Research Paper
Expressional Study of Permeability Glycoprotein and Multidrug Resistance Protein 1 in Drug-resistant Mesial Temporal Lobe Epilepsy

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ABSTRACT

Introduction: About 30% of patients with epilepsy do not respond to anti-epileptic drugs, leading to refractory seizures. The pathogenesis of drug-resistance in mesial temporal lobe epilepsy (MTLE) is not completely understood. Increased activity of drug-efflux transporters might be involved, resulting in subclinical concentrations of the drug at the target site. The major drug-efflux transporters are permeability glycoprotein (*P-gp*) and multidrug-resistance associated protein-1 (*MRP-1*). The major drawback so far is the expressional analysis of transporters in equal numbers of drug-resistant epileptic tissue and age-matched non-epileptic tissue.

Methods: We have studied *P-gp* and *MRP-1* drug-efflux transporters in the sclerotic hippocampal tissues resected from the epilepsy surgery patients (n=15) and compared their expression profile with the tissues resected from non-epileptic autopsy cases (n=15).

Results: Statistically significant over expression of both *P-gp* ($P<0.0001$) and *MRP-1* ($P=0.01$) at gene and protein levels were found in the MTLE cases. The fold change of *P-gp* was more pronounced than *MRP-1*. Immunohistochemistry of the patient group showed increased immunoreactivity of *P-gp* at blood-brain barrier and increased reactivity of *MRP-1* in the parenchyma. The results were confirmed by confocal immunofluorescence microscopy.

Conclusion: Our results suggested that *P-gp* in association with *MRP-1* might be responsible for the multi-drug resistance in epilepsy. *P-gp* and *MRP-1* could be important determinants of bio availability and tissue distribution of anti-epileptic drugs in the brain which can pharmacologically inhibited to achieve optimal drug penetration to target site.

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Highlights

• *P-gp* and *MRP-1* are found to be over expressed in most of the MTLE cases.

• Over expression of *P-gp* was more pronounced than that of *MRP-1*.

• These transporters act at brain barriers as well as within the brain parenchyma.

• Both are important determinants of bio availability and tissue distribution of anti-epileptic drugs in brain.

• Iincrease in efflux transporters levels can be pharmacologically inhibited to avoid surgery for seizure control.

Plain Language Summary

Epilepsy is the most prevalent neurological condition affecting approximately 69 million people worldwide. Mesial temporal lobe epilepsy (MTLE) is the common form of partial epilepsy. There are more than 20 types of anti-epileptic drugs (AEDs) available but around 20-40% of the MTLE cases are multi drug resistant. Uncontrolled seizures have been associated with the sudden unexpected death in epilepsy (SUDEP) in 6 per 1000 epilepsy patients per year. Patients with refractory epilepsy have increased risks of premature death, injuries, psychosocial dysfunction, and a reduced quality of life. Therefore, understanding drug resistance in MTLE is an urgent clinical need. Drug-resistance in MTLE is not fully understood; the pathogenesis is thought to be multifactorial. Resistance is usually against multiple drugs having different modes of action. This suggests that pathophysiological basis for developing drug resistance is non-specific. The principal factor which is thought to be involved in pharmacoresistant MTLE is removal of AEDs from epileptogenic tissue through drug-efflux transporters. Two ATP-binding cassette (ABC) drug-efflux transporters are thought to be chief players in resistant MTLE–multidrug-resistance protein 1 (MDR-1) gene encoding permeability glycoprotein (*P-gp*) and multidrug resistance associated protein 1 (*MRP-1*). Under physiological conditions both the transporters expel the harmful substance to maintain equilibrium of internal environment of brain. Therefore, evaluating the role of *P-gp* and *MRP-1* in detail and to find out the relative importance of these two in promoting drug resistance in MTLE is necessary. The increase in efflux transporters levels can be pharmacologically inhibited to achieve optimal drug penetration to target site in refractory mesial temporal lobe epilepsy and avoid surgery for seizure control.

1. Introduction

Epilepsy is the most prevalent neurological condition affecting approximately 69 million people worldwide (Rawat et al., 2020). Mesial temporal lobe epilepsy (MTLE) is the common form of partial epilepsy. More than 20 types of anti-epileptic drugs (AEDs) are available but around 20%-40% of the MTLE cases are multi-drug resistant. Uncontrolled seizures have been associated with sudden unexpected death in epilepsy in 6 per 1000 epilepsy patients per year (Laxer et al., 2014). Drug-resistant epileptic patients are at an increased risk of premature death, injuries, psychosocial dysfunction, and a reduced quality of life (Löscher & Friedman, 2020; Zavala-Tecuapetla et al., 2020). Therefore, understanding drug resistance in MTLE is an urgent clinical need.

