



# TXA497 as a topical antibacterial agent: Comparative antistaphylococcal, skin deposition, and skin permeation studies with mupirocin



Mania Dorrani <sup>a,b</sup>, Malvika Kaul <sup>c</sup>, Ajit Parhi <sup>d</sup>, Edmond J. LaVoie <sup>e</sup>, Daniel S. Pilch <sup>c,\*\*</sup>,  
Bozena Michniak-Kohn <sup>a,b,\*</sup>

<sup>a</sup> Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08855, USA

<sup>b</sup> Center for Dermal Research, Life Sciences Building, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

<sup>c</sup> Department of Pharmacology, Rutgers Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854-5635, USA

<sup>d</sup> TAXIS Pharmaceuticals, Inc., North Brunswick, NJ 08902, USA

<sup>e</sup> Department of Medicinal Chemistry, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08855, USA

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## ABSTRACT

TXA497 is representative of a new class of guanidinomethyl biaryl compounds that exhibit potent bactericidal behavior against methicillin-resistant *Staphylococcus aureus* (MRSA). In this study, we compared the anti-staphylococcal, skin deposition, and skin permeation properties of TXA497 and the topical anti-MRSA antibiotic mupirocin. The results of minimum inhibitory concentration (MIC) assays revealed that TXA497 retains potent activity against MRSA that is highly resistant to mupirocin. Using Franz diffusion cells, compound deposition into human cadaver skin was evaluated, and the results showed the skin deposition of TXA497 to be significantly greater than that of mupirocin. Moreover, unlike mupirocin, TXA497 does not pass through the entire skin layer, suggesting a minimal potential for the systemic absorption of the compound upon topical administration. Additionally, antibacterial concentrations of TXA497 showed no significant toxicity to primary human keratinocytes. Given the rising levels of mupirocin resistance among MRSA populations, our results are significant in that they highlight TXA497 as a potentially useful alternative therapy for treating MRSA skin infections that are resistant to mupirocin.

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## 1. Introduction

The skin is a major organ that protects the body from bacteria and other environmental pathogens. In injuries such as burns and trauma, the skin becomes vulnerable to microbial invasion due to the skin's loss of integrity and suppression of local and systemic immunity (Barajas-Nava et al., 2013; Church et al., 2006). As a result, local infection may not only delay the wound healing process, but also can cause systemic infection under severe conditions (Ansermino and Hemsley, 2004). The most common Gram-positive bacterial pathogen that colonizes wounds and causes infection is

*Staphylococcus aureus* (*S. aureus*). Topical antibiotics are used to treat skin infection or as prophylaxis to prevent skin from further infection (Al-Dabbagh and Dobson, 2013).

Mupirocin (Bactroban<sup>®</sup>, Centany<sup>®</sup>) is a topical FDA-approved antibiotic that is widely used in treating topical wound infections, especially those caused by gram-positive multidrug-resistant (MDR) bacteria, such as methicillin-resistant *S. aureus* (MRSA). Mupirocin reversibly binds to bacterial isoleucyl transfer-RNA synthetase and inhibits bacterial protein synthesis. Although not expected (Vizcaino-Alcaide et al., 1993), high levels of mupirocin resistance have been reported in *S. aureus* and *Staphylococcus epidermidis* strains (Cadilla et al., 2011; Hogue et al., 2010; Patel et al., 2009; Simor et al., 2007). For example, resistance mutations in the isoleucyl transfer-RNA synthetase gene have been shown to occur with a frequency of  $(7.2 \pm 0.9) \times 10^{-8}$  in *S. aureus* 8325-4 (Hurdle et al., 2004). The imposing threat from multidrug resistance highlights an urgent need to develop new classes of antibiotics.

Recently, we identified a guanidinomethyl biaryl compound [compound **13** in reference (Kaul et al., 2012)] as a bactericidal

\* Corresponding author at: Center for Dermal Research and Laboratory for Drug Delivery, NJ Center for Biomaterials, Rutgers, The State University of New Jersey, 145 Bevier Road, Piscataway, NJ 08854, USA. Tel.: +1 732 445 3589; fax: +1 732 445 5006.

