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Research article

In vitro characterization of junctional epidermolysis bullosa keratinocytes under treatment with aminoglycosides and Amlexanox

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Abstract. Junctional epidermolysis bullosa (JEB) are heterogeneous skin fragility entities that are caused by mutations in the genes involved in cell adhesion. Particularly, localized junctional epidermolysis bullosa is a sub-type of epidermolysis bullosa (EB) caused by mutations in the COL17A1 gene. Lately, novel compounds such as aminoglycosides (gentamicin, paromomycin and G418) and Amlexanox have shown therapeutic potential for these patients. On that account we proceeded to establish their effect on cell cultures, as to provide additional knowledge regarding their use. Well established colorimetric assays with tetrazolium salts were used to assess junctional epidermolysis bullosa keratinocytes response to treatment with above mentioned compounds. Our findings show that the drugs interaction with cell metabolism has potential to initiate some degree of temporary toxicity, affecting their viability and morphology. This effect seems to be alleviated overuse in time and continuous studies need to be conducted. Here we focus on these readthrough compounds effects on cell viability, their potential cellular toxicity and aim to characterize both cell morphology and cell performance under these drugs.

Key Words: keratinocytes, junctional epidermolysis bullosa, MTT assay, cytotoxicity, therapy

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Introduction

Junctional epidermolysis bullosa (JEB) represents a heterogeneous group of skin fragility entities, caused by mutations in various genes involved in cell adhesion, such as laminin-332, type XVII collagen, integrin α6β4 or integrin α3. Localized junctional epidermolysis bullosa is a subtype of epidermolysis bullosa (EB) characterized by mutations in the Collagen Type XVII Alpha 1 Chain (COL17A1) gene. Different genotype alterations render a wide range of clinical settings (Condrat et al. 2018). To date, treatment is primarily focused on the prevention of blisters due to mechanical trauma, proper wound care and avoidance of infections and other cutaneous and extracutaneous complications ('Completed EB Guidelines' n.d.). Therefore, the development of more specific and thus, more effective treatment strategies are needed (Sait et al 2022).

One state of the art approach that could incorporate both the clinical challenges and the genetic defects, is readthrough therapy which has been suggested as a potential therapeutic option in many disorders: cystic fibrosis, Duchenne muscular dystrophy (Linde and Kerem 2008), including epidermolysis bullosa (Cogan et al 2014) . Research advances in the field of molecular dermatology have showed that the suppression of stop codons and thus, enabling readthrough at this point in translation, can be therapeutically effective in above mentioned genetic disorders. Readthrough compounds or translational readthrough inducing drugs (TRIDS) represent promising therapeutic options in EB, as they showed to increase skin stability, promote wound healing and thus, improve the quality of life of these patients (Cogan et al 2014; Atanasova et al 2017; Woodley et al 2017; Lincoln et al 2018).

Aminoglycosides antibiotics (AGA), such as gentamicin, paromomycin and G418 were extensively studied. Particularly, gentamicin was shown to induce the production of full-length collagen VII (C7) in nonsense recessive dystrophic epidermolysis bullosa (RDEB) mutations (Cogan et al 2014; Woodley et al 2017), as well as restoring functional protein levels of laminin 332 in Herlitz JEB, a fatal subtype of EB caused by mutations in LAMB3 gene (Lincoln et al 2018). In JEB both gentamicin and paromomycin showed potentially therapeutic effect on certain mutations and provided restoration of collagen XVII (C17) in the dermal epidermal junction (DEJ) (Has et al 2022). Amlexanox, another investigated compound, activated readthrough processes in mutated dystrophin genes from patients with Duchene muscular dystrophy and induced full length synthesis of proteins in lung cancer cell lines characterized by nonsense mutations (Gonzalez-Hilarion et al 2012). In RDEB it induced full length stable and functional C7 (Atanasova et al 2017). However, these potential therapies have also been associated with various degrees of cytotoxicity based on their interaction with different cell lines and their bioavailability (Dabrowski et al 2015).

