Inhibitory Effect of Eslicarbazepine Acetate and S-Licarbazepine on Na\textsubscript{v}1.5 Channels

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Eslicarbazepine acetate (ESL) is a dibenzazepine anticonvulsant approved as adjunctive treatment for partial-onset epileptic seizures. Following first pass hydrolysis of ESL, S-licarbazepine (S-Lic) represents around 95% of circulating active metabolites. S-Lic is the main enantiomer responsible for anticonvulsant activity and this is proposed to be through the blockade of voltage-gated Na\textsuperscript{+} channels (VGSCs). ESL and S-Lic both have a voltage-dependent inhibitory effect on the Na\textsuperscript{+} current in N1E-115 neuroblastoma cells expressing neuronal VGSC subtypes including Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.6, and Na\textsubscript{v}1.7. ESL has not been associated with cardiotoxicity in healthy volunteers, although a prolongation of the electrocardiographic PR interval has been observed, suggesting that ESL may also inhibit cardiac Na\textsubscript{v}1.5 isoform. However, this has not previously been studied. Here, we investigated the electrophysiological effects of ESL and S-Lic on Na\textsubscript{v}1.5 using whole-cell patch clamp recording. We interrogated two model systems: (1) MDA-MB-231 metastatic breast carcinoma cells, which endogenously express the "neonatal" Na\textsubscript{v}1.5 splice variant, and (2) HEK-293 cells stably over-expressing the "adult" Na\textsubscript{v}1.5 splice variant. We show that both ESL and S-Lic inhibit transient and persistent Na\textsuperscript{+} current, hyperpolarise the voltage-dependence of fast inactivation, and slow the recovery from channel inactivation. These findings highlight, for the first time, the potent inhibitory effects of ESL and S-Lic on the Na\textsubscript{v}1.5 isoform, suggesting a possible explanation for the prolonged PR interval observed in patients on ESL treatment. Given that numerous cancer cells have also been shown to express Na\textsubscript{v}1.5, and that VGSCs potentiate invasion and metastasis, this study also paves the way for future investigations into ESL and S-Lic as potential invasion inhibitors.

Keywords: anticonvulsant, cancer, epilepsy, eslicarbazepine acetate, Na\textsubscript{v}1.5, S-licarbazepine, voltage-gated Na\textsuperscript{+} channel
INTRODUCTION

Eslicarbazepine acetate (ESL) is a member of the dibenzazepine anticonvulsant family of compounds which also includes oxcarbazepine and carbamazepine (Almeida and Soares-da-Silva, 2007). ESL has been approved by the European Medicines Agency and the United States Federal Drug Administration as an adjunctive treatment for partial-onset epileptic seizures (Sperling et al., 2015). ESL is administered orally and rapidly undergoes first pass hydrolysis to two stereoisomeric metabolites, R-licarbazepine and S-licarbazepine (S-Lic; also known as eslicarbazepine; Figures 1A, B) (Almeida et al., 2005; Almeida et al., 2008; Perucca et al., 2011). S-Lic represents around 95% of circulating active metabolites following first pass hydrolysis of ESL and is the enantiomer responsible for anticonvulsant activity (Potschka et al., 2014; Sierra-Paredes et al., 2014). S-Lic also has improved blood brain barrier penetration compared to R-licarbazepine (Alves et al., 2008). Although S-Lic has been shown to inhibit T type Ca2+ channels (Brady et al., 2011), its main activity is likely through blockade of voltage-gated Na+ channels (VGSCs) (Hebeisen et al., 2015). ESL offers several clinical advantages over other older VGSC-inhibiting antiepileptic drugs, e.g. carbamazepine, phenytoin; it has a favourable safety profile (Brown and El-Mallakh, 2010; Hebeisen et al., 2015), reduced induction of hepatic cytochrome P450 enzymes (Galiana et al., 2017), low potential for drug-drug interactions (Falcao et al., 2012; Zaccara et al., 2015), and takes less time to reach a steady state plasma concentration (Bialer and Soares-da-Silva, 2012).

VGSCs are composed of a pore-forming α subunit in association with one or more auxiliary β subunits, the latter modulating channel gating and kinetics in addition to functioning as cell adhesion molecules (Catterall, 2014). There are nine α subunits (Na1.1-Na1.9), and four β subunits (β1-4) (Goldin et al., 2000; Brackenbury and Isom, 2011). In postnatal and adult CNS neurons, the predominant α subunits are the tetrodotoxin-sensitive Na1.1, Na1.2, and Na1.6 isomorphs (Van Wart and Matthews, 2006) and it is therefore on these that the VGSC-inhibiting activity of ESL and S-Lic has been described. In the murine neuroblastoma N1E-115 cell line, which expresses Na1.1, Na1.2, Na1.3, Na1.6, and Na1.7, ESL and S-Lic both have a voltage-dependent inhibitory effect on the Na+ current (Bonifacio et al., 2001; Hebeisen et al., 2015). In this cell model, S-Lic has no effect on the voltage-dependence of fast inactivation, but significantly hyperpolarises the voltage-dependence of slow inactivation (Hebeisen et al., 2015). S-Lic also has a lower affinity for VGSCs in the resting state than carbamazepine or oxcarbazepine, thus potentially improving its therapeutic window over first- and second-generation dibenzazepine compounds (Hebeisen et al., 2015). In acutely isolated murine hippocampal CA1 neurons, which express Na1.1, Na1.2 and Na1.6 (Westenbroek et al., 1989; Yu et al., 2006; Royeck et al., 2008), S-Lic significantly reduces the persistent Na+ current, a very slow-inactivating component ~1% the size of the peak transient Na+ current (Saint, 2008; Doeser et al., 2014). Moreover, in contrast to carbamazepine, this effect is maintained in the absence of β1 (Uebachs et al., 2010; Doeser et al., 2014).