The International League Against Epilepsy defines drug-resistant epilepsy as "failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom" (Kwan et al., 2010). The mechanism of drug resistance in MTLE is poorly understood; therefore, the pathogenesis is considered to be multifactorial. Resistance is usually against multiple drugs with different modes of action. This suggests that the pathophysiological basis for developing drug resistance is non-specific (Kwan & Brodie, 2005). The principal factors thought to be involved in pharmacoresistant MTLE include the etiology of the disease, seizure activity progression under drug treatment, the complex temporal patterns, abnormalities in neuronal networks, changes in drug uptake across the blood-brain barrier (BBB), changes in AEDs targets, and elimination of AEDs from patient tissue through drug-efflux transporters (Lachos et al., 2011).

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This is further explained well by two widely accepted hypotheses–target and transporter hypotheses. The most accepted explanation is the transporter hypothesis. This proposes that drug resistance occurs due to the up-regulation of efflux transporters in the brain capillaries of BBB. It prevents adequate drug entry to the target location in the brain by actively expelling the drugs (Sisodiya et al., 2002).

Two adenosine 5′-triphosphate (ATP)-binding cassette (ABC) drug-efflux transporters are thought to be chief players in resistant MTLE–multidrug-resistance protein 1 (*MDR-1*), gene encoding permeability glycoprotein (*P-gp*), and *MRP-1*. *P-gp* is a transmembrane glycoprotein that is primarily distributed in the organs associated with absorption, metabolism, and excretion. It is found in the brain at the BBB (Xiong et al., 2015). *MRP-1* is an organic ion-transporter; it shares 15% of its amino acid sequence with *P-gp*. Unlike *P-gp*, *MRP-1* is found in the choroid plexus epithelium and ependymal epithelium cells for the protection of the blood-cerebrospinal fluid barrier (BCB) (Keppler, 2011). Under physiological conditions, both the transporters expel the harmful substance to maintain the equilibrium of the internal environment of the brain.

Various animal trials have shown an increase in the expression of the *P-gp* in refractory epilepsy (Rizzi et al., 2002; Volk et al., 2005; Volk et al., 2004). Similarly, *MRP-1* is overexpressed in animal models of refractory MTLE (Feldmann et al., 2013; Sisodiya et al., 2002). A study conducted on blood samples collected from epileptic patients showed increased expression of these efflux transporters in drug-resistant epileptics compared to patients controlled by AEDs. Most available efflux transporter studies on MTLE patient samples lack suitable control tissue to compare the data which is essential for result interpretation (Dombrowski et al., 2001; Kubota et al., 2006; Sisodiya et al., 2002; Tishler et al., 1995; Weidner et al., 2018). For control, most studies have used either disease-adjacent tissue or tissue obtained from surgeries done for other pathologies, such as arteriovenous malformation, aneurysms, etc. *P-gp* and *MRP-1* dysfunction has also been reported in many neurological diseases, including tumors (de Klerk et al., 2010; Ginguené et al., 2010; Jablonski et al., 2014; Kortekaas et al., 2005; Lam et al., 2001; Sakata et al., 2011; Vogelgesang et al., 2006; Wijesuriya et al., 2010). Therefore, the inclusion of control tissue from brains without known neurological disease is necessary to validate the results.

The current study was designed to analyze the expression of *P-gp* and *MRP-1* in detail and to find out the relative importance of these two in promoting drug resistance in MTLE. The study was conducted using hippocampal tissues obtained from drug-resistant MTLE patients undergoing surgery and age-matched control tissue obtained from the autopsy. We have studied *P-gp* and *MRP-1* at both gene and protein levels. Moreover, immuno-histochemical analysis and confocal fluorescence microscopy were used to determine the cellular localization of these transporters.

2. Materials and Methods

Subjects

The hippocampal tissue was obtained from 15 drug-resistant MTLE patients who underwent MTLE surgery in the Department of Neurosurgery of the Postgraduate Institute of Medical Education and Research, Chandigarh, India. Table 1 presents the clinical data of the patients. The tissue was taken from the patients with pathologically proven hippocampal sclerosis (HS) only. The exclusion criteria included patients with seizure types other than partial seizures and tissues showing any structural intracranial insults, for example, gliomas, meningitis, and neurofibromatosis.