The main goal of our study was to evaluate the various effects of certain drugs, particularly TRIDS, and especially gentamicin,

paromomycin, G418 and amlexanox on JEB keratinocytes,in order to better understand the response of these cells to these potentially cytotoxic agents. Evaluation of cell viability and proliferation status could create the background for future in vitro assays (Aslantürk 2017).

Material and methods

Cell Culture

Human keratinocytes were harvested from patients with JEB, after obtaining their written consent, in accordance with the ethical standards and with the Helsinki Declaration. Cell lines were immortalized with E6E7 genes and then cultured in keratinocyte growth medium (KGM, Invitrogen) at 37 degrees Celsius and 5% CO2. JEB keratinocytes were investigated under different external factors, particularly for screening under different drugs: gentamicin, paromomycin, G418 and amlexanox.

Cell treatment

To determine the potential cytotoxic effect of TRIDS on JEB keratinocytes, we subjected normal human keratinocytes and JEB keratinocytes with different compounds to a well-established keratinocyte assay. Different dosages were explored.

We used two concentrations of drugs, which we labelled "low" and "high". Low concentrations included 100 μg/ml gentamicin, 500 μg/ml paromomycin, 2 μg/ml of G418 and 25 μM of Amlexanox. High concentrations were represented by 500 μg/ml gentamicin, 2000 μg/ml paromomycin, 8 μg/ml of G418 and 250 μM of Amlexanox.

Experiment design

Tetrazolium salts, such as MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assays were used to measure either cell proliferation rate or the reduction in cell viability. Particularly, yellow tetrazolium MTT is reduced in metabolically active cells due to the action of dehydrogenase enzymes. As a result, a compound is formed which dissolved in dimethyl sulfoxide (DMSO) renders a purple colour with a spectrophotometric absorption rate of 570 nm. The obtained colour is directly proportional to the cell number and thus, an indicator of cell viability (Supplimentary Figure 1A).

We used two 96 well microtiter plater reader (I and II) with 650 and 570 nm filter, flat bottomed, and seeded normal human keratinocytes (NHK) and JEB keratinocytes with two cell concentrations, 2500 cells/well and 5000 cells/well.

MTT assay

After seeding, we incubated the cells for 24 hours to recover from handling. Then 10 μl of MTT Reagent (from MTT cell proliferation assay kit from ATCC) was put into each well, including controls. The plates were returned to cell culture incubator for 4 hours, until the presence of intracelullar purple punctate precipitate was observed under the microscope (fig 1B).

When the precipitate was observed, we added 100 μl of Detergent Reagent to all wells, including controls. The plates were covered overnight. Next day, the absorbance was measured in each well, including blanks at 570 nm in a microtiter plate reader. If the readings were low, longer incubation in the dark were allowed. Plate I was read after 24 hours. Plate II was read after 4 days of treatment with the above mentioned concentrations of drugs. Medium was changed every 2 days.

Images of the JEB keratinocytes were photographed with the Nikon Bio station.

Statistical analysis

One way ANOVA tests were performed for all data recorded. Newman-Keuls Multiple Comparison Test was performed to determine the statistical difference betwen the non treated versus treated groups and between different drugs tested versus non treated group. For all statistical tests, a 0.05 confidence level was used, and the two-tailed p-value was computed. All statistical analyses were performed using GraphPad Prism, version 9.2.0. Significance was defined as a p-value < 0.05 and $p<0.01$.

Results

1.Viability and morphology changes in keratinocytes treated with TRIDS

Normal human keratinocytes (NHK) are compact, typical epithelial cell which tend to form colonies with a cobblestone morphology in cultures (Fig 1A). Similar to normal human keratinocytes, the non-treated (NT) JEB keratinocytes showed good colony forming, but due to the disease carrying gene mutation some of them displayed a spindle form and enlarged with spheroid structure, suggesting distress. Treatment with aminoglycosides determined a modified cell structure with elongated silhouette (Fig 1D), spheroid shape (Fig 1 C and E) and poor colony forming pattern (Fig 1F).

