest in the presence of 6 μg/mL gentamicin (**12**) represented as OD_{600} of Δ*yjeK* cells at 240 min. Data are means ± SD (n = 3). Unpaired t-test with Welch's correction vs. control was performed, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

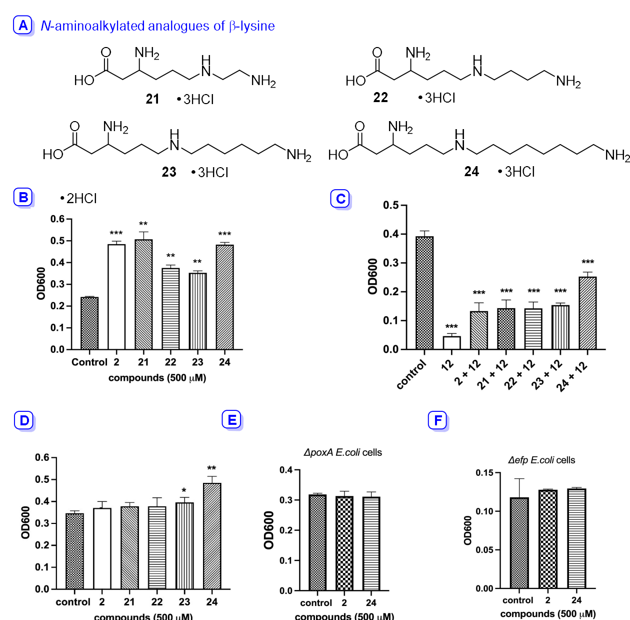

Figure 4. A: Structures of *N*-aminoalkylated analogues of β-lysine **21-24**. **B:** Differences in doubling times of a culture of Δ*yjeK E. coli* cells discernible by OD_{600} in the presence of compounds **21-24** at 240 min. Data are means ± SD (n = 3). **C:** Restoration of growth by **21-24** following translational arrest in the presence of 6 μg/mL gentamicin (**12**) represented as OD_{600} of Δ*yjeK* cells at 240 min. Data are means ± SD (n = 3). **D:** OD_{600} of wild-type *E. coli* cells incubated for 240 min in the presence of candidate molecules **21-24** Data are means ± SD (n = 3). **E:** Doubling times of a culture of Δ*poxA E. coli* cells in terms of OD_{600} in the presence of **24** at 240 min. Data are means ± SD (n = 3). **F:** Doubling times of a culture of Δ*efp E. coli* cells in terms of OD_{600} in the presence of **24** at 240 min. Data are means ± SD (n = 3). Unpaired t-test with Welch's correction vs. control was performed, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, not significant values not indicated.

The next tranche of candidate molecules **18-20** possessed the secondary amino moiety useful for activity and were equipped with *N*-alkyl substituents designed to potentially extend into the peptide transfer site and disrupt acyl transfer (Figure 3A). These species – including the *N*-hexyl variant **20** – instead served as promoters of Δ*yjeK E. coli* growth (Figure 3B) and restored growth in the presence of gentamicin in a similar fashion to **2** (Figure 3C). No effect on the growth of wild type cells was discernable.

With the same goal of generating a β-lysine derivative which could serve as a substrate for PoxA while incorporating a chain capable of reaching and influencing the peptide-transfer site, we synthesised **21-24** (Figure 4A). These are characterised by an alkyl chain of increasing length through the series and a terminal primary amine - which it was postulated could maximise the opportunity for interaction with the components at the ribosomal peptide transfer site. When Δ*yjeK E. coli* cells were exposed to these compounds a similar augmentation of growth rates to that observed using β-lysine supplementation was observed using **21** and **24**, with less improvement in proliferation (yet substantially greater than that associated with the control) seen in the presence of **22** and **23** (Figure 4B). Remarkably, a repeat of these experiments in the presence of gentamicin revealed that **24** restored a rate of growth substantially greater than that obtained using β-lysine (Figure 4C). The long-chained analogue **24** is also able to increase the rate of growth of wild type *E. coli* – the first time in the study that these cells proved phenotypically responsive to the actions of EFP modified with an unnatural substrate (Figure 4D). To establish that the observed effect is a result of the actions of the compound after installation onto EFP, assays involving *ΔpoxA* and *Δefp* cells were undertaken (Figure 5E-F, respectively). Derivative **24** had no effect on eubacterial growth in these experiments, which strongly indicates the reliance on the EFP pathway for the observed faster growth rates of wild type eubacteria in the presence of this compound.