\*\* Corresponding author. Tel.: +1 732 235 3352; fax: +1 732 235 4073.

E-mail addresses: [pilchds@rwjms.rutgers.edu](mailto:pilchds@rwjms.rutgers.edu) (D.S. Pilch), [michniak@dls.rutgers.edu](mailto:michniak@dls.rutgers.edu), [bomichniak@gmail.com](mailto:bomichniak@gmail.com) (B. Michniak-Kohn).

agent with potent activity against MDR bacterial strains, including MRSA. This compound (hereafter denoted as TXA497) is associated with a mutational resistance frequency in *S. aureus* 8325-4 of  $(<3.1 \pm 0.8) \times 10^{-9}$  (Kaul et al., 2012). Thus, in contrast to mupirocin, TXA497 is associated with a minimal potential for the emergence of mutational resistance in *S. aureus*. TXA497 is a relatively small hydrophobic molecule (Fig. 1) that has the potential to permeate and deposit in skin layers, making it suitable for treating topical wound infections. The objective of this study was to first compare the antibacterial effects of TXA497 and mupirocin against both MRSA and methicillin-sensitive *S. aureus* (MSSA), and then to compare the ability of these agents to permeate and deposit in human skin layers.

## 2. Materials and methods

### 2.1. Compounds, bacterial strains, and media

TXA497 was synthesized as previously described (Kaul et al., 2012). Mupirocin and oxacillin were obtained from Sigma–Aldrich, Co. (St. Louis, MO). *S. aureus* 8325-4 and Mu3 were gifts from Dr. Glenn W. Kaatz (John D. Dingell, VA Medical Center, Detroit, MI) (Kaatz et al., 2000) and Dr. George M. Eliopoulos (Beth Israel Deaconess Medical Center, Boston, MA) (Sakoulas et al., 2002), respectively. All other *S. aureus* strains (33,591 and BAA1708) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cation-adjusted Mueller-Hinton (CAMH) broth was obtained from Becton Dickinson and Co. (Franklin Lakes, NJ).

### 2.2. Minimum inhibitory concentration (MIC) assays

MIC assays were conducted in accordance with clinical and laboratory standards institute (CLSI) guidelines for broth microdilution (CLSI, 2009). Briefly, log-phase *S. aureus* bacteria were added to 96-well microtiter plates (at  $5 \times 10^5$  CFU/mL) containing two-fold serial dilutions of TXA497 or mupirocin in CAMH broth. Compound concentrations (each concentration being present in duplicate) ranged from 256  $\mu\text{g/mL}$  to 0.0625  $\mu\text{g/mL}$ . The final volume in each well was 0.1 mL, and the microtiter plates were incubated aerobically for 24 h at 37 °C. Bacterial growth was then monitored by measuring OD<sub>600</sub> using a Versa Max<sup>®</sup> plate reader (Molecular Devices, Inc., Sunnyvale, CA), with the MIC being defined as the lowest compound concentration at which growth was  $\geq 90\%$  inhibited compared to compound-free control. As

recommended by CLSI, the CAMH broth was supplemented with 2% NaCl in all MRSA experiments (CLSI, 2009).

### 2.3. Preparation of drug formulations

TXA497 was dissolved in propylene glycol (PG) at concentrations of 1.4 mg/mL and 5.6 mg/mL and vortexed to allow efficient dissolution. Mupirocin was dissolved in PG at a concentration of 5.6 mg/mL. The PG vehicle was used as control in the permeation test for each compound formulation.