The most notable effect is seen in the cell line treated with Amlexanox (Fig 1F) with extensively apoptotic cells, without any colony formation. Cells became quite large, round and did

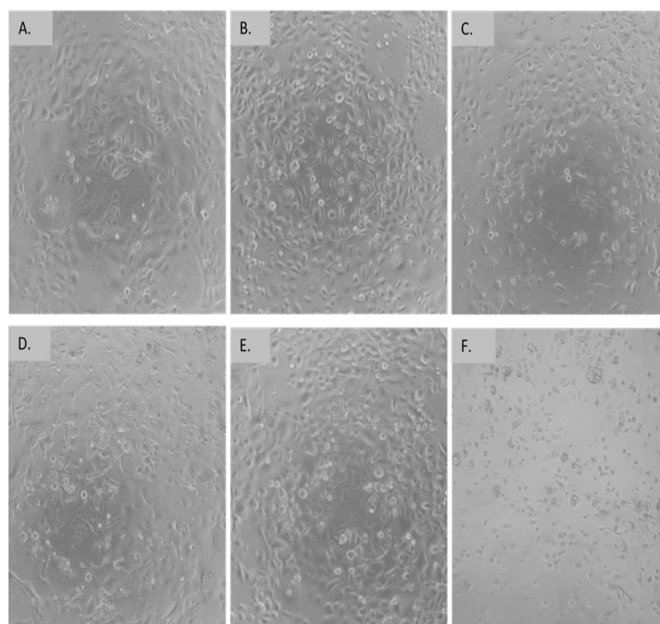

Figure 1. Keratinocytes morphology during treatment with various compounds.

A. Norman Human Kertinocytes (NHK) Not Treated (NT); B. JEB cell line NT; C. JEB cell line treated with Gentamicin; D. JEB cell line treated with Paromomycin; E. JEB cell line treated with G418; F. JEB cell line treated with Amlexanox. Note the different cell morphologies in pictures C –F due to treatment. Cells become spheroid with lack of dendritic extensions (arrow). Picture F depicts increased apoptosis in keratinocytes and thus, a decreased cell number.

not develop dendritic elongation, thus highlighting the potential negative effect of Amlexanox (Wong et al 2019).

2.TRIDS cytotoxicity assays on JEB keratinocytes

Different cell counts of JEB keratinocytes were treated with various amounts of TRIDS and their response in cell metabolic activity was evaluated with MTT assays. We performed tests with both 5000 cells/vial (Fig 2A) and 2500 cells/vial (Fig 2B) in order to achieve greater colonies of cells, due to the distress of the cells observed during treatment beforehand (Fig 1). When there were a high count of cells/vial, both HNK and NT JEB keratinocytes had similar absorbance rates, 62% versus 58%, whereas in the low count cell experiment (2500 cells/vial), NHK had a lower absorbance rate then JEB NT, 28% versus 40% (Fig 2). In both cell count settings, as shown in figure 2, treated JEB keratinocytes showed low absorbance rates compared to both NHK and NT JEB, suggesting marked decrease in cell activity. JEB keratinocytes treated with 500 μg/ml gentamicin, have had higher absorbance rates compared to 2000 μg/ml paromomycin and 250 μM Amlexanox, but lower than both NHK and NT JEB ($p<0.01$, 95% confidence interval). Amlexanox treated JEB keratinocytes showed the lowest absorbance rates during evaluation, compared to NT JEB, 22% versus 58%, almost a 3-fold decrease in cell activity. These findings show that in high doses, all drugs tested showed a decrease in cell proliferation rate, with Amlexanox being the most toxic on cell proliferation, on keratinocytes already suffering from JEB.