Controls

The hippocampal tissue taken from the autopsies (n=15) performed in the Department of Forensic Medicine of the Postgraduate Institute of Medical Education and Research was used as control tissue. Samples were collected from the autopsies performed within 4 hours after death with only non-neurological causes. Death due to non-neurological causes was considered for the study while patients with any severe head injury, hemorrhage, etc. were not included in the study. Table 2 presents clinical data.

Tissue was collected only after written informed consent from the relatives of patients and controls.

Processing of hippocampal tissue

Under sterile conditions, the collected tissue was immediately placed in the saline. The collected tissue was then divided into sections, one section was fixed in ribonucleic acid (RNA) later at -20°C, one section was frozen at -80°C and another section was fixed in 10% formalin overnight at room temperature.

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In each case, a part of the fixed tissue was processed for histology. Using the standard procedure, hematoxylin and eosin staining was performed and the slides were then assessed by a histopathologist to identify HS.

Real-time polymerase chain reaction (RT-PCR)

The trizol method (Ambion) was used to isolate total RNA by taking an amount of 0.5-1 g of tissue kept in the RNA later. Spectrophotometric measurements (Biotek Epoch) were performed to assess RNA quality and quantity. The absorbance taken for measurements was at A260/A280. Complementary DNA (cDNA) was synthesized using a BioradiScript cDNA synthesis kit as per instructions. For RT-PCR, primers at 250 nM concentration were used–human MDR-1 forward 5'-TCAGCTGTTGTCTTTGGTGC-3'; reverse 5'-GGTCGGGTGGGATAGTTGAA-3', human *MRP-1* forward 5'-TTGGTGATATTCGCCATTGA-3'; reverse 5'-ACGCATAGTGGATGGCTTTC-3', and human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-TGAACGGGAAGCTCACTGG-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. Evaluation of gene expression was performed using SYBR Green chemistry on ABi step one plus RT-PCR system. The data was standardized (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). The data was represented in the form of plots and a standard criterion was followed to describe the gene's status whether up-regulated or down-regulated.

Western blot (WB)

Hippocampal tissue was properly thawed and washed with phosphate-buffered saline (PBS). Total proteins were extracted ice cold 1X RIPA lysis buffer. One mL of 1X RIPA lysis buffer was added along with 2 µL protease inhibitor (Sigma-Aldrich). The bicinchoninic acid (BCA) method was used to analyze protein concentration using the BCA kit (BioRad). After heating, the samples were resolved on sodium-dodecyl sulphate polyacrylamide gel using 4% stacking and 8% separating gel. Polyvinylidene difluoride (PVDF) membrane was used to blot the resolved proteins. The blocking was performed in the BSA at 25°C. Blotted PVDF membranes were incubated with specific primary antibodies overnight at 4°C, mouse monoclonal MDR-1 at a concentration of 1:200 (Santa-Cruz Biotech., sc-71557), mouse monoclonal *MRP-1* at a concentration of 1:50 (Abcam, ab24102), and mouse monoclonal β-actin (an internal control) at a concentration of 1:5000 (Thermo fisher, M5A-15739). After proper washing with tris-buffered saline, 0.1% Tween 20 (TBST) buffer, blotted membranes were incubated with respective secondary antibodies at room temperature–rabbit anti-mouse (1:10,000, Abcam, ab97046). Enhanced chemiluminescence (ECL) kit (BioRad) was used to visualize blotted membranes. To interpret data, ImageJ Plus software was used for the densitometry analysis.

Immunohistochemistry (IHC)

Immunohistochemical analysis was performed to confirm RT-PCR and WB results by evaluating the protein localization and expression. After processing with standard protocol, 5 µm thick sections were taken on poly-L-lysine coated slides. Deparaffinization was done in xylene (three 10-minute changes) followed by hydration of sections through graded alcohols (absolute or 100% ethanol, 70% ethanol, 50% ethanol, and distilled water for 5 minutes each). Hydrogen peroxide and deionized water in a ratio of 1:9 were used for endogenous blocking by incubating sections for 10-15 minutes. Antigen retrieval was performed using tris-ethylenediaminetetraacetic acid (EDTA) buffer at pH 9.0 to increase the immunogenicity. The sections were placed in an incubator at 55°C for 4-8 hours. Incubation with primary antibodies-mouse monoclonal MDR-1 (1:200, Santa-Cruz Biotech., sc-71557) and mouse monoclonal *MRP-1* (1:20, Abcam, ab24102) was performed by an overnight incubation at 4°C. Primary antibody was omitted in the negative control. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibody (1:2000, Abcam, ab97046) was used at room temperature. A total of 0.5% 3, 3'-diaminobenzidine was used to develop peroxidase activity. Hematoxylin was used as a counterstain. The slides were properly cleaned and coverslipped after mounting with di-n-butyl phthalate. The observation was done under a light microscope by two unbiased observers.