The wild type cell-active species **24** consists of a β-lysine core *N*-substituted with an octylamine moiety. This invites the hypothesis that when installed on EFP, that the chain terminus has reached a region in space at the peptide-transfer site where it can exert influence, and that the further extension of the chain length was warranted. Accordingly, the analogous *N*-decylamino and *N*-dodecylamino variants (**25-26**, Figure 5A) were prepared and evaluated. These compounds behaved differently: the growth of *Δyjek* cells was no longer promoted in a similar fashion to that observed using β-lysine, and in the case of **26**, growth rates were inferior to the control cells (Figure 5B). Intriguingly, dramatic effects were observed using wild type cells (Figure 5C) – use of **25** greatly reduced *E. coli* growth rates relative to either the control or cells in the presence of **18**; while exposure to **26** led to an almost complete arrest of growth. The difference in growth rates between *Δyjek* and wild-type cells in the presence **25-26** is difficult to rationalise at this juncture and may imply the involvement of YjeK in the retardation of proliferation of wild type cells. It is noteworthy however that the data trend (*i.e.* growth: **18** > **25** > **26**) is the same in both experiments. The critical importance of chain length in these systems is exemplified by the fact that **25** has no effect on *ΔpoxA* cells, while **26** - a compound 2 methylene units longer - halts their growth. This indicates that **25** operates *via* the EFP pathway while the activity of **26** is off target (Figure 5D). Very similar results were obtained using *Δefp* cells (Figure 5E).

The β-amino acid of intermediate chain length (*i.e.* the undecylamine-substituted **27**, Figure 5A) was next prepared. This compound exhibited behaviour in between that associated with **25** and **26**. It promoted the growth of *Δyjek* cells with less efficiency that β-lysine itself (Figure 5F), reduced proliferation rates of wild type cells less than **26** yet more than **25** (Figures 5G) and demonstrably operated *via* the EFP pathway (Figure 5 H-I).

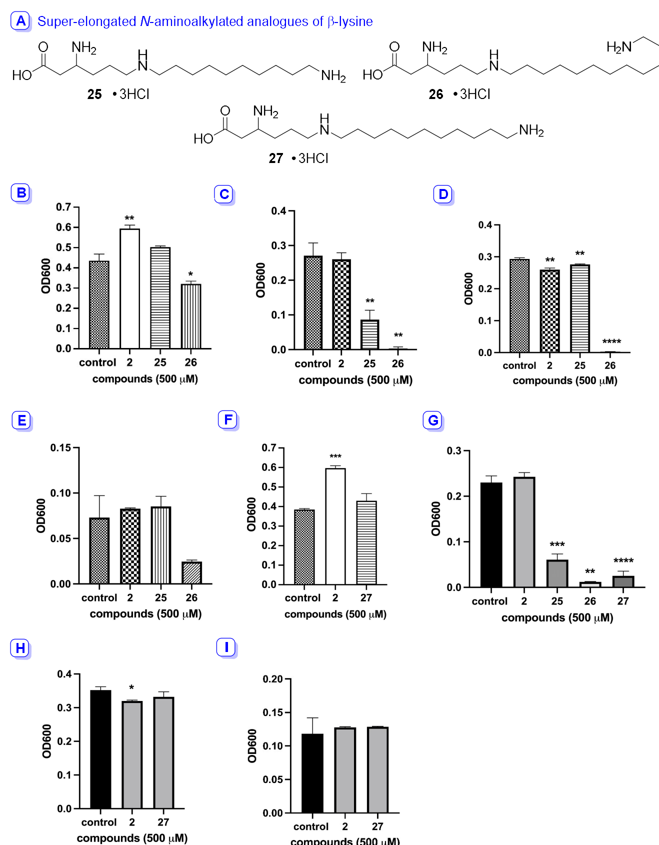

Figure 5. A: Structures of *N*-aminoalkylated analogues of β-lysine **25-27**. **B:** Differences in doubling times of a culture of Δ*yjeK E. coli* cells discernible by OD_{600} in the presence of compounds **25-26** at 240 min. Data are means ± SD (n = 3). **C:** Differences in doubling times of a culture of wild type *E. coli* cells discernible by OD_{600} in the presence of compounds **25-26** at 240 min. Data are means ± SD (n = 3). **D:** Differences in doubling times of a culture of Δ*poxA E. coli* cells discernible by OD_{600} in the presence of compounds **25-26** at 240 min. Data are means ± SD (n = 3). **E:** Doubling times of a culture of Δ*efp E. coli* cells in terms of OD_{600} in the presence of **25-26** at 240 min. Data are means ± SD (n = 3). **F:** Doubling times of a culture of Δ*yjek E. coli* cells in terms of OD_{600} in the presence of **27** at 240 min. Data are means ± SD (n = 3). **G:** OD_{600} of wild-type *E. coli* cells incubated for 240 minutes in the presence of candidate molecules **25**, **26** and **27**. Data are means ± SD (n = 3). **H:** OD_{600} of a culture of Δ*poxA E.coli* cells in the presence of **27** at 240 minutes. Data are means ± SD (n = 3). **I:** OD_{600} of a culture of Δ*efp E.coli* cells in the presence of **27** at 240 minutes. Data are means ± SD (n = 3). Unpaired t-test with Welch's correction *vs.* control was performed, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, not significant values not indicated.