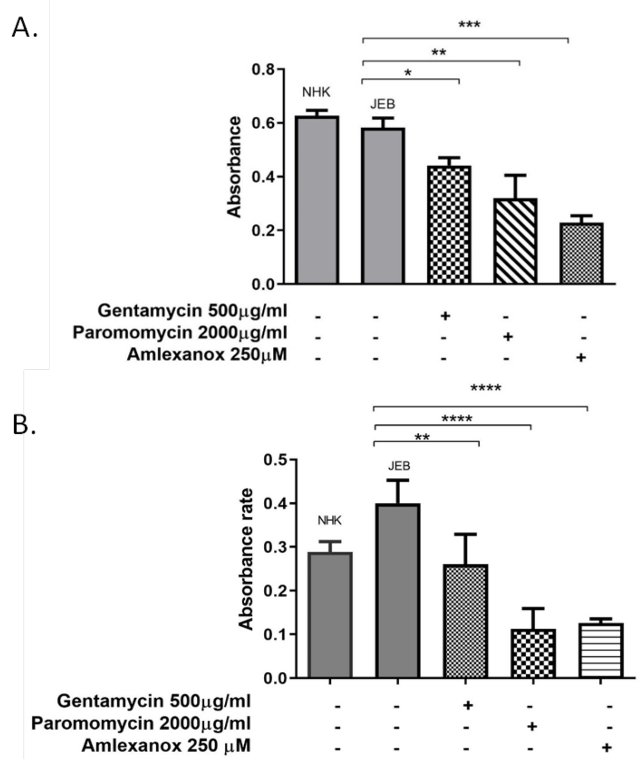

Figure 2: MTT Assay on JEB Keratinocytes, A represents 5000 cells/vial and B represents 2500 cells/vial. Cells were treated directly in the 96 flat bottom microtiter plate with 500 μg/ml gentamicin, 2000 μg/ml paromomycin, 250 μM Amlexanox for 4 days with every 2 days change of the KGM media. Column 1 represents NHK, column 2 represent NT-JEB keratinocytes and column 3-5 the effects of AGA on JEB keratinocytes. Aminoglycosides such as Gentamicin and Paromomycin in high concentrations, have a cytotoxic effect. Amlexanox has the most toxic effect, with a significant decrease in cell proliferation. (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001)

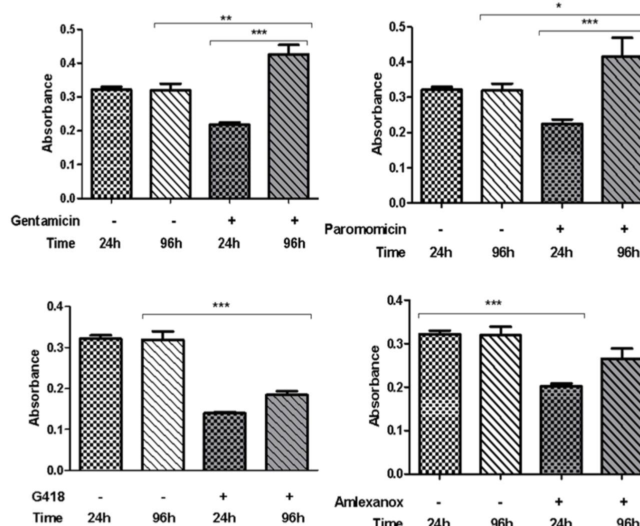

Figure 3: JEB keratinocytes behavior in different time points of the treatment with TRIDS. First and second columns represent NT JEB keratinocytes. Here, cells were treated before seeding into the 96 flat bottom microtiter plate for 4 days with 100 μg/ml gentamicin, 500 μg/ml paromomycin, 2 μg/ml G418 and 25 μM Amlexanox.

Furthermore, we evaluated the cells behavior with lower concentrations and in different time points of the treatment with TRIDS (Fig 3). Lower dose of 100 μg/ml Gentamicin showed that the absorbance rate decreased after 24 h by 20%, but increased over 96h by 36%, suggesting an overall 2-fold increase during treatment ($p<0.05$, 95 confidence interval). Treatment with 500 μg/ml Paromomycin showed similar absorbance rates, with a decrease compared to NT after 24h by 20% but increase after 96h by the same percentage. Similarly, after 4 days of treatment cells doubled their absorbance rates ($p<0.05$, 95 confidence interval) and performed better compared to NT JEB keratinocytes. 2 μg/ml G418 treatment was slightly different, with a decrease in the cells absorbance rates 24 h after treatment by 60%, and a humble increase after 96h by only 10% compared to 24h. These findings may suggest that the cells could have been affected during treatment and could not recover in such a short time.

Lastly Amlexanox, the fourth TRID evaluated, showed a decrease by 37% compared to NT ($p<0.05$, 95% confidence interval). Afterwards, the increase was only by 18% compared to 24h, and the cells did not show an overall improvement in absorbance rates after 96h.

