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Title Page

Effect of Topical Neuromodulatory Medications on Oral and Skin Keratinocytes

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Abstract

Background

Neuromodulatory medications (NMs), such as Amitriptyline, Carbamazepine, and Gabapentin are used as topical preparations for management of neuropathic orofacial pain (NOP) and have produced promising preliminary results. The aim of this study was to investigate the effects of three aforementioned NMs on cell lines relevant to the orofacial tissues *in vitro* as no published studies have examined the effect of these topical NMs.

Methods

Cellular viability was measured using alamarBlue®, testing cumulative and specific time-point effects of NMs on human skin keratinocytes and oral keratinocytes. Effects of the NMs on cell counts were investigated by CCK-8 assay. Drug concentrations released from NM orabase pastes after 30 min incubation were measured by High-Performance Liquid Chromatography. Using these clinical concentrations, morphological changes and cytokine expression were investigated using Scanning Electron Microscopy (SEM) and Human Inflammatory Antibody Array (AAH) respectively.

Results

Cumulative and specific time-point viability and cell count methods revealed that Amitriptyline caused a significant decrease in cellular viability and counts in both cell lines. Carbamazepine also had significant effects after long-term exposure and at higher concentrations, whilst Gabapentin had little demonstrable effect. SEM confirmed the cytotoxicity of Amitriptyline, whilst AAH revealed no significant changes in cytokine expression following Amitriptyline, Carbamazepine or Gabapentin exposure compared with control.

Conclusions

The results raise concerns about the safety of topical Amitriptyline as it was cytotoxic to skin and oral keratinocytes in both exposure-times and concentrations, whilst Carbamazepine was cytotoxic only at high concentrations and after longer exposure-times and Gabapentin had no demonstrable effects.

Introduction

Orofacial pain (OFP) is “pain perceived in the face and/or oral cavity caused by diseases or disorders of regional structures, by dysfunction of the nervous system, or through referral from distant sources” (1). The pathophysiology of persistent OFP is varied, but includes neuropathy (2) which can be due to macro or microscopic trauma, a disorder, or a disease affecting the trigeminal nerve (3). There are several persistent OFP conditions thought to be of neuropathic origin including Persistent Dento-Alveolar Pain disorder (PDAP) and Trigeminal Neuralgia (4, 5). Neuropathic pain affecting the trigeminal region is known to exert significant impacts on individual’s daily lives (6) and following root canal treatment or extraction PDAP is thought to occur in 1.6% (7) of cases while trigeminal neuralgia is thought to occur in 12.7 per 100,000 people (8).

Contemporary evidence-based management of neuropathic pain suggests sequential phases of treatment according to the primary diagnosis, efficacy, and side effect profile of the medication(s) employed (9). Core classes of medications recommended for their efficacy in neuropathic (orofacial) pain are antidepressants and anticonvulsants with evidence of their efficacy largely being derived from studies focussing on neuropathic pain elsewhere in the body (10). Management of neuropathic OFP follows this evidence base, but one of the disadvantages of this approach is the necessity to deliver medication, such as antidepressants and anticonvulsants, systemically. This route of administration has concomitant side effects that are known to affect patient’s adherence to drug regimens especially in neuropathic OFP (6). As opposed to systemic administration, topical administration of medication may help improve drug delivery to the site of need and also the side-effect profile of the medication; thereby potentially improving adherence to and efficacy of therapy (11). This approach has already shown promise in neuropathies outside the orofacial region using medications, such as capsaicin and lignocaine patches (12). Sporadic reports of clinical experience employing compounded, topical, forms of antidepressants and anticonvulsants (neuromodulatory medication [NM]) in neuropathic OFP have emerged in the literature with largely promising results (13, 14). The analgesic effect of NMs attributed to peripheral targets, Amitriptyline has a blocking effect on ion gated channels (Na^+ , Ca^{++} and K^+

channels), along with NMDA-receptors and modification of adenosine-receptors functions (15-17). Carbamazepine and Gabapentin affinity to block sodium channels and calcium subunit ($\alpha_2\delta$ -1) respectively made them as adjunctive analgesics in chronic pain conditions topically, and systemically (15, 18).

While hepatic, renal, and neuronal tissue cytotoxicity testing has been conducted for the systemic administration of NMs, there are no such data for their topical application to orofacial tissues or thoroughly investigation of the inflammatory profile including cytokine expression except clinical notes (19) following topical application. The effect of NMs exposure on cytokine expression has been investigated following systemic administration and *in vitro* studies. The exposure or treatment was generally found to be associated with suppression of specific pro-inflammatory cytokines (IL-1 β and TNF- α), in addition to over expression of anti-inflammatory cytokine (IL-10). Carbamazepine exposure and treatment was associated with the over expression of the following groups of cytokines: IL-2, IL-5, IL-6 and IL-10 (20-25) .

