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14. ABSTRACT We have solved the structures of bacterial PanKs from <i>S. aureus</i> , <i>K. pneumoniae</i> and <i>E. coli</i> in complex with substrate analogs (N5-Pan and N7-Pan) as well as the structure of human PanK3 in complex with N7-Pan. With the finding that the Pan analog binding site of SaPanK is different from human PanK, we synthesized two new compounds that fit to the Pan analog binding site of SaPanK. Similarly, we are currently synthesizing a drug-like chemical series against KpPanK (or EcPanK) by comparing the N7-Pan binding pockets of KpPanK (or EcPanK) and human PanK3. Afterward, we will optimize the conditions of bacterial colony and cytotoxicity MTT assays for newly synthesized compounds. These studies will result in three sets of new compounds that inhibit the activities of PanKs from three different bacteria. Predictably, each compound set will specifically hamper the growth of respective bacteria, but has minimal effect on human cells. These new compounds will be the basis for further testing in animal models and clinical trials to develop a novel class of narrow-spectrum antibiotics active against the multidrug resistant strains of <i>S. aureus</i> , <i>K. pneumoniae</i> , and <i>E. coli</i> frequently found in battlefield wounds sustained in Iraq and Afghanistan.					
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INTRODUCTION:

A goal of the studies is to develop antimicrobial compounds with novel modes of action to treat the battlefield infections from *Staphylococcus aureus*, *Klebsiella pneumoniae* and extended-spectrum beta-lactamase enterobacteriaceae including *E. coli*. N-substituted pantothenate analogs have been developed and shown to inhibit the growth of *S. aureus* and *E. coli* (and probably also *K. pneumoniae*) but have not been tested in preclinical and clinical set-ups, primarily because of their possible interference with human cells. We propose to elucidate differences in the architecture of the compound-pantothenate kinase binding site between humans and bacteria using x-ray crystallographic techniques and exploit these differences to develop new compounds specific for the drug-resistant bacterial strains.

BODY:

Pantothenate kinase gene cloning The genes of *K. pneumoniae* and *E. coli* pantothenate kinase (KpPanK and EcPanK) were chemically synthesized by GenScript, the gene of *S. aureus* pantothenate kinase (SaPanK) was from its genomic DNA library, and the gene of human pantothenate kinase (hPanK) Isoform 3 was from the Mammalian Gene Collection. These PanK genes were sub-cloned into a bacterial expression vector pET28a, as described in our previous publications¹⁻³. Each of the resulting plasmids contains the NH₂-terminal His₆ tag fused to the PanK protein, which can be purified by nickel-nitrilotriacetic acid chromatography based on the interaction between a transition Ni²⁺ ion immobilized on a matrix and the histidine side chains.

Protein expression and purification *E. coli* BL21(DE3) (Stratagene) was transformed with each of the resulting plasmids, and recombinant PanK proteins (i.e., KpPanK, EcPanK, SaPanK and hPanK3) were overexpressed upon 1.0 mM isopropyl 1-thio- β -D-galactopyranoside induction for 18 h at 18 °C. The proteins were purified using nickel-nitrilotriacetic acid affinity and Superdex 200 (GE Healthcare) gel-filtration columns. Purified PanK samples were concentrated to up to 35 mg/ml, and stored at -80 °C in the gel filtration buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10 mM dithiothreitol).

Protein crystallization, data collection and structure determination For co-crystallization, the purified PanK was incubated at room temperature for overnight in the presence of two different N-substituted pantothenate analogs (N5-Pan and N7-Pan) and then set up crystallization trays using the sitting-drop vapor diffusion method with droplets of protein solution (0.5 μ l) and reservoir solution (0.5 μ l). The best diffracting crystals were obtained from conditions as follows. For SaPanK + N5 and SaPanK + N7, a reservoir solution contained 30% PEG4K, 0.2 M MgCl₂, 0.1 M Tris and pH 8.5. For hPanK + N7, a reservoir solution contained 25% P3350, 0.2 M LiSO₄, 0.1 M HEPES and pH 7.5. For KpPanK + N5, a reservoir solution contained 20% PEG3350, 0.2M tri-lithium Citrate and 0.2 μ l butanediol. For EcPanK + N5, a reservoir solution contained 30% PEG2K, 0.2 M KBr and 20 μ M Urea. Crystals were cryo-protected with 50% Paratone-N, 50% mineral oil and were frozen directly in liquid nitrogen for x-ray data collection. Data collection was carried out using a home x-ray source and at the Advanced Photon Source.