Discussion

In this study we performed various treatments on junctional epidermolysis bullosa mutated genes carrying cells with readthrough compounds such as aminoglycosides and amlexanox. The main goal of our study was to evaluate their effects on JEB keratinocytes, in order to better understand the response of these cells to these presumably cytotoxic agents (Dabrowski et al 2018).

AGA are known to cause oto- and nephrotoxic side effects and their accumulation in cells leads to apoptosis and necrosis of these cells (Lopez-Novoa et al 2011; Dabrowski et al 2018). The exact mechanism by which they induce cell death is not yet fully established. A few valuable data have been nevertheless recorded. AGA can form aggregates in the internal lysosomal membranes, which leads to phospholipidosis, a condition associated with oto- and nephrotoxicity. There is also the secondary formation of reactive oxygen species after treatment with AGA that can affect the membrane permeability and disturb the activity of enzymes, modify ion channels and receptors, and lead subsequently to cell apoptosis (Xie et al 2011). Another possible explanation is intervention by oxidative damage on mitochondrial enzyme called aconitase that can lead to an overproduction of free ferrous iron and in turn induces the cell apoptosis (Shulman et al 2014). Our cell lines developed apoptosis during treatment (Fig 1) with modifications in the morphological state of the cells. Their behaviour has also been altered, which was noticed by a lack of colonies forming abilities and lack of formation of dendritic extensions.

Our subsequent findings (Fig 2) showed that treatment with high doses of gentamicin, paromomycin and G418, has a cytotoxic effect, with decreased absorbance rates compared to both normal human keratinocytes and non-treated junctional epidermolysis bullosa keratinocytes. Later, we established that gentamicin and paromomycin, even though they have in the beginning a killing-like effect on the cells, after 96 h the cells recover and improve their viability (Figure 3). Not the same effect could be observed with G418 and Amlexanox. Lincoln et al (2018) also investigated the effect of Gentamicin on JEB keratinocytes but with LAMB3 mutations. They reported that the compound was able to correct the cells morphology, their proliferation status and hypermotility, resembling close to NHK (Lincoln et al 2018).

Other cell lines were investigated under Gentamicin drug treatment, HEI-OC1 (house ear institute-organ of Corti1), HEK-293 (human embryonic kidney cells) and HeLa (human cervical cancer cells) and their response was both quantitatively and qualitatively different. Similarly, in the present study, Gentamicin decreased cell viability at 24 h , but was recovered and even increased in 48h (Kalinec et al 2016). When Amlexanox was studied in cancer cell lines, it showed that concentrations higher than 25 µM interfered with cell morphology and possibly with cellular metabolism. (Gonzalez-Hilarion et al 2012)

Our results showed that all TRIDS have some degree of detectable cytotoxicity on JEB keratinocytes, but these are cell line specific and should be addressed accordingly. Further studies are needed to better evaluate and understand these effects, such as caspase assay, flow cytometry, evaluation of cell division, senescence, and autophagy.

Despite these side effects, 6-month treatment with AGA in patients with Duchenne muscular dystrophy was not associated with any impairment to renal or hearing function (Dabrowski et al 2018). Moreover, AGA could be administered topically in JEB, thus decreasing the risk for potential systemic side effects. Further research regarding mode of drug administration, dosage and therapy duration are required. The stimulation of translational readthrough is a transitory phenomenon. Therefore, the therapeutic use of these drugs require probably consistent and lifelong administration (Dabrowski et al 2018).

Conclusion

Treatment with AGA, particularly gentamicin and paromomycin on JEB keratinocytes showed initially some extent of toxicity on cell lines but was transitory and afterwards improved cells tolerability to the treatment. Low concentrations were safer than higher concentrations, but an important condition of the treatment should be their efficacy on readthrough, which is cell lines specific. They could be applied topically in JEB and provide a safe therapeutic option, without any systemic side effects, even though more studies are needed to reach an appropriate and significant conclusion. Amlexanox still needs more data in order to assess its toxicity, provided that its effect on readthrough is established for the genes carrying mutation cells.

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