This study investigates the effects of three NMs (Amitriptyline, Carbamazepine and Gabapentin) on the relevant oral and skin cell-line models and provides preliminary data on drug release from common compounded formulations of these drugs *in vitro*.

Materials and Methods

Four groups of experiments were conducted as part of this paper:

- 1) Cell viability and median lethal dose calculations.
- 2) Cell count experiments.
- 3) Drug release and cellular morphology experiments.
- 4) Cytokine expression experiments.

The NMs included and their concentrations were decided after a literature search (13, 14), and an informal survey of the international members of the University of California, Los Angeles (UCLA) OFP list-serve about the more common compounded medications used in neuropathic OFP. The only exceptions to this were the cellular morphology and cytokine expression experiments, which used concentrations determined from the drug release experiment.

Keratinocyte non-cancer cell lines were utilised in this study as representative of the target orofacial tissues for topical NMs. For more comparative reproducible results, the use of cell lines has been approved in cytotoxicity and dental biocompatibility investigations (26, 27). The cell lines employed were: immortalized skin keratinocytes (HaCat) (28) and immortalized keratinocyte cell line (OKF6-TERT1) from oral mucosa (29).

Preparation of drug solutions

Pure Amitriptyline hydrochloride (AMI), Carbamazepine (CBZ) and Gabapentin (GAB) were obtained from Sigma-Aldrich, UK. Amitriptyline and Gabapentin were dissolved in sterile deionized water to stock concentrations of 63.7 mM and 29.1 mM respectively, before further dilution in fresh culture media to the working concentrations of 200 μ M and 1.8 mM, and 150 μ M and 5.54 mM respectively. Carbamazepine was dissolved in 100% ethanol to a stock concentration of 84.6 mM before further dilution in fresh culture medium to two final working solutions of 100 μ M and 1.7 mM (Table 1, outlines the equivalent clinical concentrations of these laboratory values). The final concentration of ethanol was <2% in the final working solutions and this concentration of ethanol alone did not affect the viability of any cell line. For antibody array experiments, serum free media were used to prepare the drug solutions.

Cell Culture

Immortalized HaCat cells (passage 67) and OKF6-TERT1 cells (passage 7) were maintained and passaged as described previously (29, 30).

1. Cellular viability and medial lethal dose:

AlamarBlue® (AbD Serotec, Oxford, UK) was used to investigate the effect of NMs on cell viability *in vitro*. This test works by measuring the colour change from blue to pink as a result of Resazurin's chemical reduction, in response to cellular metabolic activities during cellular growth and multiplication. This colour change was measured by absorbance at a wavelength of 570/600nm.

Experiments examined the: (a) cumulative effect of NMs on cellular viability, and (b) the effect of NMs observed at specific time points of cellular exposure.

Median lethal dose (LD₅₀) was calculated using absorbance values obtained by the alamarBlue® method, using semi-log graph paper for all three drugs and for both

exposure methods (cumulative and specific time points), applying manufacturer instructions.

a: Cumulative NMs effect: HaCat and OKF6-TERT1 cells were seeded at densities of 1×10^4 and 2×10^4 cells/well respectively, in 96-well plates with fresh culture media to a final volume of 200µl per well. Cells were incubated at 37°C overnight to ensure cell attachment. The media were then replaced with 200µl of previously prepared media containing pure drug solution at different concentrations of Amitriptyline, Gabapentin or Carbamazepine in media plus 10% (v/v) alamarBlue®. After incubation, the absorbance was measured after 30min, 1h, 2h, 4h and 24h exposure using a plate reader (Synergy HT, BioTek, Winooski, VT) at two wavelengths (570/600)nm.

b: Effect of NMs at specific time points: HaCat and OKF6-TERT1 cells were seeded at densities of 1×10^4 and 2×10^4 cells/well respectively in 96-well plates containing fresh culture media up to a volume of 100µl per well. Cells were incubated at 37°C overnight before media replacement with 100µl of previously prepared media containing pure drug solution at the different low and high concentrations of Amitriptyline, Gabapentin or Carbamazepine. After incubation for 30min and 24h the effects of the NM's effect were stopped by washing the cells in Phosphate Buffed Saline (PBS) before further incubation with 100µl of fresh media, plus 10% (v/v) alamarBlue®. Absorbance was measured after 4h incubation.