### 2.4. In vitro skin deposition and permeation assay

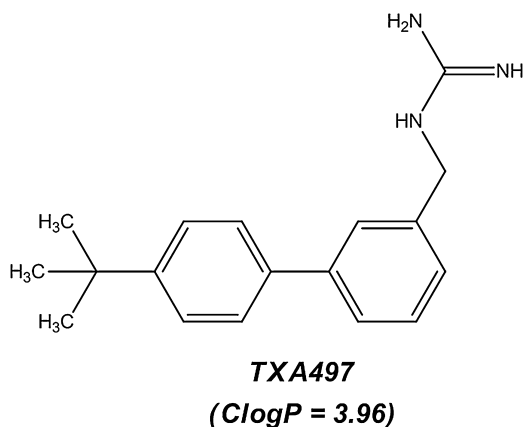
The *in vitro* skin permeation study using human cadaver skin was conducted using static vertical glass Franz diffusion cells with a donor area of 0.64 cm<sup>2</sup> and a receptor volume of 5.0 mL (Pomegear Inc., Hellertown, PA). At the time of experiment, dermatomed (500  $\mu\text{m}$ ) freshly excised skin samples, which were obtained from The New York Firefighters Skin Bank (New York, NY) from the posterior thigh of a 58 years old male, were cut into appropriately sized pieces, slowly thawed and hydrated in filtered phosphate-buffered saline (PBS) (pH 7.4), and then mounted onto the Franz diffusion cells. The receptor compartment of each cell was filled with filtered PBS (pH 7.4) and maintained at 37 °C, and the surface of the skin was open to the environment. The receptor medium was under synchronous continuous stirring using a magnetic stirrer. Prior to applying the compound formulations or the vehicle control, diffusion cells were allowed to equilibrate for 30 min. At time zero, 100  $\mu\text{L}$  of each compound formulation was added to the donor compartment of the Franz diffusion cell and left uncovered for uniform drying of the formulation on skin surface. Sink conditions were maintained for both TXA497 and mupirocin. The entire receptor was collected at time intervals of 0 h, 3 h, 6 h, 12 h, and 24 h. All compound formulations were prepared in triplicate, and the data are presented as means  $\pm$  s.d.

### 2.5. Drug extraction from the skin samples

In order to investigate the amount of deposited compound in the skin at each time point, the skin surface was carefully wiped using Kimwipes and then washed thoroughly 5 times with distilled water to clean the skin surface of any remaining formulation. Prior to compound extraction, the weight of each skin that was in contact with the receptor compartment was recorded. The compound content was recorded as ng of compound per mg of skin. The compounds were extracted into 3 mL of methanol:water [80:20 (v/v)] with skin homogenization. Skin sections were homogenized at 2500 rpm using a Polytron<sup>™</sup> PT 10/35 homogenizer from Kinematica, Inc. (Bohemia, NY) for 15 min in the methanol:water. The suspension was centrifuged for 3 min at 13,200 rpm, and the resulting supernatant was directly analyzed chromatographically as detailed in the next section.

### 2.6. Reverse-phase high performance liquid chromatography (HPLC)

The HPLC method for analysis of TXA497 was developed in-house, and was validated in terms of linearity, range, limit of detection (LOD), limit of quantification (LOQ), inter-day precision, and intra-day precision. For all HPLC measurements, a reverse-phase SPHER-100C18 column (Princeton Chromatography, Inc., Cranbury, NJ) was used on a Shimadzu LC-20AT liquid chromatograph equipped with a Shimadzu SPD-20AV UV/vis detector set at 254 nm for detection of TXA497 and at 223 nm for detection of mupirocin. The column size was 150 mm  $\times$  4.6 mm with the particle and pore sizes of 5  $\mu\text{m}$  and 100 Å, respectively. A 20  $\mu\text{L}$  sample of each experimental solution was injected and a flow rate



**Fig. 1.** Chemical structure of TXA497 (MW = 281.4 g/mol). The indicated *ClogP* value was calculated using the weighted method (VG = KLOP = PHYS = 1) in the Marvin 5.12 Software Suite (ChemAxon, Ltd.), with Cl<sup>-</sup> and Na<sup>+</sup>/K<sup>+</sup> concentrations being set at 0.1 mol/dm<sup>3</sup>.

of 1 mL/min was applied, along with a gradient of 10–90% acetonitrile [containing 0.1% (v/v) trifluoroacetic acid (TFA)] and water in the mobile phase. The total run time was 20 min, with the sampling frequency and response time being 2 Hz and 1 s, respectively. Under these conditions, TXA497 eluted approximately 12.2 min after sample injection and mupirocin eluted approximately 10.9 min after sample injection. Peak areas were determined using the Shimadzu EZStart 7.4 SP3 software package.