Semi-quantitative analysis of immunohistochemistry (IHC) using histo (H)-score

The cells with clearly outlined nuclei were observed and counted in 10 random fields of view in each section of the patient (n=15) and non-epileptic control (n=15) at scale bars 100 µm for the detailed analysis. Histo-score (H-score) was calculated using the Equation 1:

1. H-score=(1×[% Cells with 1+]+2×[% Cells with 2+]+3×[% Cells with 3+])

Where 1+, 2+ and 3+ are the intensity scores of immunostaining according to the appearance (Cheon et al., 2001).

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Each section was examined under a light microscope by two unbiased observers in a blinded manner. It was calculated in the 10 high power fields in the section and the average was taken as H-score. Based on the H-score, the immunoreactivity (IR) was categorized as mild (<50), moderate (50-100), or strong (>150). The selected sections were photographed using an Olympus microscope with ProgRes® Capture Pro software, version 2.9.01.

Immunofluorescence

For immunolabelling, tissues were processed with standard protocol. Sections with a thickness of 5 μm were taken on poly-L-lysine coated slides. Deparaffinization was performed in xylene (three 10-minute changes) followed by hydration of sections through graded alcohols (absolute or 100% ethanol, 70% ethanol, and 50% ethanol for 5 minutes each). The tissue sections were then washed in the running water (deionized) for one minute. After PBS rinsing, antigen retrieval was done to increase the immunogenicity using tris-EDTA buffer at pH 6.1 in the oven for 12 minutes. The slides were incubated with two different primary antibodies (used in IHC) mixed in the immunofluorescence (IF) buffer and incubated at room temperature for one hour. Antibody binding was observed by incubation with appropriate Alexa Flour (647-red) anti-mouse conjugated secondary antibodies (Thermo-fisher Scientific A21235, dilution 2 μ/mL) diluted in the immunofluorescence buffer in a dark for one hour. After washing 2-3 times with PBS, the slides were counterstained with 60 μL 4′,6-diamidino-2-phenylindole (DAPI) and mounted using glycerol. Slides were stored in the dark by covering them with aluminum foil at 4°C before analysis using a confocal immunofluorescence microscope (Evos FL auto BX53). All the samples were processed on the same day to avoid confounding with the quantification. Fluorescence of cells and images were determined at scale bars 100 μm using the ImageJ plus software. The color channels were split by selecting the image. After making the composite and splitting the different channels, the images were stacked. An image montage was created as displayed images. For densitometric analysis, z-stacks of the photographed images were converted to grayscale. Signal intensity was quantified as integrated density by simply marking a circle around the cell. For further analysis, the mean gray value, and integrated density were calculated. Corrected total cell fluorescence (CTCF) was interpreted using the Equation 2:

2. CTCF=Integrated density (Measured area of the selected cell×Mean fluorescence of background)

Background signals were measured in signal-free areas.

Statistical analysis

The software used for data analysis and graph preparation was GraphPad Prism software, version 8 and SPSS software, version 23. Statistical significance was determined using the student's t-test comparing control versus patients. Mean±SD, Mean±SE, and the range were calculated for age, onset age of seizure, epilepsy duration, and seizure frequency. The drug-efflux transporters, *P-gp* and *MRP-1* were correlated with the duration of epilepsy, duration of drug resistance, and seizure frequency. Correlation was determined by calculating Spearman's rank correlation coefficient, ρ. P≤0.05 was considered significant. Unpaired student's t-test was applied to determine any significant difference in H-score and of immunofluorescence (CTCF values), between MTLE cases and non-epileptic controls.

3. Results

Clinical data

Tables 1 and 2 present the clinical and demographic data of the patients and controls. In the patient group (9 men and 6 women), the mean age of the patients was 29.27±10.12 years, the onset age of seizures was 12.13±6.26 years, the duration of epilepsy was 15.13±10.70 years and seizure frequency was 8.60±5.86 month. The whole data of patients are expressed compared to the age-matched controls. In the non-epileptic control group (13 men and 2 women), the mean age of the controls was 40.47±13.68 years.