In healthy volunteers, ESL has not been associated with cardiotoxicity and the QT interval remains unchanged on treatment (Vaz-Da-Silva et al., 2012). However, a prolongation of the PR interval has been observed (Vaz-Da-Silva et al., 2012), suggesting that caution should be exercised in patients with cardiac conduction abnormalities (Zaccara et al., 2015). Prolongation of the PR interval suggests that ESL may also inhibit the cardiac Na1.5 isoform, although this has not previously been studied. Na1.5 is not only responsible for the initial depolarisation of the cardiac action potential (George, 2005), but is also expressed in breast and colon carcinoma cells, where the persistent Na+ current promotes invasion and...
metastasis (Roger et al., 2003; Fraser et al., 2005; House et al., 2010; Nelson et al., 2015a). Inhibition of Na$_{1.5}$ with phenytoin or ranolazine decreases tumor growth, invasion and metastasis (Yang et al., 2012; Drifort et al., 2014; Nelson et al., 2015b). Thus, it is of interest to specifically understand the effect of ESL on the Na$_{1.5}$ isoform.

In the present study we investigated the electrophysiological effects of ESL and S-Lic on Na$_{1.5}$ [1] endogenously expressed in the MDA-MB-231 metastatic breast carcinoma cell line, and [2] stably over-expressed in HEK-293 cells. We show that both ESL and S-Lic inhibit transient and persistent Na$^+$ current, hyperpolarise the voltage-dependence of fast inactivation, and slow the recovery from channel inactivation. These findings highlight, for the first time, the potent inhibitory effects of ESL and S-Lic on the Na$_{1.5}$ isoform.

**MATERIALS AND METHODS**

**Pharmacology**

ESL (Tokyo Chemical Industry UK Ltd) was dissolved in DMSO to make a stock concentration of 67 mM. S-Lic (Tocris) was dissolved in DMSO (0.45%) and made to a stock concentration of 300 mM. Both drugs were diluted to working concentrations of 100–300 µM in extracellular recording solution. The concentration of DMSO in the recording solution was 0.45% for ESL and 0.1% for S-Lic. Equal concentrations of DMSO were used in the control solutions. DMSO (0.45%) had no effect on the Na$^+$ current (Supplementary Figure 1).

**Cell Culture**

MDA-MB-231 cells and HEK-293 cells stably expressing Na$_{1.5}$ (a gift from L. Isom, University of Michigan) were grown in Dulbecco’s modified eagle medium supplemented with 5% FBS and 4 mM L-glutamine (Simon et al., 2020). Molecular identity of the MDA-MB-231 cells was confirmed by short tandem repeat analysis (Masters et al., 2001). Cells were confirmed as mycoplasma-free using the DAPI method (Uphoff et al., 1992). Cells were seeded onto glass coverslips 48 h before electrophysiological recording.

**Electrophysiology**

Plasma membrane Na$^+$ currents were recorded using the whole-cell patch clamp technique, using methods described previously (Yang et al., 2012; Nelson et al., 2015a). Patch pipettes made of borosilicate glass were pulled using a P-97 pipette puller (Sutter Instrument) and re-polished to a resistance of 3–5 MΩ when filled with intracellular recording solution. The extracellular recording solution for MDA-MB-231 cells contained (in mM): 144 NaCl, 5.4 KCl, 1 MgCl$_2$, 2.5 CaCl$_2$, 5.6 D-glucose, and 5 HEPES (adjusted to pH 7.2 with NaOH). For the extracellular recording solution for HEK-293 cells expressing Na$_{1.5}$, the extracellular [Na$^+$] was reduced to account for the much larger Na$^+$ currents and contained (in mM): 60 NaCl, 84 Choline Cl, 5.4 KCl, 1 MgCl$_2$, 2.5 CaCl$_2$, 5.6 D-glucose, and 5 HEPES (adjusted to pH 7.2 with NaOH). The intracellular recording solution contained (in mM): 5 NaCl, 145 CsCl, 2 MgCl$_2$, 1 CaCl$_2$, 10 HEPES, 11 EGTA, (adjusted to pH 7.4 with CsOH) (Brackenbury and Djamgoz, 2006). Voltage clamp recordings were made at room temperature using a Multiclamp 700B or Axopatch 200B amplifier (Molecular Devices) compensating for series resistance by 40–60%. Currents were digitized using a Digidata interface (Molecular Devices), low pass filtered at 10 kHz, sampled at 50 kHz and analysed using pCLAMP 10.7 software (Molecular Devices). Leak current was subtracted using a P/6 protocol (Armstrong and Bezanilla, 1977). Extracellular recording solution ± drugs was applied to the recording bath at a rate of ~1.5 ml/min using a ValveLink 4-channel gravity perfusion controller (AutoMate Scientific). Each new solution was allowed to equilibrate in the bath for ~4 min following switching prior to recording at steady state.