All of the diffraction data were processed using the HKL2000 package⁴. Structures were solved by the molecular replacement method using PHASER⁵ with search model

based on the coordinates of corresponding PanK (PDB codes 2EWS for the SaPanK structures, 2I7P for the hPanK3 structure and 1SQ5 for the KpPanK and EcPanK structures). The models underwent several rounds of model building, refinement, and validation with COOT ⁶, REFMAC5 ⁷, and MOLPROBITY ⁸, respectively. Data collection and refinement statistics are given in Table 1.

Table 1. Data collection and refinement statistics of SaPanK, hPanK3, KpPanK and EcPanK in complex with N-substituted pantothenate analogs.

PanK compound ⁺	SaPanK+N5	SaPanK+N7	hPanK3+N7	KpPanK+N5	EcPanK+N5
PDB code	Refinement in progress	Refinement in progress	3SMS	Refinement in progress	Refinement in progress
Space group	C2	C2221	P3121	P212121	P212121
Resolution (outer shell, Å)	30.00-1.80 (1.85-1.80)	40.00-1.45 (1.49-1.45)	25.00-2.20 (2.26-2.20)	48.04-2.05 (2.16-2.05)	38.85-2.20 (2.32-2.20)
Unique observed HKLs	48878 (3589)	49833 (3561)	20590 (1524)	201488 (28707)	77343 (11035)
Completeness (%)	99.4 (99.0)	97.4 (95.8)	99.8 (99.8)	99.7 (98.1)	99.8 (98.6)
Friedel Redundancy	7.1 (7.1)	11.0 (7.5)	10.8 (10.6)	7.0 (6.4)	7.6 (7.3)
Rsym ^a	0.079 (0.950)	0.090 (0.838)	0.129 (0.647)	0.157 (1.046)	0.175 (0.877)
<I>/<σ>	14.9 (2.0)	14.7 (2.2)	16.7 (4.3)	12.9 (2.6)	9.1 (2.4) ^a
Refinement resolution (Å)	26.53-1.80	35.55-1.45	25.00-2.20	40.0-2.06	30.00-2.20
Number of reflections work/free	46722/2072	47190/2511	19464/1044	188312/9999	75138/2087
Rwork/Rfree ^b	0.194/0.249	0.230/0.254	0.190/0.243	0.205/0.243	0.234/0.269
RMSD bond lengths(Å)/angles(°)	0.023/2.0	0.025/2.2	0.013/1.4	0.011/1.265	0.015/1.7
Ramachandran residues in most favored regions (%) ^c	91.2	89.6	92.1	91.8	91.6
Additional allowed	8.8	10.0	7.3	8.0	8.1
Generously allowed	none	none	none	0.3	0.4
Disallowed	none	0.4	0.7	None	none

$$^a R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I.$$

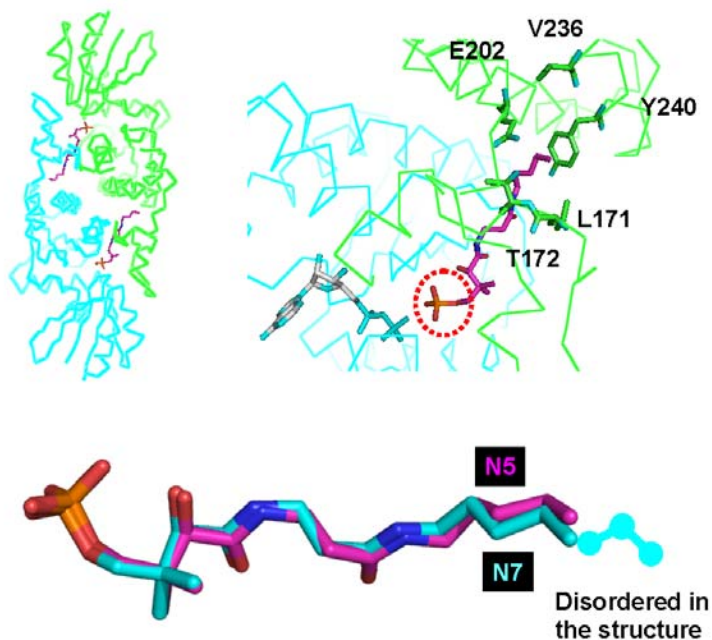
^b $R_{\text{work}} = \sum ||F_0| - |F_c|| / \sum |F_0|$, where F_0 and F_c are the observed and calculated structure factors, respectively. R_{free} was calculated as R_{work} using ~5.0% of the data set aside for an unbiased test of the progress of the refinement.