Expression of transcripts of *P-gp* and *MRP-1* genes in hippocampal tissue

Figure 1 (a-d) shows relative messenger ribonucleic acid (mRNA) levels of *P-gp* and *MRP-1* genes of drug-resistant MTLE patients and non-epileptic controls. Both *P-gp* and *MRP-1* were detected in the hippocampal tissue. It has been observed that mRNA levels of *P-gp* and *MRP-1* were higher in the patient tissues compared to the control tissues (Figure 1b and 1d). The Mean±SD (ΔCt value) of the patient group was 10.12±5.47 and for the control group was 0.51±0.73 for *P-gp*. And Mean±SD (ΔCt) of the patient group was 2.99±2.40 and for the control group was 0.97±0.69 for *MRP-1*. The fold change was calculated for each patient (n=15), 66% of the patients showed overexpression of *P-gp*. Eleven out of Fifteen patients showed overexpression of *MRP-1* (Figure 1a and 1c). Elevated levels of *P-gp* and *MRP-*

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Table 1. Clinical characteristics of patients of MTLE with HS and AEDs taken before surgery

Line	Age/Sex	Onset Age (y)	Surgery Age (y)	Epilepsy Duration (y)	Seizure Frequency (Episodes/Month)	AEDs Before Surgery	Side of Surgery
1	31/F	13	31	18	8-9	LV, TG, FR	Right
2	54/M	11	54	43	8-9	LP, TG, FR	Right
3	29/M	15	29	14	3-4	LP, TG, FR	Right
4	10/M	5	10	5	9-10	LP, TG, CL	Right
5	33/F	12	33	2	1-2	LV, CB, CL	Left
6	23/F	5	23	10	20-21	CB, LP, LV, CL, LA	Right
7	21/M	5	21	14	4-5	CB, CL, LV, LP, TG	Right
8	27/F	19	27	8	12-13	LV, GD	Right
9	29/M	20	29	9	2-3	CB, CL, LV, VP	Right
10	32/M	2	32	30	3-4	TG, FR, LC	Left
11	26/M	7	26	19	8-10	LV, CL, CB, VP	Left
12	35/M	15	35	20	7-8	LV, CL, CB, VP	Left
13	28/F	21	28	7	8-9	CL, CB, VP, LV	Right
14	42/F	20	41	21	15-20	ZX, LP, FR, LC	Left
15	19/M	12	19	7	1-2	LP, TG, FR	Right

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Abbreviations: AEDs: Anti-epileptic drugs; F: Female; M: Male; LV: Levetiracetam; TG: Tegretol; FR: Frisium, LP: Levipil; LA: Lamotrigine; CB: Carbamazepine; CL: Clobazam; GD: Gardenal; VP: Valproate; ZX: Zenoxa.

1 in the patients were between 4-6 folds compared to the controls. The results were statistically significant for *P-gp* (P<0.0001) and *MRP-1* (P=0.01). Fold change seen for *P-gp* in the MTLE patients was more pronounced than *MRP-1*.

Expression and localization of *P-gp* and *MRP-1* proteins in hippocampal tissue

WB analysis further revealed increased protein expression of both *P-gp* and *MRP-1* with an apparent molecular weight of 170 and 172 kDa, respectively in the patients and controls using sc-71557 and ab24102 antibodies (Figure 1e). The densitometric analysis showed that the results using β-actin as standard were statistically significant (Figure 1f). The Mean±SD of the ratio of the area was 0.60±0.02 in the patients and 0.42±0.08 in the controls for *P-gp* (P=0.006) and 0.67±0.04 in the patients and 0.36±0.04 in the controls f*or MRP-1* (P<0.001).