### 2.7. Cell proliferation assay

HaCaT human primary keratinocyte cells were cultured at 37 °C (10% CO<sub>2</sub>, RH 95%) in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were then seeded in 96-well plates at a density of 10,000 cells per well. After 24 h of incubation at 37 °C, the media was changed to include TXA497 at concentrations ranging from 0.3 µg/mL to 2 µg/mL. DMSO was used as the drug vehicle control. Treated cells were incubated at 37 °C for 24 h and cell proliferation was measured using the AlamarBlue<sup>®</sup> metabolic assay kit and protocol (AbD Serotec, Inc.). After addition of the AlamarBlue<sup>®</sup> reagent and incubation for 3 h, the fluorescence intensity was measured from 560 nm to 590 nm (manual gain 100%) using an Infinite<sup>®</sup> M200 plate reader (Tecan Group, Ltd.). The untreated cell control was used as a measure of 100% viability. All compound formulations were prepared in triplicate, and the data are presented as means ± s.d.

### 2.8. Data analysis

The amount of compound in each experimental sample was determined from the HPLC peak area. Flux ( $J$ ) was calculated using the following formula:  $J = M/S - t$ , where:  $M$  is the amount (in µg) of collected compound in the receptor,  $S$  is surface area of the skin that was in contact with the receptor (0.64 cm<sup>2</sup>) and  $t$  is time (in hours). Lag time ( $t_{lag}$ ) was calculated by extrapolating the steady state of the graph to the  $x$ -axis, and is reported in hours. Data are presented as means ± s.d. ( $n = 3$ ).

## 3. Results and discussion

### 3.1. TXA497 is active against mupirocin-resistant MRSA

The antibacterial activity of TXA497 against *S. aureus* was investigated and compared to that of mupirocin. One MSSA strain (8325-4) and three different MRSA strains (33,591, Mu3, and BAA1708) were used in these studies, the results of which are summarized in Table 1. Inspection of these data reveals that mupirocin is active (MIC = 0.125 µg/mL) against three of the *S. aureus* strains examined (MSSA strain 8325-4 and MRSA strains 33,591 and Mu3). A notable exception is MRSA strain BAA1708, which is highly resistant to mupirocin (MIC > 256 µg/mL).

**Table 1**  
Comparison of the antibacterial activities of TXA497 and mupirocin against MSSA and MRSA.

Compound	MIC (µg/mL)			
	MSSA	MRSA		
	8325-4	33591	Mu3	BAA1708
TXA497 <sup>a</sup>	1.0	1.0	1.0	2.0
Mupirocin	0.125	0.125	0.125	>256
Oxacillin	0.125	>256	>256	>256

<sup>a</sup> MIC values for TXA497 against strains 8325-4, 33591, and Mu3 are taken from reference (Kaul et al., 2012).

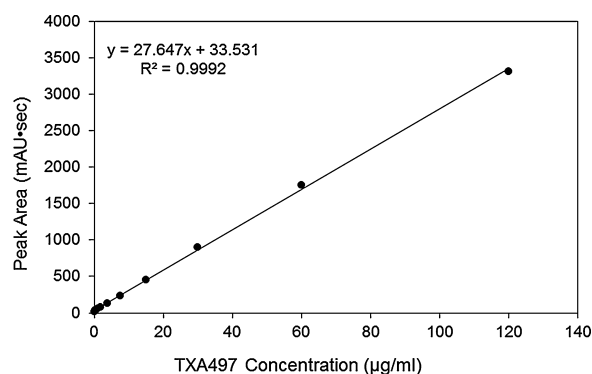
TXA497 is also active against MSSA strain 8325-4 and MRSA strains 33,591 and Mu3, though with a somewhat reduced potency (MIC = 1.0 µg/mL) compared to mupirocin. Significantly, however, TXA497 retains its activity against MRSA strain BAA1708 (MIC = 2.0 µg/mL), which is resistant to mupirocin. Thus, TXA497 remains active against MRSA, even in cases where mupirocin fails due to resistance. As expected, all MRSA strains were resistant (MIC > 256 µg/mL) to the control antibiotic oxacillin.