^c Program PROCHECK was used ⁹.

Co-crystal structure of the *S. aureus* pantothenate kinase with substrate analogs This section is in accordance with Statement of Work, Tasks 1.1 and 1.2. We solved the structures of SaPanK in complex with N5-Pan or N7-Pan. The N5-Pan analog is bound to one molecule of dimeric SaPanK as well as a loop region of the second molecule (Fig. 1, top left). The co-crystallization of SaPanK with N5-Pan was carried out in the presence of ATP molecule, but the resulting N5-Pan was in phosphorylated form and ADP, instead of ATP, was found at the binding site (Fig. 1, top right), suggesting that ATP was converted to ADP with the phosphorylation of N5-Pan (Pho-N5-Pan). This finding is consistent with the fact that N5-Pan is a substrate of SaPanK. The hydrophobic portions of multiple residues (Leu171, Thr172, Glu202, Val236 and Tyr240) form a non-polar tunnel that accommodates the pentyl tail of Pho-N5-Pan (Fig. 1, top right).

N7-Pan was co-crystallized with SaPanK in the presence of ATP. Similar to N5-Pan, ATP and N7-Pan were converted to ADP and the phosphorylated N7-Pan (Pho-N7-Pan), respectively. The binding sites of SaPanK for the two analogs almost overlap each other. The mode of binding between Pho-N5-Pan and Pho-N7-Pan in both crystal structures of SaPanK was identical (Fig. 1, bottom), which is well reflected in the same orientation of the surrounding residues. N7-Pan analog has two more methyl groups at the N-substituted tail than that of N5-Pan. Electron density corresponding to these two methyl groups of N7-Pan is invisible and thus disordered in the structure due to no contact with SaPanK residues. Therefore, the extra two methyl groups of N7-Pan are not needed for the binding to SaPanK (Fig. 1, bottom).

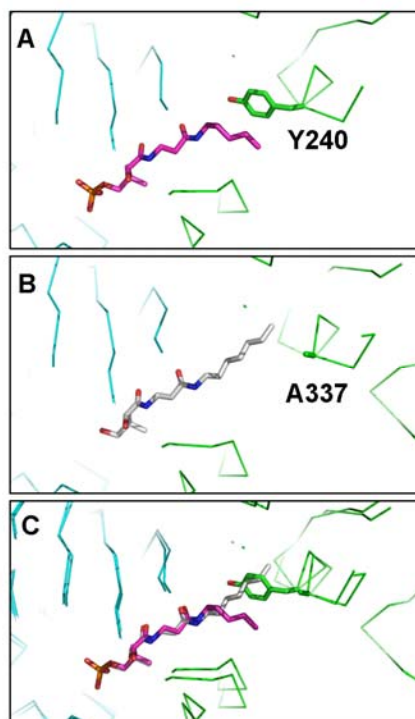
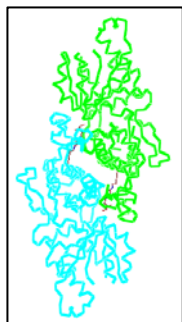
Figure 1. **Structures of SaPanK in complex with N-substituted Pan analogs.** **Top left**, N5 (magenta, stick) is bound to both molecules of a dimeric SaPanK (cyan and green, ribbon). **Top right**, the pentyl moiety of N5 forms non-polar interactions with the 2nd molecule residues (green). Dotted circle indicates the phosphorylated N5, transferred from the nucleotide (carbon in grey). **Bottom**, the conformations of N5 and N7 are similar to each other when bound to SaPanK. The last two methyl groups of N7 disordered in the structure are indicated (cyan).



Structural comparison of SaPanK and human PanK This section is in accordance with Statement of Work, Task 1.5. To compare the binding properties of Pan-analogs with SaPanK and hPanK, we solved the structure of the hPanK isoform 3 complexed with N7-Pan analog. In the structure, the N-7 Pan analog was bound to hPanK3, similar to SaPanK. The bound N7 analog interacted with the beta-barrel from one molecule of dimeric hPanK3 and also with the loop region of the other molecule (Fig. 2 insert). Unlike in the SaPanK structures complexed with the phosphorylated Pan analogs, however, the N7 analog bound to hPanK3 was not phosphorylated.