**Voltage Clamp Protocols**

Cells were clamped at a holding potential of -120 mV or -80 mV for ≥250 ms, dependent on experiment (detailed in the Figure legends). Five main voltage clamp protocols were used, as follows:

1. To assess the effect of drug perfusion and wash-out on peak current in real time, a simple one-step protocol was used where cells were held at -120 mV or -80 mV for 250 ms and then depolarised to -10 mV for 50 ms.
2. To assess the voltage-dependence of activation, cells were held at -120 mV for 250 ms and then depolarised to test potentials in 10 mV steps between -120 mV and +30 mV for 50 ms. The voltage of activation was taken as the most negative voltage which induced a visible transient inward current.
3. To assess the voltage-dependence of steady-state inactivation, cells were held at -120 mV for 250 ms followed by prepulses for 250 ms in 10 mV steps between -120 mV and +30 mV and a test pulse to -10 mV for 50 ms.
4. To assess recovery from fast inactivation, cells were held at -120 mV for 250 ms, and then depolarised twice to 0 mV for 25 ms, returning to -120 mV for the following intervals between depolarisations (in ms): 1, 2, 3, 5, 7, 10, 15, 20, 30, 40, 50, 70, 100, 150, 200, 250, 350, 500. In each case, the second current was normalized to the initial current and plotted against the interval time.

**Curve Fitting and Data Analysis**

To study the voltage-dependence of activation, current-voltage (I-V) relationships were converted to conductance using the following equation:

$$G = I/(V_m - V_{rev})$$

where G is conductance, I is current, $V_m$ is the membrane voltage and $V_{rev}$ is the reversal potential for Na$^+$ derived from the Nernst equation. Given the different recording solutions used, $V_{rev}$ for Na$_{1.5}$ was +85 mV for MDA-MB-231 cells and +63 mV for HEK-Na$_{1.5}$ cells. The voltage-dependence of conductance and availability were normalized and fitted to a Boltzmann equation:

$$G = G_{max}/[1 + \exp((V_{1/2} - V_m)/k)]$$

where $G_{max}$ is the maximum conductance, $V_{1/2}$ is the voltage at which the channels are half
activated/inactivated, $V_m$ is the membrane voltage and $k$ is the slope factor.

Recovery from inactivation data ($I_t/I_{i-o}$) were normalized, plotted against recovery time ($\Delta t$) and fitted to a single exponential function:

$$\tau = A_1 + A_2 \exp(-t/t_0),$$

where $A_1$ and $A_2$ are the coefficients of decay of the time constant ($\tau$), $t$ is time and $t_0$ is a time constant describing the time dependence of $\tau$.

The time course of inactivation was fitted to a double exponential function:

$$I = A_1 \exp(-t/\tau_s) + A_2 \exp(-t/\tau_s) + C,$$

where $A_1$ and $A_2$ are maximal amplitudes of the slow and fast components of the current, $\tau_s$ and $\tau_f$ are the fast and slow decay time constants and $C$ is the asymptote.

**Statistical Analysis**

Data are presented as mean and SEM unless stated otherwise. Statistical analysis was performed on the raw (non-normalized) data using GraphPad Prism 8.4.0. Pairwise statistical significance was determined with Student’s paired t-tests. Multiple comparisons were made using ANOVA and Tukey post-hoc tests, unless stated otherwise. Results were considered significant at $P < 0.05$.

**RESULTS**

**Effect of Eslicarbazepine Acetate and S-Licarbazepine on Transient and Persistent Na⁺ Current**

Several studies have clearly established the inhibition of neuronal VGSCs (Nav1.1, Nav1.2, Nav1.3, Nav1.6, Nav1.7 and Nav1.8) by ESL and its active metabolite S-Lic (Bonifacio et al., 2001; Doesper et al., 2014; Hebeisen et al., 2015; Soares-da-Silva et al., 2015). Given that ESL prolongs the PR interval (Vaz-Da-Silva et al., 2012), potentially via inhibiting the cardiac Nav1.5 isoform, together with the interest in inhibiting Nav1.5 in carcinoma cells to reduce invasion and metastasis (Drifort et al., 2014; Martin et al., 2015; Nelson et al., 2015b; Elajnaf et al., 2018; Djamgoz et al., 2019), it is also relevant to evaluate the electrophysiological effects of ESL and S-Lic on this isoform. We therefore evaluated the effect of both compounds on Nav1.5 current properties using whole-cell patch clamp recording, employing a two-pronged approach: (1) recording Nav1.5 currents endogenously expressed in the MDA-MB-231 breast cancer cell line (Roger et al., 2003; Fraser et al., 2005; Brackenbury et al., 2007), and (2) recording from Nav1.5 stably over-expressed in HEK-293 cells (HEK-Na1.5) (Patino et al., 2011).