### 3.2. TXA497 is associated with an enhanced propensity for human skin deposition relative to mupirocin

We have previously demonstrated that the antibacterial activity of TXA497 is strongly dependent on the hydrophobicity of this compound (Kaul et al., 2012). These results motivated us to evaluate the ability of the TXA497 to permeate and deposit in human skin. As in the antibacterial studies discussed above, mupirocin was used as a comparator antibiotic in the skin permeation studies. The outermost layer of the skin, the stratum corneum, consists of dead corneocytes, and is the major barrier against topical and transdermal drug delivery (Prausnitz and Langer, 2008). It has frequently been reported that small hydrophobic molecules are ideal compounds for passive topical delivery (Arora et al., 2008; Geusens et al., 2011). Therefore, we designed the next set of experiments to examine whether TXA497 can permeate through the stratum corneum and reach the inner layers of the skin.

For measurement of TXA497 concentration, we first validated an HPLC method with regard to linearity, range, LOD, LOQ, and precision. Linearity was studied in the range of 0.11–120 µg/mL, with each concentration being assayed in triplicate. Linearity was observed over the range of 0.23–120 µg/mL, with an  $R^2$  value of 0.9992 (Fig. 2). LOD and LOQ values were determined to be 0.036 µg/mL and 0.12 µg/mL, respectively. Precision is the degree of agreement among individual test results when the method is applied repeatedly to the same or similar samples. The relative standard deviation (RSD %) of six replicates at a concentration of 60 µg/mL, 7.5 µg/mL, and 0.93 µg/mL were calculated. Based on the observed results (listed in Table 2), the repeatability (intra-day and inter-day precision) of determined concentrations had RSD values < 2%. Using the same approach, the HPLC method for analysis of mupirocin was also validated.

After method validation, we exposed human cadaver skin to TXA497 at concentrations of 1.4 mg/mL and 5.6 mg/mL and extracted the compound deposited in the skin at different time points ranging from 1 h to 24 h. The results demonstrated that TXA497 could be detected in the skin within 1 h of exposure (Fig. 3A). Furthermore, the rate of compound deposition in the skin was dependent on compound concentration. This observation



**Fig. 2.** Standard curve for quantitation of TXA497 using HPLC.

**Table 2**  
Intra-day and inter-day precision analysis of TXA497<sup>a</sup>.

TXA497 concentration (µg/mL)	Intra-day analysis		Inter-day analysis	
	Response area (mAU s)	RSD (%)	Response area (mAU s)	RSD (%)
60	1771.14 ± 20.74	1.17	1763.26 ± 19.04	1.08
7.5	237.00 ± 2.28	0.96	235.62 ± 3.57	1.51
0.94	53.03 ± 0.15	0.28	52.85 ± 0.24	0.45

<sup>a</sup> Response areas represent the average of 6 injections, and are presented as mean ± standard deviation (SD). RSD denotes the relative standard deviation.

implies that as the compound concentration in the formulation increases, the rate of compound diffusion through the skin also increases. The basis for this effect can be attributed to Fick's first law, which postulates that the flux ( $J$ ) is proportional to the amount of material ( $M$ ) that follows through a section unit ( $S$ ) over a unit of time ( $t$ ):

$$J = \frac{dM}{S \cdot dt}$$

Therefore, by keeping the receptor compartment under sink condition and increasing the amount of the compound in the donor compartment, the flux is expected to increase (Notman and Anwar, 2013).