To analyze the interaction mode of SaPanK and hPanK with the Pan analogs, we superimposed the structures of N7-Pan bound SaPanK and hPanK3 (Fig. 2). The pantothenate moieties of the N7-Pan analogs were well overlapped while the hydrophobic tail regions were poorly superimposed. This conformational difference between SaPanK and hPanK3 could be explained by the difference of residues lining the hydrophobic tail binding pocket. Among these residues, we specially paid attention to Tyr240 of SaPanK, which is replaced by Ala337 of hPanK3 (Fig. 2). Structurally, the bulky Tyr240 of SaPanK was oriented toward the N7 tail, bending the N7 tail to avoid a steric hindrance and exposing the last two methyl groups of the N7 tail to solvent. In contrast, Ala337 of hPanK3 accommodated the N7 tail for binding.

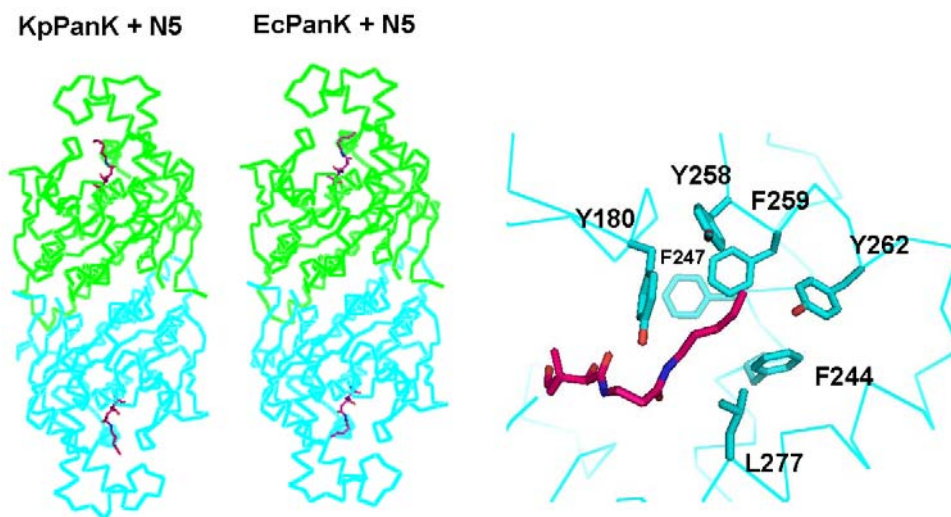
Figure 2. **The binding sites of SaPanK and hPanK3 for N-substituted Pan analogs.** **A)** The penty/heptyl moiety of N5/N7 (magenta, carbon) is bent and interacts with the side chain of Y240 of SaPanK. **B)** The heptyl moiety of N7 (grey, carbon) is straight in hPanK3 due to the corresponding amino acid changed to alanine (A337). The small side chain of A337 does not cause the bending of the heptyl moiety of N7. **C)** when overlaid, the heptyl moiety of N7 of hPanK3 clashes into Y240 of SaPanK, suggesting that the bent conformations of N5 and N7 are required for the binding to SaPanK.



Structural comparison of KpPanK and EcPanK This section is in accordance with Statement of Work, Tasks 1.3 and 1.4. We solved the structures of KpPanK and EcPanK complexed with N5-Pan. In KpPanK, the aliphatic tail of N5-Pan is bent towards a hydrophobic pocket containing multiple aromatic residues of KpPanK (Y180, F244, F247, Y258, F259, Y262 and L277) (Fig. 3). Similar to KpPanK, the hydrophobic tail of N5-Pan in the EcPanK structure was bent to form a U-shape (data not shown). This bent conformation can be compared with the N7-Pan molecule bound to hPanK3, in which the hydrophobic chain of the bound N7-Pan adopted a straight conformation. The

conformational differences of the analogs between KpPanK/EcPanK and hPanK3 as well as the amino acid differences lining the pentyl/heptyl moiety of N-substituted Pan analog will be the basis to develop new compounds specifically interfering with the activities of KpPanK and EcPanK.

Figure 3. **Structures of KpPanK and EcPanK in complex with N5 Pan analog.** *Left*, both the structures of KpPanK and EcPanK are dimeric (green and cyan). N5 is bound to only one molecule, different from SaPanK and hPanK3 that involve two molecules to form the binding pocket for N5 or N7. *Right*, a zoomed-in view of the N5 binding site of KpPanK shows the bending of N5, similar to that of SaPanK, but different from the straight conformation of N7 in hPanK3. Non-polar aromatic residues of KpPanK interacting with the pentyl moiety of N5 are labeled.