2. Cell counting assay: The CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) is based on cellular activity, specifically dehydrogenases and mitochondrial activity, resulting in reduction of Tetrazolium salts (yellow) to Formazan (orange) that can be quantified. The CCK-8 assay was used to investigate changes in cultured cell counts, in response to exposure the NMs. HaCat and OKF6-TERT1 cells were seeded and treated as before alongside a range of control cells seeded at different cell densities to establish a calibration curve. After incubation for 30min and 24h, the effect of the NM was again stopped by washing the cells in PBS. Cells were then incubated with 100µl fresh media plus 10% (v/v) CCK-8 reagent. Absorbance was measured after incubation for 150min using a plate reader (BioTek, Winooski, VT) at 450nm wavelength.

3. Drug release and cellular morphology experiments

A sterile Orabase® paste without additives (ConvaTec, Deeside, UK) was mixed with the required amount of the pure medication powder using a doubling up technique (31) in order to prepare NMs at clinically published concentrations (13, 14).

Approximately 2g from the resultant Amitriptyline (2% w/v, 63.7mM), Gabapentin (4% w/v, 169.3mM) and Carbamazepine (4% w/v, 233.6mM) topical paste was then used to coat the inner walls of a 6 well-plate. The wells were then filled with 6ml of culture medium. Thirty minutes of incubation were used in both the drug release and cellular morphology experiments as it was felt this was a realistic time for a topical medication to be present in the oral cavity once applied to the buccal mucosa. After 30min incubation, the media were aspirated and sent for High-Performance Liquid Chromatography (HPLC) analysis to establish the concentration of drug released from the topical paste. Replicate samples were analysed (Cardiff Toxicology Laboratories, Llandough hospital, Penarth, UK).

Cellular morphology

HaCat and OKF6-TERT1 cells were seeded on coverslips at 1×10^5 and 2×10^5 per well respectively in a 12 well plate with 2ml of media as the final volume per well. After overnight incubation, the media were discarded and replaced with 2ml of new media alone (control) or media containing one of the NMs at a concentration that reflected the HPLC findings in the drug release experiment. After 30min incubation, media were then aspirated and stored at -20°C to be used later in Antibody Array Assay. The cover slips were fixed and processed for SEM as described previously (32).

4. Cytokine expression

An exploratory Human Inflammation Antibody Array C1 (RayBiotech®, Norcross, USA) was used to investigate a number of cytokines expressed in duplicate samples of conditioned media that had been collected from cells exposed to NMs for 30min. The cytokines present on the array were Eotaxin-1, Eotaxin-2, G-CSF, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12p40, IL-12p70, IL-13, I-309, TIMP-2. Membranes were treated following the manufacturer's instructions before being developed on highly sensitive Amersham Hyperfilm ECL for 2 min.

Statistical analysis: Simple descriptive statistics and one-way ANOVA followed by Bonferroni *post hoc* tests were conducted in SPSS version 22 (SPSS Inc., Chicago, IL).

Results

1. Cell Viability

A: Cumulative NMs effect: *In vitro* effects of pure NMs on HaCat cellular viability are shown in Figure 1. HaCat cellular viability was significantly reduced by both low (200 μ M) and high (1.8mM) Amitriptyline concentrations compared to untreated controls ($p < 0.05$). Carbamazepine exposure significantly reduced cellular viability only at high (1.7mM) concentrations and after 2h of exposure ($p < 0.05$), while Gabapentin significant reduced viability only at high (5.54mM) concentrations ($p < 0.05$). HaCat cell exposure to low concentrations of Carbamazepine and Gabapentin at all time points produced no significant changes compared with controls ($p > 0.05$).

In oral keratinocyte cells, (OKF6-TERT1, Figure 2) significant reductions in viability ($p < 0.05$) were observed after exposure to Amitriptyline at low (200 μ M) concentrations for 4h and high (1.8mM) concentrations for 2h. Similar observations were found for Carbamazepine after exposure to high (1.7mM) concentrations for 4h. Neither concentration of Gabapentin significantly affected cellular viability compared with controls ($p > 0.05$).