As noted above, the main barrier for effective topical delivery in skin is the stratum corneum. Depending on the location and level of moisture, the thickness of this skin layer in humans ranges between 10 µm and 20 µm. The stratum corneum contains lipids and non-viable corneocytes that are cross-linked together by keratin (Cilurzo et al., 2014). Molecules <600 Da have been reported to successfully cross this layer with no assistance from physical or chemical enhancement (Barry, 2001). TXA497 is a small hydrophobic molecule with a molecular weight of 281.4 Da and is therefore likely to diffuse through the stratum corneum. Furthermore, TXA497 has a lipophilicity (ClogP = 3.96) that should enable the compound to permeate the stratum corneum via the lipoidal route (Moghadam et al., 2013). The small size and lipophilic nature of TXA497 may thus explain why this molecule was able to permeate through the stratum corneum successfully in our study. In addition to the presence of hydrophobic moieties in TXA497, the presence of guanidine group (pK<sub>a</sub> 11.60) may also play a significant role in its enhanced skin deposition. The guanidine structure becomes positively charged inside the skin, which has a pH in the range of 5.4–5.9. The presence of such a positively charged functionality could help TXA497 permeate and internalize into skin cells better than mupirocin. It has been previously demonstrated that the extent of skin permeation and retention of positively charged molecules is greater than that of negatively

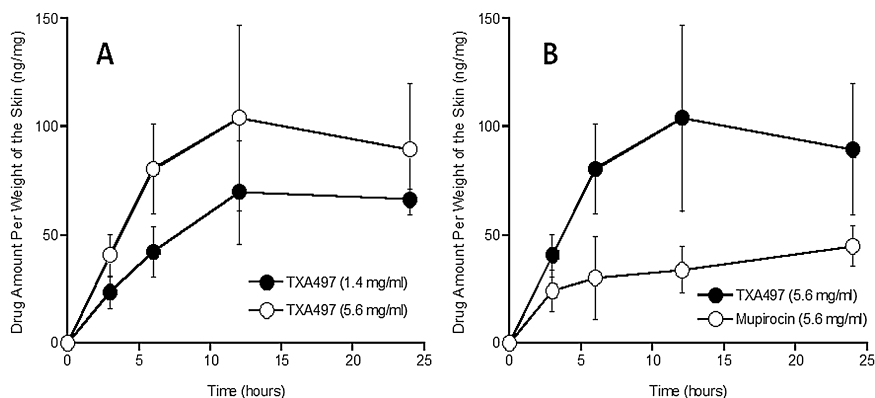
charged or neutral molecules, as positively charged molecules can interact with negative surface charges on the cell membranes and become internalized into skin cells (Caon et al., 2014; Rodriguez-Cruz et al., 2013). Overall, it is likely that the presence of both hydrophobic and cationic moieties in TXA497 contribute to the contributed to the skin permeation and deposition of the compound.

The results shown in Fig. 3B reveal that, at an equivalent concentration (5.6 mg/mL), TXA497 is associated with a two- to three-fold greater extent of skin deposition than mupirocin. Thus, not only does TXA497 retain activity against mupirocin-resistant MRSA, it also exhibits an enhanced propensity for human skin deposition relative to mupirocin.

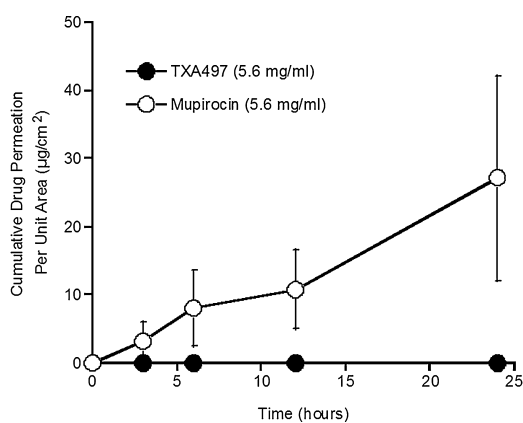
### 3.3. Unlike mupirocin, TXA497 does not pass through the entire skin layer

In the next step, we examined whether TXA497 traversed through the entire skin layer and could be detected in the receptor compartment. The sampling data (Supplementary Fig. 1) from the receptor compartment revealed that TXA497 remained in the skin and was undetectable in the receptor compartment.