New compound design and validation This section is in accordance with Statement of Work, Tasks 2.1, 2.2 and 2.3. Based on the differences of the N7-Pan heptyl group binding sites between SaPanK and hPanK3, we synthesized two new compounds and tested them as well as N5 and N7-Pan analogs (Fig. 4) against proliferating human HepG2 liver cells for assessing their cytotoxicity potential. The cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum. The cells were incubated at 37°C incubator in a 5% CO₂ atmosphere until a confluent monolayer was achieved. The liver cells were trypsinized from the culture flasks and washed in fresh culture medium. The cells were counted with a hemocytometer and diluted in culture medium. Fifty microliters of inoculum (10,000 cells/well) were added to each of the wells of the two 96-well flat-bottom plates. N5-Pan, N7-Pan and the two newly designed compounds were prepared in different concentrations using serial dilutions in the cell medium. Fifty microliters of diluted drugs in cell media were added to each well achieving the final concentrations of 5000, 1000, 500, 100, 50, 10, 5 μ M. The plates were incubated at 37°C in 5% CO₂ for 24 hrs. Following compound exposure, viabilities of the cells were determined with MTT dye (Thiazolyl Blue Tetrazolium Bromide) in two time intervals: 24 and 48 hrs. MTT is reduced to purple formazan in living cells; the absorbance of the formazan was quantified by measuring it at a wavelength of 570 nm by spectrophotometer. Only N7-Pan at concentration of $\sim$ 600 μ g/ml showed 50% decrease in the cell viability (Fig. 5a), indicating that none of the tested compounds including N7-Pan significantly inhibit the growth of the human cells.

To confirm the non-cytotoxic effects on HepG2 liver cells, the cytotoxicity potential of N5-Pan and N7-Pan were tested against proliferating human adenocarcinomic alveolar basal epithelial cells (A549 cells, American Type Culture Collection). The cells were grown in RPMI media 1640 (Gibco) supplemented with 10% fetal bovine serum incubated at 37°C incubator in a 5% CO₂ atmosphere until a confluent monolayer is achieved. Trypsin/EDTA solution was used to detach the cells from the culture flask for cell passage. The analogs were prepared as a double dilution series in 96-well flat-bottom plates with RPMI 1640–10% fetal bovine serum. Fifty microliters of inoculum (1.5×10^4 cells/well) were added to each of the wells of the microtiter plate. The plate was incubated at 37°C in 5% CO₂ for 72 hrs. Following drugs exposure, cell viability was determined with the thiazolyl blue tetrazolium bromide dye (MTT, Sigma Aldrich). For this purpose, the media was removed and replaced with fifty microliters of RPMI 1640- 10% FBS without phenol red supplemented with 1 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The plate was incubated for another two hours so that the live cells reduce yellow MTT to purple formazan. The resulting formazan solubilized by the solubilization buffer (Isopropanol, 10% Triton X-100, 0.1N HCl); the absorbance was measured at 570 nm and corrected for 650 nm background. The 50% toxic concentrations of the analogs are the lowest concentrations of the compounds, which cause a $\geq 50\%$ decrease in cell viability compared to analog-free control containing cells and culture media only. Figure 5b shows that the IC₅₀ value of N7 for A549 lung cells is ~ 350 $\mu\text{g/ml}$, similar to that of N7 for HepG2 liver cells. Testing the cytotoxicity of the two new compounds for A549 cells is in progress.

For assessing the bacterial-killing capability of Pan analogs, we determined the MICs (Minimum Inhibitory Concentration) of N5-Pan and N7-Pan against *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 by using a broth microdilution assay. Briefly, bacterial cultures were prepared by inoculating 5 ml of Laura broth with a small amount of the desired glycerol stock of each bacterial strain and allowed to grow at 37°C to an A_{600} of ~ 0.1 . 96-Well plates were prepared containing 50 μl of two-fold serial dilutions (20 mg/ml - 9.76 $\mu\text{g/ml}$) of Pan analogs and then inoculated with another 50 μl of the desired culture (the desired final inoculum size is 5×10^5 cfu/ml) and allowed to incubate at 37°C for 18 h. The optical density was measured for all cultures at A_{600} , and the MIC was determined from the concentration of compounds at which no growth was observed. All MIC determinations were performed in duplicate. The results of the MIC assays are as follows (Fig. 6): the MIC for *E. faecalis* was determined as 156 $\mu\text{g/ml}$ for N5-Pan and 29 $\mu\text{g/ml}$ for N7-Pan, respectively. In the case of *S. aureus*, however, its MIC values to both Pan analogs could not be determined, because cells did not grow even at the given minimal concentration (9.76 $\mu\text{g/ml}$) of both Pan analogs. So, we are planning to repeat the MIC assay for *S. aureus* with less than 10 $\mu\text{g/ml}$ of Pan analogs. The MIC test for *P. aeruginosa* did not go well due to the fact that the *P. aeruginosa* cells were clotted during broth culture, making it impossible for optical density measurement. Shortening the incubation time may prevent cell clotting, which will be tried. More importantly, we will measure the MICs of the new compounds against *S. aureus*, *K. pneumoniae* and *E. coli* in the near future.