The median lethal dose (LD₅₀) of Amitriptyline in HaCat cells (Figure S1, in the supplementary materials) was 512 μ M and 186 μ M at 30min and 24h respectively. For Carbamazepine, the LD₅₀ value for HaCat cells was 398 μ M after 24h exposure (Figure S2). Gabapentin had no LD₅₀ effect (Figure S3). In OKF6-TERT1 cells, the only LD₅₀ for Amitriptyline was 630 μ M after 24h exposure.

b: Effect of NMs at specific time points: Exposure of HaCat cells to 200 μ M and 1.8mM Amitriptyline for 30min and 24h significantly reduced their viability compared with controls ($p < 0.05$). Exposure to high concentrations of Carbamazepine (1.7mM) for 24h also resulted in a significant loss of viability ($p < 0.05$) (Figure 3). Gabapentin showed no significant effect on cellular viability compared with control.

Short-term (30 min) exposure of OKF6-TERT1 cells to Amitriptyline, Carbamazepine and Gabapentin at all concentrations significantly decreased cellular viability compared with untreated controls ($p < 0.05$, Figure 4). Both Amitriptyline concentrations and high concentrations of Carbamazepine and Gabapentin significantly reduced the viability of OKF6-TERT1 cells after longer exposure (24h), ($p < 0.05$, Figure 4).

The only LD₅₀ value was for Amitriptyline exposure at 30min (141 μ M), (Figures S4-S6).

2. Cell counting assay:

Using the calibration curve the estimated number of HaCat cells was less than 5,000 after 30min exposure to Amitriptyline at low (200 μ M) and high (1.8mM) concentrations. At the 24h time point, the cell count was less than 1,000 (Figure S7). Exposure to low (100 μ M) and high (1.7mM) concentrations of Carbamazepine for 30 min caused a similar reduction in cell counts. However, after 24h exposure to 100 μ M Carbamazepine, cell counts were similar to controls. High concentration Carbamazepine reduced cell counts to less than 1000 (Figure S8). Gabapentin exposure for 30 mins had no significant effects on cell counts, but after 24h exposure to 150 μ M Gabapentin, cell counts were less than 10,000 (Figure S9).

In OKF6-TERT1 cells, exposure to low concentration Amitriptyline (200 μ M) for 30 min reduced cell counts by half, to 10,000 (Figure S10). High concentration Amitriptyline (1.8mM) reduced the cell count to <2,000 after 30 mins, whilst after exposure to high and low concentrations for 24h cell counts reduced to less than 200.

Exposure to Carbamazepine at both low (150 μ M) and high (1.7mM) concentration for 30min had no effects on cell counts. After 24h exposure to both concentrations, cell counts dropped to 15,000 (Figure S11). Exposure to Gabapentin at low (150 μ M) and high (5.45mM) concentrations for 30 min or 24h had no effects on cell counts (Figure S12).

3. Drug release experiment and cellular morphology experiments

HPLC analysis of the aspirated media after 30min incubation with Orabase paste containing Amitriptyline (2% w/v), Carbamazepine (4% w/v), and Gabapentin (4%

w/v), demonstrated transfer of 226 μ M, 123.9 μ M and 5.54mM drug concentrations respectively in the media.

SEM revealed considerable changes in the cellular attachment and morphology of HaCat and OKF6-TERT1 cells exposed to 226 μ M Amitriptyline only (Figure 5).

4. Global cytokine expression

There were no significant differences in the expression of twenty different inflammatory cytokines between controls and both cell lines after 30min exposure to the NMs at the concentrations determined by the drug release experiment.

Discussion

Neuropathic OFP can be challenging to manage because of the limited number of proven interventions available (33). This difficulty and patient frustration with side effects (6) may have driven the move towards topical compounded NMs, based upon a reasonable hypothesis about the advantages this route of administration may offer (34). However, compounded formulations are not without their problems (35) and represent a challenge and a responsibility to the prescriber from a safety and efficacy perspective (36).

Prior to commencing any Phase I clinical trial for a new drug, the U.S. Food and Drug Administration (FDA) requires detailed preclinical data for that drug that includes cytotoxicity (37). In these *in vitro* cytotoxicity studies, the typical tissue regarding systemically administered drugs are target, metabolism and excretion tissues. In the case of topical NMs that are applied on orofacial tissues in higher concentrations, however these drugs are already approved systemic medications, it is necessary to examine their toxic effects on these tissues as changing the route of administration suggested different pharmacokinetic and pharmacodynamics reactions(38).

This *in vitro* study tested the effect of three NMs on cellular viability, count and morphology in addition to inflammatory response. Regarding viability, two exposure methods were used: cumulative drug and specific time-point effects.