Initially, we evaluated the effect of both compounds on the size of the peak Na⁺ current in MDA-MB-231 cells. Na⁺ currents were elicited by depolarising the membrane potential ($V_m$) to -10 mV from a holding potential ($V_h$) of -120 or -80 mV. Application of the prodrug ESL (300 μM) reversibly inhibited the transient Na⁺ current by 49.6 ± 3.2% when the $V_h$ was -120 mV ($P < 0.001$; $n = 13$; ANOVA + Tukey test; Figures 2A, D). When $V_h$ was set to -80 mV, ESL (300 μM) reversibly inhibited the transient Na⁺ current by 79.5 ± 4.5% ($P < 0.001$; $n = 12$; ANOVA + Tukey test; Figures 2C, E). We next assessed the effect of ESL in HEK-Na1.5 cells. Application of ESL (300 μM) inhibited Na1.5 current by 74.7 ± 4.3% when $V_h$ was -120 mV ($P < 0.001$; $n = 12$; Figures 2F, I) and by 90.5 ± 2.8% when $V_h$ was -80 mV ($P < 0.001$; $n = 14$; Figures 2H, J). However, the inhibition was only partially reversible ($P < 0.001$; $n = 14$; Figures 2F, H–J). Application of ESL at a lower concentration (100 μM) elicited a similar result (Supplementary Figures 2A–J and Supplementary Table 1). Together, these data suggest that ESL preferentially inhibited Na1.5 in the open or inactivated state, since the current inhibition was greater at more depolarised $V_h$.

We next tested the effect of the active metabolite S-Lic. S-Lic (300 μM) inhibited the transient Na⁺ current in MDA-MB-231 cells by 44.4 ± 6.1% when the $V_h$ was -120 mV ($P < 0.001$; $n = 9$; ANOVA + Tukey test; Figures 3A, D). When $V_h$ was set to -80 mV, S-Lic (300 μM) inhibited the transient Na⁺ current by 73.6 ± 4.1% ($P < 0.001$; $n = 10$; ANOVA + Tukey test; Figures 3C, E). However, the inhibition caused by S-Lic (300 μM) was only partially reversible ($P < 0.05$; $n = 10$; ANOVA + Tukey test; Figures 3A, C–E). In HEK-Na1.5 cells, S-Lic (300 μM) inhibited Na1.5 current by 46.4 ± 3.9% when $V_h$ was -120 mV ($P < 0.001$; $n = 13$; ANOVA + Tukey test; Figures 3F, I) and by 74.0 ± 4.2% when $V_h$ was -80 mV ($P < 0.001$; $n = 12$; ANOVA + Tukey test; Figures 3H, J). Furthermore, the inhibition in HEK-Na1.5 cells was not reversible over the duration of the experiment. Application of S-Lic at a lower concentration (100 μM) elicited a broadly similar result (Supplementary Figures 3A–J & Supplementary Table 1). Together, these data show that channel inhibition by S-Lic was also more effective at more depolarised $V_h$. However, unlike ESL, channel blockade by S-Lic persisted after washout, suggesting higher target binding affinity for the active metabolite and/or greater trapping of the active metabolite in the cytoplasm.

We also assessed the effect of both compounds on the persistent Na⁺ current measured 20–25 ms after depolarisation to -10 from -120 mV. In MDA-MB-231 cells, ESL (300 μM) inhibited the persistent Na⁺ current by 77 ± 34% although the reduction was not statistically significant (P = 0.13; $n = 12$; paired t test; Figure 2B, Table 1). In HEK-Na1.5 cells, ESL (300 μM) inhibited persistent current by 76 ± 10% ($P < 0.01$; $n = 12$; paired t test; Figure 2G, Table 1). S-Lic (300 μM) inhibited the persistent Na⁺ current in MDA-MB-231 cells by 66 ± 16% ($P < 0.05$; $n = 9$; paired t test; Figure 3B, Table 2). In HEK-Na1.5 cells, S-Lic (300 μM) inhibited persistent current by 35 ± 16% ($P < 0.05$; $n = 11$; Figure 3G, Table 2). Application of both compounds at a lower concentration (100 μM) elicited a similar result (Supplementary Table 1). In summary, both ESL and S-Lic also inhibited the persistent Na⁺ current.