Figure 4. **N-substituted Pan analogs.** MT-0183 and MT-0190 are two newly synthesized compounds.

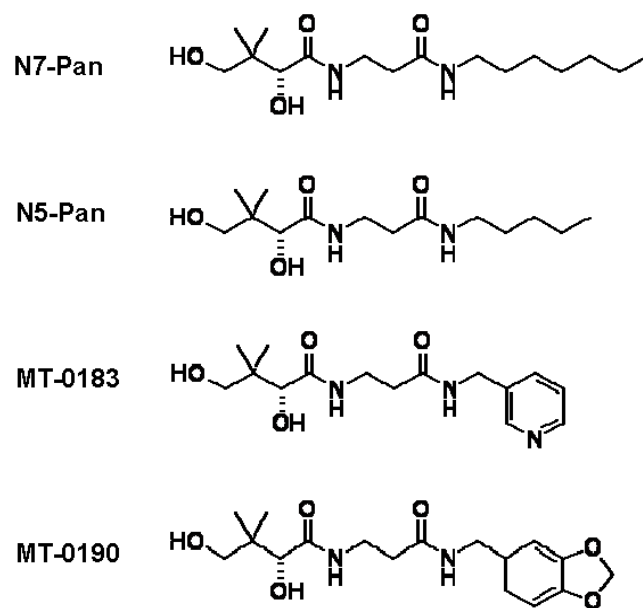


Figure 5. **Measurement of the metabolic activities of HepG2 liver and A549 lung cells.** **A)** Measurement after 24 and 48 hours (top and bottom panels, respectively). Even after 48 hours, HepG2 liver cells were viable in N5, MT-0183 and MT-0190, but not in N7. The IC₅₀ value of N7 is 2 mM (~600 µg/ml). **B)** A549 lung cells were viable in N5, but not in N7. The IC₅₀ value of N7 is ~350 µg/ml.