There are limited reports in the literature regarding the *in vitro* cytotoxicity of Amitriptyline, but those that are present demonstrate cytotoxicity in both human and animal cell lines attributed to different mechanisms including: mitochondrial dysfunction; increased production of reactive oxygen species leading to alteration in cellular metabolism and permeability and elevated intracellular oxidative stress (25, 39). In the current study, Amitriptyline reduced cellular viability and cell counts in both cell lines at all concentrations and all time points and SEM examination confirmed these findings. The cytotoxicity of Amitriptyline was also demonstrated in its LD₅₀ values, which were low in both cell lines and after short exposure times.

The apoptotic and anti-proliferative effects of Carbamazepine have been investigated mainly in neurone and glial cell culture, using much lower concentrations than presented here (40, 41). Comparing to Amitriptyline, in the current study, Carbamazepine was less cytotoxic in both cell lines, and major

changes in viability only occurred at high concentrations and longer exposure times. SEM revealed no major changes in cellular morphology and attachment. The LD₅₀ values were also confirmatory as they were calculable only after a cumulative exposure of 24h, which is not clinically realistic for the topical application of a gel in the oral cavity.

Gabapentin appeared to be the least cytotoxic of the agents tested. Only a high concentration and long exposure time (24h) caused a demonstrable reduction in cellular viability. A LD₅₀ was not calculable. This is consistent with previous few reports in literature (42), that employed lower concentrations of Gabapentin *in vitro* than the current study.

In our experiments, there was a very low level of detected cytokine expression, with all NMs, and the cytokine expressions did not differ to control. This could be due to the short exposure time (30min) used and the method of measuring cytokine expression was employed, because it allowed multiple cytokines to be analysed at the same time.

The major limitation of this study is its use of a monolayer culture, which is conceivably more susceptible to the cytotoxic effects of medications than a more complex three-dimensional tissue model. However, the laboratory concentrations of NMs used in viability and counting experiments were much lower than those used in clinical practice and yet still demonstrated cytotoxicity. The concentrations employed also mirrored the ranges of drug concentrations used in the drug release experiment. Within the limits of this investigation, the clinical implications for future topical *in vivo* NM trials are that low Gabapentin and Carbamazepine concentrations are unlikely to adversely affect oral mucosa and skin. However, if Amitriptyline is to be trialled *in vivo*, short exposure times and very low concentrations with careful clinical assessment of skin and oral mucosa after repeated exposure are advisable.

Conclusions

Viability, cell counting, and SEM experiments revealed that Amitriptyline was cytotoxic to both oral and skin keratinocyte cells *in vitro*. Gabapentin and Carbamazepine affected cellular viability to a lesser extent, even at high concentrations and long exposures. Caution should be exercised in the topical application of Amitriptyline to skin and oral mucosa.

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Conflict of interest

Authors declare no conflict of interest.

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Table 1: Laboratory and equivalent clinical concentrations of Amitriptyline (AMI), Carbamazepine (CBZ) and Gabapentin (GAB).

Neuromodulatory medications (NMs)	Low concentration		High concentration	
	Laboratory concentration (μM)	Equivalent clinical concentration (%)	Laboratory concentration (mM)	Equivalent clinical concentration (%)
AMI	200	0.006	1.8	0.05
CBZ	100	0.002	1.7	0.04
GAB	150	0.002	5.54	0.09

Figure Legends:

Figure 1: Viability of HaCat cells exposed to Amitriptyline (AMI), Carbamazepine (CBZ) and Gabapentin (GAB), measured using alamarBlue®. Mean values $\pm$ SEM (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line) by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

Figure 2: Viability of OKF6-TERT1 cells exposed to Amitriptyline (AMI), Carbamazepine (CBZ) and Gabapentin (GAB), measured using alamarBlue®. Mean values $\pm$ SEM (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line) by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

Figure 3: Viability of HaCat cells exposed to Amitriptyline (AMI), Carbamazepine (CBZ) and Gabapentin (GAB) at 30min and 24h time points, measured using alamarBlue®. Mean values $\pm$ SEM (n=6) are shown for each experiment. Compared with untreated control cells (black dotted line) by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

Figure 4: Viability of OKF6-TERT1 cells exposed to Amitriptyline (AMI), Carbamazepine (CBZ) and Gabapentin (GAB) at 30min and 24h time points, measured using alamarBlue®. Mean values $\pm$ SEM (n=6) are shown for each experiment. Compared with untreated control cells (black dotted line) by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

Figure 5: SEM images at 100 X magnification of OKF6-TERT1 and HaCat cells after 30min exposure to NMs at concentrations determined by HPLC.