Two of the compounds exhibiting antibacterial profiles (*i.e.* **25** and **26**) along with β-lysine (**2**, control) and *N*-hexyl β-lysine (**20**) were evaluated for toxicity in human embryonic kidney 293 (HEK-293) cells at various concentrations to allow the determination of IC_{50} (Figure 6). While this assay confirmed the lack of toxicity associated with β-lysine supplementation in mammals, the candidate molecules **20**, **25** and **26** displayed dose-dependent toxicity. The promising compound **25** was calculated to have an IC_{50} value of 250 μM. The survival rate of the cells was 81% when the concentration of **25** was 50 μM; however, the survival rate dipped to 0% when **25** was employed at 500 μM; which is the concentration at which antibacterial toxicity had been observed (Figure 6). These data imply that **25** is highly toxic to mammalian cells and lacks the biocompatibility properties required for the development of a therapeutic agent. It was posited

that the cytotoxicity of this compound could be due to its structural similarity to the eukaryotic substrate hypusine which could result in an interaction with deoxyhypusine hydroxylase – an enzyme required for the activity of eIF5A, an essential protein in eukaryotes. Another mode of toxicity could be the lipophilic nature of this compound, resulting in cell membrane toxicity. Similarly, **26** is highly toxic to the cells used in this assay: at concentrations as low as 50 μM, the cell survival rate is 14%. Additionally, **20** appears to be toxic (IC_{50} value of 110 μM), again implying that the toxicity associated with these compounds could be a result of their lipophilic nature; implicating the alkyl side chain at the ε-amino group in the toxicity of these compounds to mammalian cells.

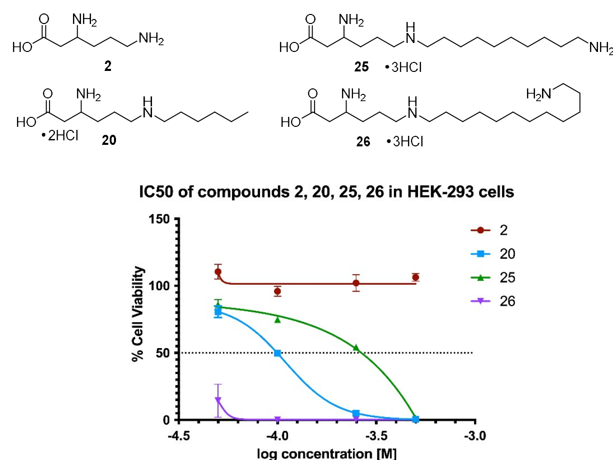

Figure 6. Determination of IC_{50} cells for compounds **20**, **25,** and **26** with **2** as a control in HEK-293 cells. IC_{50} values for **20** were determined to be ~110 μM and for **25** ~ 250 μM. Data was generated from three independent repeats..

In summary, it has been shown for the first time that the promiscuity of PoxA is considerable – the enzyme recognises the β-lysine motif and appears capable of catalysing the modification of EFP with a range of exogenous β-lysines equipped with terminal *N*-substituents, some 10-12 carbon-carbon bonds in length. Both alkyl and aminoalkyl substituents are compatible. Assays initially focused on the effect of potential substrates for PoxA on Δ*yjek E. coli* cells – as these are devoid of the β-lysine natural substrate. It was found that an unnatural chain extended homologue of the natural substrate (*i.e.* **9**) could promote faster growth than that associated with control cells (while having no effect on wild type cells), and also restore growth in the presence of gentamicin (eubacterial resistance to which is associated with EFP) with efficacy similar to β-lysine. Variants of this homologue with either terminal dimethylamino- or ether functionality were devoid of activity, while it was shown that β-lysine itself could be *N*-substituted without damaging activity once one N-H bond remained at the terminus. This, in addition to the activity of a non-basic, amidated analogue **17** points to hydrogen bond donation being the dominant interaction with the target at this position.

Both *N*-alkyl and *N*-aminoalkyl chain extended β-lysines **18-23** exhibited similar behaviour – they accelerated the growth of Δ*yjek* cells but not wild type *E. coli*, while being able to restore the rate of growth of gentamicin-treated cells with similar efficacy to that associated with β-lysine **2**. The *N*-octylamino β-lysine **24** displayed intriguing properties: use of this compound was associated with an increase in growth rates of even wild type cells which have β-lysine available. The compound was inactive in the absence of either EFP or PoxA, meaning that it must be operating *via* covalent modification of EFP - leading to an improvement in the modified protein's functionality. Further extension of the aminoalkyl chain length by 2 and 4 carbon units (**25** and **26**) led to very different activity: these materials inhibited eubacterial growth even in wild type cells – **25** *via* the EFP pathway exclusively and **26** *via* off target effects. The opposing sense of biological activity associated with the closely structurally related **24** and **25** strongly indicates that the aminoalkyl chain in these species protrudes into a region in space on the ribosome proximal to the peptide forming event.

It was found that a representative sample of the chain-extended β-lysines were toxic to mammalian cells, thereby limiting their potential use as chemotherapeutic agents. It seems clear however, that modification of the core β-lysine structure is possible without losing PoxA compatibility - which allows the design of compounds which can either enhance or retard the growth of wild type eubacterial cells *via* the EFP pathway. Studies to further elucidate the mode of action of these compounds and to obviate toxicity are underway in our laboratories.

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Supplementary Material

Experimental details, characterization data.