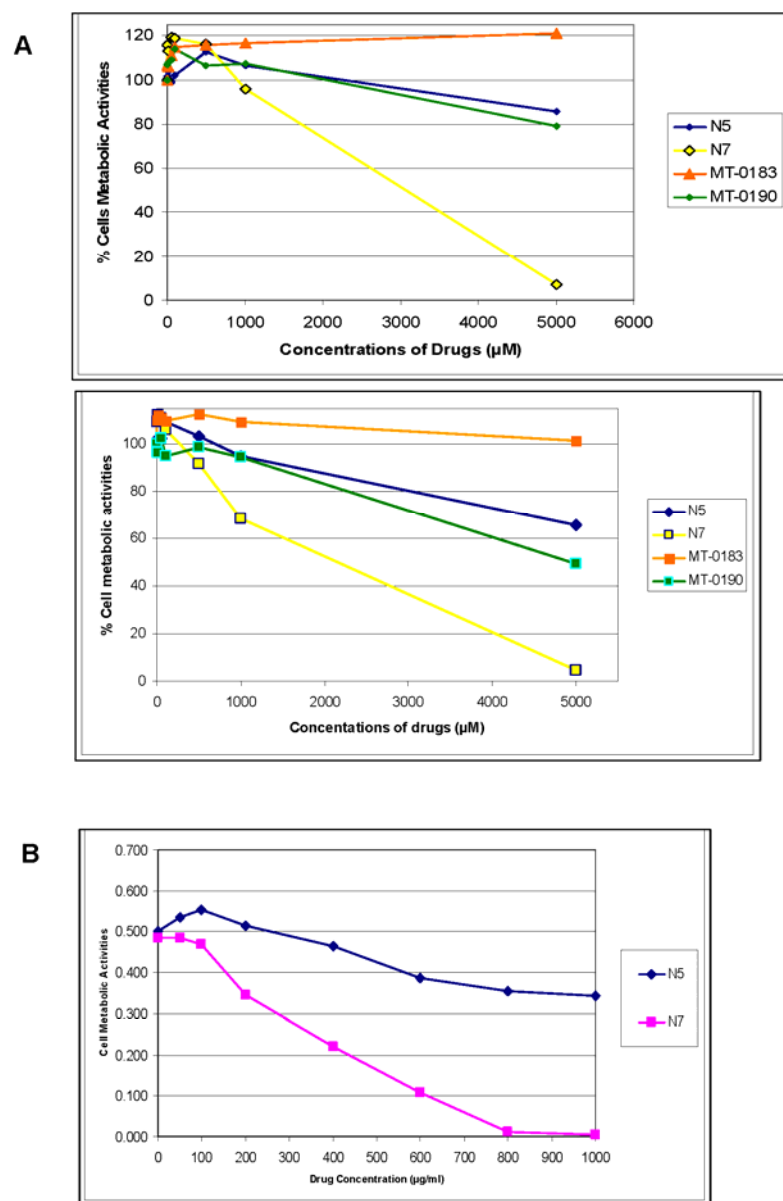
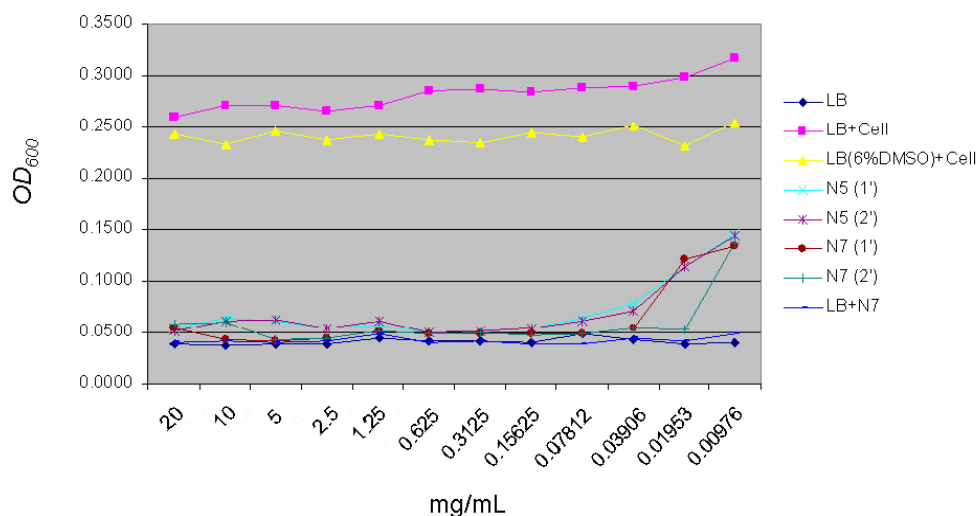
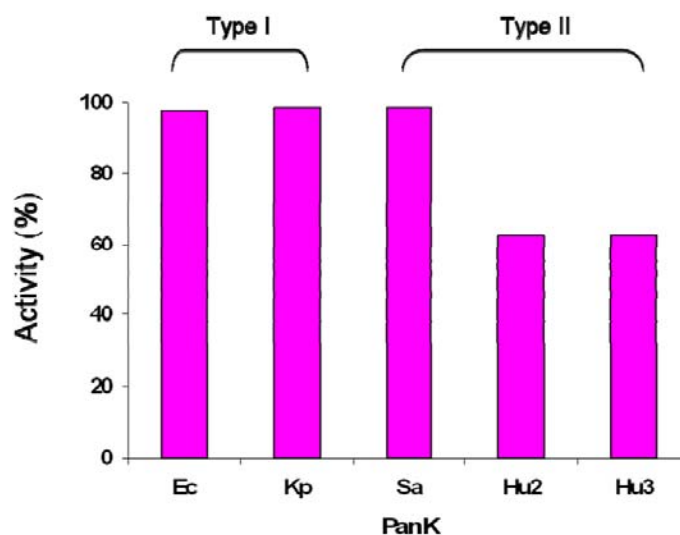


Figure 6. **Minimum Inhibitory Concentrations of N5- and N7-Pan analogs.** LB: media only, LB+Cell: media with bacterial cells, LB (6% DMSO) + Cell: media containing 6% DMSO with bacterial cells, N5 (1') and N5 (2'): duplicate experiments with N5-Pan dissolved in 6% DMSO, N7(1') and N7(2'); duplicate experiments with N7-Pan dissolved in 6% DMSO, LB +N7: media + N7-Pan without bacterial cells.



In vitro pantothenate kinase activity assay To confirm that all recombinant PanK proteins expressed in *E.coli* are fully active, the enzymatic activities were measured using the pyruvate kinase/lactate dehydrogenase (PK/LDH) coupled assay and relatively compared to that of SaPanK². Both the type I and II bacterial PanKs are fully active (Fig. 7). The activities of human PanK isoforms 2 and 3 are relatively low, probably due to the partial occupancy of the acetyl-CoA inhibitor co-purified from *E.coli* cells. These results suggest that the recombinant PanK proteins are fully active and we can use the PK/LDH coupled assay for investigating *in vitro* inhibitory effects of the Pan analogs.