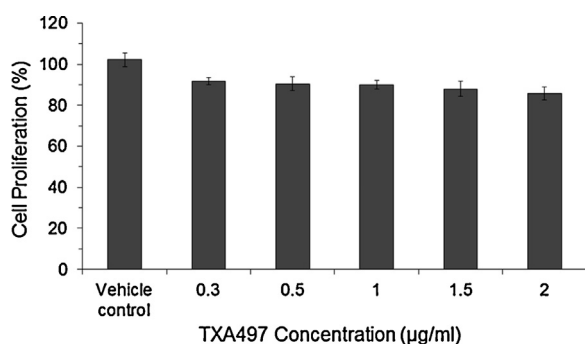
In striking contrast to TXA497, mupirocin did pass through the entire skin layer (Fig. 4). The calculated lag time for mupirocin to permeate through the entire skin layer was 1.02 h, with a flux of  $1.13 \pm 0.62 \mu\text{g}/\text{cm}^2 \text{h}$ . Below the stratum corneum is a layer of viable epidermal cells that are joined together through tight junctions, leaving little or no intercellular space (Denis et al., 2008). Highly lipophilic compounds such as TXA497 (ClogP = 3.96) are expected to slow down when reaching the relatively aqueous environment of the epidermis. As a result, they tend to be retained in this layer and hence concentrate in the skin rather than passing into the systemic circulation (Naik et al., 2000). In contrast, less lipophilic molecules like mupirocin (ClogP = 2.45) can easily diffuse through the skin and reach the receptor compartment. Since the purpose of topical drug delivery for treating infection is local therapy, the drug should ideally remain in the skin layers, thereby



**Fig. 3.** (A) Concentration dependence of the deposition of TXA497 into human skin. (B) Comparison of the deposition of TXA497 and mupirocin. Skin deposition was studied over 24 h and is reported per skin weight unit.



**Fig. 4.** Time dependence of TXA497 and mupirocin permeation through the skin into the receptor compartment.



**Fig. 5.** Impact of TXA497 treatment on the proliferation of human HaCaT epithelial cells.

minimizing absorption into the systemic circulation. Otherwise, significant adverse effects due to systemic toxicity can result, as is the case with many topical steroids (Garg et al., 2014).

### 3.4. At antibacterial concentrations, TXA497 does not adversely impact the growth of cultured human primary keratinocytes

Previous reports have indicated that antibacterial concentrations of mupirocin are not toxic to cultured human skin cells (Boyce et al., 1995; Casewell and Hill, 1987). We sought to determine whether the same is true for TXA497. To this end, human epithelial HaCaT cells were exposed to differing concentrations of TXA497 in the range of its antistaphylococcal MICs. Significantly, TXA497 did not adversely impact the growth on HaCaT cells in this concentration range (Fig. 5). Although these preliminary cytotoxicity results for TXA497 are encouraging, additional studies are currently underway to determine how a broader concentration range of the compound formulated as a topical dosage form (e.g., a cream or an ointment) impacts skin cells in both full-layered engineered skin tissue samples as well as *in vivo* animal skin models.

## 4. Conclusions

The results of this study demonstrate that TXA497 is able to effectively permeate through the stratum corneum and deposit in human skin. In comparison with mupirocin, which is the only commercially available topical antibiotic for the treatment of MRSA skin infections, TXA497 appears to have more effective skin deposition properties and retains antistaphylococcal activity even against mupirocin-resistant strains. Furthermore, TXA497 has no

toxicity on human keratinocytes in the concentration range of its antistaphylococcal MICs. These collective properties make TXA497 an appealing candidate for use against MRSA skin infections, particularly in cases of mupirocin resistance. An important next step will be to develop a topical formulation (e.g., a cream) of TXA497 and evaluate the formulation with regard to not only efficacy, but also toxicity.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpharm.2014.09.033>.

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