**Effect of Eslicarbazepine Acetate and S-Licarbazepine on Voltage Dependence of Activation and Inactivation**

We next investigated the effect of ESL (300 μM) and S-Lic (300 μM) on the I-V relationship in MDA-MB-231 and HEK-Na1.5 cells. A $V_h$ of -120 mV was used for subsequent analyses to ensure that the elicited currents were sufficiently large for...
FIGURE 2 | Effect of eslicarbazepine acetate on Nav1.5 currents. (A) Representative Na⁺ currents in an MDA-MB-231 cell elicited by a depolarisation from -120 to -10 mV in physiological saline solution (PSS; black), eslicarbazepine acetate (ESL; 300 μM; red) and after washout (grey). Dotted vertical lines define the time period magnified in (B). (B) Representative persistent Na⁺ currents in an MDA-MB-231 cell elicited by a depolarisation from -120 to -10 mV. (C) Representative Na⁺ currents in an MDA-MB-231 cell elicited by a depolarisation from -80 to -10 mV. (D) Normalized Na⁺ currents in MDA-MB-231 cells elicited by a depolarisation from -120 to -10 mV. (E) Normalized Na⁺ currents in MDA-MB-231 cells elicited by a depolarisation from -80 to -10 mV. (F) Representative Na⁺ currents in a HEK-Nav1.5 cell elicited by a depolarisation from -120 to -10 mV in PSS (black), ESL (300 μM; red) and after washout (grey). Dotted vertical lines define the time period magnified in (G). (G) Representative persistent Na⁺ currents in a HEK-Nav1.5 cell elicited by a depolarisation from -120 to -10 mV. (H) Representative Na⁺ currents in a HEK-Nav1.5 cell elicited by a depolarisation from -80 to -10 mV. (I) Normalized Na⁺ currents in HEK-Nav1.5 cells elicited by a depolarisation from -120 to -10 mV. (J) Normalized Na⁺ currents in HEK-Nav1.5 cells elicited by a depolarisation from -80 to -10 mV. Results are mean ± SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; one-way ANOVA with Tukey tests (n = 12–14). NS, not significant.
FIGURE 3 | Effect of S-licarbazepine on Nav1.5 currents. (A) Representative Na⁺ currents in an MDA-MB-231 cell elicited by a depolarisation from -120 to -10 mV in physiological saline solution (PSS; black), S-licarbazepine (S-Lic; 300 µM; red) and after washout (grey). Dotted vertical lines define the time period magnified in (B). (B) Representative persistent Na⁺ currents in an MDA-MB-231 cell elicited by a depolarisation from -120 to -10 mV. (C) Representative Na⁺ currents in an MDA-MB-231 cell elicited by a depolarisation from -80 to -10 mV. (D) Normalized Na⁺ currents in MDA-MB-231 cells elicited by a depolarisation from -120 to -10 mV. (E) Normalized Na⁺ currents in MDA-MB-231 cells elicited by a depolarisation from -80 to -10 mV. (F) Representative Na⁺ currents in a HEK-Nav1.5 cell elicited by a depolarisation from -120 to -10 mV in PSS (black), S-Lic (300 µM; red) and after washout (grey). Dotted vertical lines define the time period magnified in (G). (G) Representative persistent Na⁺ currents in a HEK-Nav1.5 cell elicited by a depolarisation from -120 to -10 mV. (H) Representative Na⁺ currents in a HEK-Nav1.5 cell elicited by a depolarisation from -80 to -10 mV. (I) Normalized Na⁺ currents in HEK-Nav1.5 cells elicited by a depolarisation from -120 to -10 mV. (J) Normalized Na⁺ currents in HEK-Nav1.5 cells elicited by a depolarisation from -80 to -10 mV. Results are mean ± SEM. *P ≤ 0.05; ***P ≤ 0.001; one-way ANOVA with Tukey tests (n = 9-13). NS, not significant.
analysis of kinetics and voltage dependence, particularly for MDA-MB-231 cells, which display smaller peak Na+ currents (Tables 1, 2). Neither ESL nor S-Lic had any effect on the threshold voltage for activation (Figures 4A-D; Tables 1, 2). ESL also had no effect on the voltage at current peak in either cell line (Figures 4A-D; Tables 1, 2). Although S-Lic had no effect on voltage at current peak in MDA-MB-231 cells, it was significantly hyperpolarised by ESL from -120 mV. Results are mean ± SEM. Statistical comparisons were made with paired t-tests.

### Table 1 | Effect of eslicarbazepine acetate (300 μM) on Na+ current characteristics in MDA-MB-231 and HEK-Na1.5 cells.

| Parameter                      | Control | ESL | P value | N  |
|--------------------------------|---------|-----|---------|----|
| Vthres (mV)                    | -45.7 ± 1.7 | -45.0 ± 1.4 | 0.038 | 13 |
| Vpeak (mV)                     | 3.1 ± 2.1  | -3.9 ± 2.7  | 0.056   | 13 |
| Activation V½ (mV)             | -19.3 ± 1.4 | -22.0 ± 1.5 | 0.085   | 12 |
| Activation k (mV)              | 10.6 ± 0.7  | 9.3 ± 0.8   | 0.076   | 12 |
| Inactivation V½ (mV)           | -80.6 ± 0.7 | -86.7 ± 1.2 | < 0.001 | 13 |
| Inactivation k (mV)            | -4.8 ± 0.4  | -7.4 ± 1.7  | 0.139   | 13 |
| Peak current density at -10 mV (pA/pF) | -14.8 ± 3.9 | -8.0 ± 2.5  | < 0.001 | 13 |
| Persistent current density at -10 mV (pA/pF) | -0.15 ± 0.05 | -0.02 ± 0.07 | 0.13   | 12 |
| Tp at -10 mV (ms)              | 2.1 ± 0.2  | 1.9 ± 0.2   | < 0.01  | 13 |
| t½ at -10 mV (ms)              | 1.3 ± 0.1  | 1.3 ± 0.2   | 0.964   | 13 |
| t½ at -10 mV (ms)              | 10.0 ± 2.3 | 6.9 ± 2.0   | 0.289   | 13 |
| t½ (ms)                        | 6.0 ± 0.5  | 8.7 ± 0.7   | < 0.05  | 10 |