Figure 7. **Relative activities of recombinant pantothenate kinases.** The activities are normalized to that of SaPanK. Ec: EcPanK, Kp: KpPanK, Sa: SaPanK, Hu2: hPanK2, Hu3: hPanK3.



KEY RESEARCH ACCOMPLISHMENTS:

- Structure determination of both N5- and N7-Pan bound *S. aureus* PanK.
- Structure determination of N5-Pan bound *K. pneumoniae* PanK
- Structure determination of N5-Pan bound *E. coli* PanK
- Structure determination of N7-Pan bound human PanK3
- Two newly synthesized compounds showing no cytotoxicity to HepG2 liver and A549 lung cells
- Minimal inhibitory concentrations of N5-Pan and N7-Pan analogs against *S. aureus* lower than 10 µg/ml.

REPORTABLE OUTCOMES:

(1) An Oral presentation at 1st European Conference of Microbiology and Immunology in Budapest, Hungary between May 12-14, 2011, entitled “Crystal structure of *Staphylococcus aureus* pantothenate kinase in complex with a substrate analog N5-Pan.”

(2) An invited presentation at Konkuk University in Korea on Sep 14th, 2011, entitled “Targeted delivery of toxic vitamin B5 analogs into the bacterial cells”.

CONCLUSION:

We have solved the structures of bacterial PanKs (SaPanK, KpPanK and EcPanK) in complex with substrate anaogs (N5-Pan and N7-Pan) as well as the structure of hPanK3 in complex with N7-Pan. With the finding that the Pan analog binding site of SaPanK is different from hPanK, we synthesized two new compounds that fit to the Pan analog binding site of SaPanK. Similarly, we will design and synthesize a drug-like chemical series against KpPanK (or EcPanK) by comparing the Pan binding pockets of KpPanK (or EcPanK) and hPanK3. Afterward, we will perform bacterial colony and cytotoxicity MTT assays for newly synthesized compounds.

These studies will result in three new sets of compounds that inhibit the activities of PanKs from three bacteria (*S. aureus*, *K. pneumoniae*, and *E. coli*). Predictably, each compound set will specifically hamper the growth of respective bacteria, but has minimal effect on human cells. These compounds will be the basis for further testing in animal models and clinical trials to develop a novel class of narrow-spectrum antibiotics active against the multidrug resistant strains of *S. aureus*, *K. pneumoniae*, and *E. coli* frequently found in battlefield wounds sustained in Iraq and Afghanistan.

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APPENDICES:

Abstract for European Conference of Microbiology and Immunology

Crystal structure of *Staphylococcus aureus* pantothenate kinase in complex with a substrate analog N5-Pan.

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An objective of these studies is to advance targeted therapeutic intervention of staphylococcal infections by utilizing the pantothenate (vitamin B₅) substrate analogs of pantothenate kinase (PanK), the first step in the Coenzyme A biosynthetic pathway. The *S. aureus* PanK activity is inhibited by N-substituted pantothenate analogs, N-pentylpantothenamide (N5-Pan) and N-peptylpantothenamide (N7-Pan), and the growth rate of *S. aureus* cells is sensitive to N5-Pan and N7-Pan. The elucidation of differences between the human and bacterial enzymes, especially at the Pan analog binding site, will be of particular interest for the design of novel therapeutic compounds. The gene encoding *S. aureus* PanK was subcloned into a bacterial expression vector pET28a and PanK was expressed in *E. coli* BL21(DE3) cells. The protein was purified by a Ni-agarose affinity column and a S-200 Superdex gel filtration column on a FPLC system. Before crystallization, *S. aureus* PanK was mixed with ATP and N5-Pan. Crystals of the complex were obtained in 20% PEG5000 and 200mM KPO₄ and diffracted to 2.1 Å. The structure was determined by the molecular replacement method using the structure of *S. aureus* PanK excluding bound AMPPNP as a search model (PDB code 2EWS). The resulting structure of *S. aureus* PanK bound to N5-Pan identified residues involved in N5 recognition and provided the framework for understanding the detailed mechanism of the specificity of *S. aureus* PanK for N5-Pan. The N5-Pan-bound structure is being used for the design of novel therapeutic compounds against antibiotic-resistant stains of *Staphylococcus aureus*.