| Parameter                      | Control | S-Lic | P value | N  |
|--------------------------------|---------|-------|---------|----|
| Vthres (mV)                    | -34.4 ± 2.0 | -35.7 ± 2.0 | 0.603 | 7  |
| Vpeak (mV)                     | 11.43 ± 4.4 | 10.9 ± 4.9  | 0.818   | 7  |
| Activation V½ (mV)             | -12.9 ± 3.3 | -13.7 ± 1.4 | 0.371   | 7  |
| Activation k (mV)              | 11.0 ± 0.5  | 11.9 ± 0.8  | 0.520   | 7  |
| Inactivation V½ (mV)           | -7.18 ± 2.5 | -7.86 ± 2.2 | < 0.05  | 7  |
| Inactivation k (mV)            | -6.8 ± 0.9  | -6.0 ± 1.2  | 0.302   | 7  |
| Peak current density at -10 mV (pA/pF) | -12.0 ± 3.1 | -6.9 ± 2.5  | < 0.001 | 7  |
| Persistent current density at -10 mV (pA/pF) | -1.3 ± 0.4  | -0.6 ± 0.2  | < 0.05  | 7  |
| Tp at -10 mV (ms)              | 4.5 ± 0.4  | 5.1 ± 0.7   | 0.103   | 9  |
| t½ at -10 mV (ms)              | 3.8 ± 1.1  | 3.2 ± 0.4   | 0.553   | 7  |
| t½ at -10 mV (ms)              | 25.7 ± 7.0 | 27.1 ± 12.0 | 0.920   | 7  |
| t½ (ms)                        | 6.8 ± 0.4  | 13.5 ± 1.0  | < 0.01  | 7  |

### Table 2 | Effect of S-licarbazepine (300 μM) on Na+ current characteristics in MDA-MB-231 and HEK-Na1.5 cells.

| Parameter                      | Control | S-Lic | P value | N  |
|--------------------------------|---------|-------|---------|----|
| Vthres (mV)                    | -80.6 ± 7.0 | -77.2 ± 17.0 | < 0.001 | 13 |
| Vpeak (mV)                     | -180.5 ± 4.2 | -30.0 ± 5.6  | 0.009   | 9  |
| Activation V½ (mV)             | -32.8 ± 3.1 | -40.5 ± 3.4 | < 0.01  | 9  |
| Activation k (mV)              | 5.9 ± 0.9   | 4.5 ± 1.1   | < 0.05  | 9  |
| Inactivation V½ (mV)           | -75.9 ± 2.6 | -79.3 ± 4.1  | 0.116   | 9  |
| Inactivation k (mV)            | -6.5 ± 0.4  | -8.1 ± 0.5  | < 0.05  | 9  |
| Peak current density at -10 mV (pA/pF) | -140.9 ± 26.8 | -77.2 ± 17.0 | < 0.001 | 13 |
| Persistent current density at -10 mV (pA/pF) | -0.9 ± 0.2  | -0.5 ± 0.2  | < 0.05  | 11 |
| Tp at -10 mV (ms)              | 1.8 ± 0.5  | 2.3 ± 0.6   | < 0.01  | 13 |
| t½ at -10 mV (ms)              | 1.0 ± 0.4  | 1.3 ± 0.06  | < 0.001 | 11 |
| t½ at -10 mV (ms)              | 6.3 ± 0.5  | 7.3 ± 0.5   | < 0.05  | 11 |
| t½ (ms)                        | 5.7 ± 0.7  | 8.0 ± 1.2   | < 0.01  | 10 |

1ESL, eslicarbazepine acetate (300 μM); Vthres, threshold voltage for activation; Vpeak, voltage at which current was maximal; V½, half (in)activation voltage; k, slope factor for (in) activation; Tp, time to peak current; t½ fast time constant of inactivation; t½, slow time constant of inactivation; t½, time constant of recovery from inactivation. The holding potential was -120 mV. Results are mean ± SEM. Statistical comparisons were made with paired t-tests.

2S-Lic, S-licarbazepine (300 μM); Vthres, threshold voltage for activation; Vpeak, voltage at which current was maximal; V½, half (in)activation voltage; k, slope factor for (in) activation; Tp, time to peak current; t½, fast time constant of inactivation; t½, slow time constant of inactivation; t½, time constant of recovery from inactivation. The holding potential was -120 mV. Results are mean ± SEM. Statistical comparisons were made with paired t-tests.
Effect of Eslicarbazepine Acetate and S-Licarbazepine on Activation and Inactivation Kinetics

We next studied the effect of both compounds on kinetics of activation and inactivation. In MDA-MB-231 cells, ESL (300 μM) significantly accelerated the time to peak current (Tp), upon depolarisation from -120 to -10 mV, from 2.1 ± 0.2 to 1.9 ± 0.2 ms (P < 0.01; n = 13; paired t test; Table 1). However, in HEK-Nav1.5 cells, ESL significantly slowed Tp from 1.4 ± 0.2 to 1.5 ± 0.2 ms (P < 0.001; n = 14; paired t test; Table 1). S-Lic (300 μM) had no significant effect on Tp in MDA-MB-231 cells but significantly slowed Tp in HEK-Nav1.5 cells from 1.8 ± 0.5 to 2.3 ± 0.6 ms (P < 0.01; n = 13; paired t test; Table 1). To study effects on inactivation kinetics, the current decay following depolarisation from -120 to -10 mV was fitted to a double exponential function to derive fast and slow time constants of inactivation (τf and τs). Neither ESL nor S-Lic had any significant effect on τf or τs in MDA-MB-231 cells (Tables 1, 2). However, in HEK-Nav1.5 cells, ESL significantly slowed τf from 0.9 ± 0.1 to 1.2 ± 0.1 ms (P < 0.001; n = 12; paired t test; Table 1) and slowed τs from 6.6 ± 0.8 to 20.8 ± 8.5 ms, although this was not statistically significant. S-Lic significantly slowed τf from 1.0 ± 0.04 to 1.3 ± 0.06 ms (P < 0.001; n = 11; paired t test; Table 2) and τs from 6.3 ± 0.5 to 7.3 ± 0.5 ms (P < 0.05; n = 11; paired t test; Table 2). In summary, both ESL and S-Lic elicited various effects on kinetics in MDA-MB-231 and HEK-Nav1.5 cells, predominantly slowing activation and inactivation.

Effect of Eslicarbazepine Acetate and S-Licarbazepine on Recovery From Fast Inactivation

To investigate the effect of ESL and S-Lic on channel recovery from fast inactivation, we subjected cells to two depolarisations from Vh of -120 to 0 mV, changing the interval between these in which the channels were held at -120 mV to facilitate recovery. Significance was determined by fitting a single exponential curve to the normalized current/time relationship and calculating the time constant (τr). In MDA-MB-231 cells, ESL (300 μM) significantly slowed τr from 6.0 ± 0.5 to 8.7 ± 0.7 ms (P < 0.05; n = 10; paired t test; Figure 6A, Table 1). Similarly, in HEK-Nav1.5 cells, ESL significantly slowed τr from 4.5 ± 0.4 to 7.1 ± 0.6 ms (P < 0.01; n = 10; paired t test; Figure 6B, Table 1). S-Lic...
(300 μM) also significantly slowed \( \tau_r \) in MDA-MB-231 cells from 6.8 ± 0.4 to 13.5 ± 1.0 ms (P < 0.01; n = 7; paired t test; Figure 6C, Table 2). Finally, S-Lic also significantly slowed \( \tau_r \) in HEK-Nav1.5 cells from 5.7 ± 0.7 to 8.0 ± 1.2 ms (P < 0.01; n = 10; paired t test; Figure 6D, Table 2). In summary, both ESL and S-Lic slowed recovery from fast inactivation of Nav1.5.

**DISCUSSION**

In this study, we have shown that ESL and its active metabolite S-Lic inhibit the transient and persistent components of Na\(^+\) current carried by Nav1.5. We show broadly similar effects in MDA-MB-231 cells, which express endogenous Nav1.5 (Roger et al., 2003; Fraser et al., 2005; Brackenbury et al., 2007), and in HEK-293 cells over-expressing Nav1.5. Notably, both compounds were more effective when \( V_h \) was set to -80 mV than at -120 mV, suggestive of depolarised state-dependent binding. In addition, the inhibitory effect of ESL was reversible whereas inhibition by S-Lic was less so. As regards voltage-dependence, both ESL and S-Lic shifted activation and steady-state inactivation curves, to varying extents in the two cell lines, in the direction of more negative voltages. ESL and S-Lic had various effects on activation and inactivation kinetics, generally slowing the rate of inactivation. Finally, recovery from fast inactivation of Na\(_{\text{a,1.5}}\) was significantly slowed by both ESL and S-Lic.

To our knowledge, this is the first time that the effects of ESL and S-Lic have specifically been tested on the Nav1.5 isoform. A strength of this study is that both the prodrug (ESL) and the active metabolite (S-Lic) were tested using two independent cell lines, one endogenously expressing Nav1.5, the other stably over-expressing Na\(_{\text{a,1.5}}\). MDA-MB-231 cells also express Na\(_{\text{a,1.7}}\), although this isoform is estimated to be responsible for only ~9% of the total VGSC current (Fraser et al., 2005; Brackenbury et al., 2007). MDA-MB-231 cells also express Na\(_{\text{a,1.7}}\), although this isoform is estimated to be responsible for only ~9% of the total VGSC current (Fraser et al., 2005; Brackenbury et al., 2007). MDA-MB-231 cells express endogenous \( \beta1, \beta2, \) and \( \beta4 \) subunits (Chioni et al., 2009; Nelson et al., 2014; Bon et al., 2016). MDA-MB-231 cells predominantly express the developmentally regulated “neonatal” Na\(_{\text{a,1.5}}\) splice variant, which differs from the “adult” variant over-expressed in the HEK-Nav1.5 cells by seven amino acids located in the extracellular linker between transmembrane segments 3 and 4 of domain 1 (Fraser et al., 2005; Brackenbury et al., 2007; Djamgoz et al., 2019). Notably, however, there were no consistent differences in effect of either ESL or S-Lic between the MDA-MB-231 and HEK-Nav1.5 cells, suggesting that the neonatal vs. adult splicing event, and/or expression of...
endogenous β subunits, does not impact on sensitivity of Na\textsubscript{1.5} to these compounds. This finding contrasts another report showing different sensitivity of the neonatal and adult Na\textsubscript{1.5} splice variants to the amide local anaesthetics lidocaine and levobupivacaine (Elajnaf et al., 2018). Our findings suggest that the inhibitory effect of S-Lic on Na\textsubscript{1.5} is less reversible than that of ESL. This may be explained by the differing chemical structures of the two molecules possibly enabling S-Lic to bind the target with higher affinity than ESL. Most VGSC-targeting anticonvulsants, including phenytoin, lamotrigine and carbamazepine, block the pore by binding via aromatic-aromatic interaction to a tyrosine and phenylalanine located in the S6 helix of domain 4 (Lipkind and Fozzard, 2010). However, S-Lic has been proposed to bind to a different site given that it was found to block the pore predominantly during slow inactivation (Hebeisen et al., 2015). Alternatively, the hydroxyl group present on S-Lic (but not ESL) may become deprotonated, potentially trapping it in the cytoplasm.

The findings presented here broadly agree with in vitro concentrations used elsewhere to study effects of ESL and S-Lic on Na\textsuperscript{+} currents. For example, using a V\textsubscript{h} of -80 mV, 300 µM ESL was shown to inhibit peak Na\textsuperscript{+} current by 50\% in N1E-115 neuroblastoma cells expressing Na\textsubscript{1.1}, Na\textsubscript{1.2}, Na\textsubscript{1.3}, Na\textsubscript{1.6}, and Na\textsubscript{1.7} (Bonifacio et al., 2001). S-Lic (250 µM) also blocks peak Na\textsuperscript{+} current by ~50\% in the same cell line (Hebeisen et al., 2015). In addition, S-Lic (300 µM) reduces persistent Na\textsuperscript{+} current by ~25\% in acutely isolated murine hippocampal CA1 neurons expressing Na\textsubscript{1.1}, Na\textsubscript{1.2}, and Na\textsubscript{1.6} (Westenbroek et al., 1989; Yu et al., 2006; Royeck et al., 2008; Doeser et al., 2014). Similar to the present study, ESL was shown to hyperpolarise the voltage-dependence of steady-state inactivation in N1E-115 cells (Bonifacio et al., 2001). On the other hand, similar to our finding in HEK-Nav1.5 cells, S-Lic has no effect on steady-state inactivation in N1E-115 cells (Hebeisen et al., 2015). Again, in agreement with our own findings for Nav1.5, S-Lic slows recovery from inactivation in N1E-115 cells (Hebeisen et al., 2015). These observations suggest that the sensitivity of Na\textsubscript{1.5} to ESL and S-Lic is broadly similar to that reported for neuronal VGSCs. In support of this, Na\textsubscript{1.5} shares the same conserved residues proposed for Nav1.2 to interact with ESL (Shaikh et al., 2014).

Notably, the concentrations used in this study are at or above those achieved in clinical use (e.g. ESL 1,200 mg once daily gives a peak plasma concentration of ~100 µM) (Hebeisen et al., 2015). However, it has been argued that the relatively high concentrations which have been previously tested in vitro are clinically relevant given that S-Lic has a high (50:1) lipid:water partition coefficient and thus would be expected to reside predominantly in the tissue membrane fraction in vivo (Bialer and Soares-da-Silva, 2012). Our
study suggests that a clinically relevant plasma concentration (100 µM) would inhibit peak and persistent Nav1.5 currents. Future work investigating the dose-dependent effects of ESL and S-Lic would be useful to aid clinical judgements.

The data presented here raise several implications for clinicians. The observed inhibition of Nav1.5 is worthy of note when considering cardiac function in patients receiving ESL (Zaccara et al., 2015). Although the QT interval remains unchanged for individuals on ESL treatment, prolongation of the PR interval has been observed (Vaz-Da-Silva et al., 2012). Further work is required to establish whether the basis for this PR prolongation is indeed via Nav1.5 inhibition. In addition, it would be of interest to investigate the efficacy of ESL and S-Lic in the context of heritable arrhythmogenic mutations in SCN5A, as well as the possible involvement of the β subunits (Brackenbury and Isom, 2008; Uebachs et al., 2010; Doeser et al., 2014; Rivaud et al., 2020). The findings presented here are also relevant in the context of Nav1.5 expression in carcinoma cells (Fraser et al., 2014). Given that cancer cells have a relatively depolarised Vm, it is likely that Nav1.5 is mainly in the inactivated state with the persistent Na⁺ current being functionally predominant (Yang and Brackenbury, 2013; Yang et al., 2020). Increasing evidence suggests that persistent Na⁺ current carried by Nav1.5 in cancer cells contributes to invasion and several studies have shown that other VGSC inhibitors reduce metastasis in preclinical models (Roger et al., 2003; Fraser et al., 2005; House et al., 2010; Yang et al., 2012; Driffort et al., 2014; Besson et al., 2015; Nelson et al., 2015a; Nelson et al., 2015b). Thus, use-dependent inhibition by ESL would ensure that channels in malignant cells are particularly targeted, raising the possibility that it could be used as an anti-metastatic agent (Martin et al., 2015). This study therefore paves the way for future investigations into ESL and S-Lic as potential invasion inhibitors.

**DATA AVAILABILITY STATEMENT**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**AUTHOR CONTRIBUTIONS**

TL, SC, and WB contributed to the conception and design of the work. TL, LB, and WB contributed to acquisition, analysis, and interpretation of data for the work. TL, SC, and WB contributed to drafting the work and revising it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.555047/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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