The cellular localization of *P-gp* and *MRP-1* was determined by immunohistochemistry using the same antibodies. The results demonstrated that these proteins were present in both diseased and control tissues. *P-gp* showed increased immunostaining in the MTLE patients compared to the controls (Figure 2). In the diseased patients, the IR was moderate to strong. Maximum staining was observed at the BBB (Figure 2b and 2c). The endothelial cells in blood vessels demonstrated cytoplasmic as well as membranous staining. The neuropil surrounding the capillaries depicted moderate immunostaining; it includes foot processes in astrocytes. Moderate to severe intensity cytoplasmic IR was also observed in the glial cells (Figure 2c). In addition, moderate IR was displayed by neurons; it was punctate in pattern and cytoplasmic as well as membranous in location. In the controls, mild IR was observed in endothelial cells of the capillaries at the BBB. Very mild cytoplasmic IR was also observed in the neurons (Figure 2a). No immunostaining glial cells were found in the control cases.
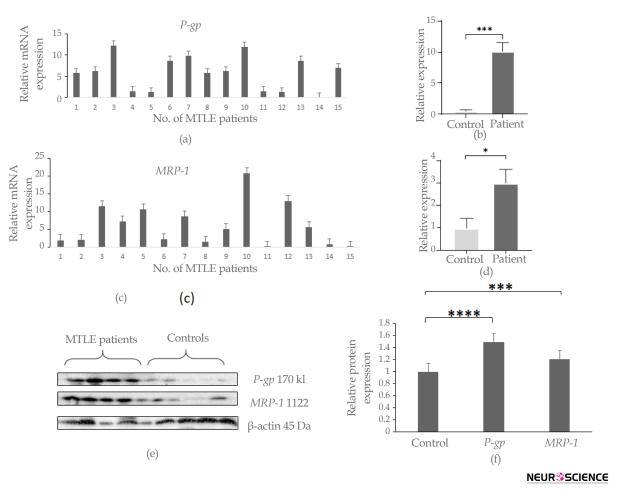
Figure 1. mRNA expression data of *P-gp* and *MRP-1* in MTLE patients compared to the non-epileptic controls
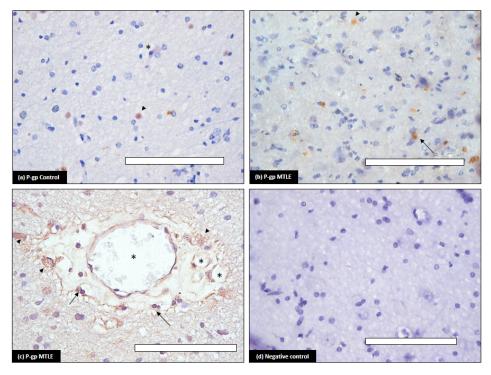
Both the efflux transporters show up regulation in the patients. a) & c) Fold change in *P-gp* and *MRP-1*, respectively in individual MTLE patients; b) & d) Relative expression (ΔΔCt) between MTLE patients and non-epileptic controls; e) *P-gp* and *MRP-1* proteins are overexpressed in MTLE patients compared to the non-epileptic controls with β-actin as standard; f) Relative density of the WB data by densitometric analysis using ImageJ software.

*P<0.05, ***P<0.001, ****P<0.0001 represents significant difference among the study groups (unpaired student t-tests), error bars show the Mean±SE.

Immunohistochemical analysis of *MRP-1* was performed and increased expression was observed in the MTLE cases compared to the non-epileptic controls (Figure 3). In the MTLE patients, IR was cytoplasmic in glial cells. Some neurons were also immunopositive (Figure 3a). In the control group, mild IR was observed in neurons and glial cells (Figure 3b). In the vessel, stained endothelial cells were observed neither in patients nor in controls (Figure 3c and 3d). Semi-quantitative analysis by H-score also confirmed the IHC results (Table 3).

Further, immunofluorescence of *P-gp* and *MRP-1* was checked in the MTLE and non-epileptic control tissue. Immunofluorescence staining confirmed the expression of *P-gp* and *MRP-1* in the hilar region of the dentate gyrus. The immunofluorescence images labeled with

P-gp (red) and *MRP-1* (red) only described the presence or absence of particular protein along with nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) (blue) in patients and controls. Increased immunofluorescence was observed in the MTLE cases for both the efflux transporters (Figure 4). The results were statistically significant for *P-gp*. The Mean±SE was 116293±11698 in the MTLE cases and 38887±2583 in the controls with a P 0.0002. The Mean±SE for *MRP-1* was 79172±5032 in the MTLE cases and 49942±6304 in the controls with a P=0.006, which was statistically significant. The number of positive cells for *P-gp* showed a significant difference in patients and controls, while positive cells in the case of *MRP-1* were significant but not significantly different between patients and controls.

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Figure 2. Immuno-histochemical staining of hippocampal tissue in the hilar region of the dentate gyrus using *P-gp* monoclonal antibody (all scale bars 100 μm)

a) In the control cases mild cytoplasmic IR was observed at the BBB in the capillary endothelial cells (asterisks) and in neurons (arrowheads); b) & c) In the MTLE cases capillary endothelial cells (asterisks) show moderately positive IR for *P-gp*. Outer to the capillary, IR can also be appreciated in glial elements (which would be foot processes of astrocytes forming BBB), Immuno-stained glial nuclei are depicted by arrows. Neurons (arrowheads) show moderate cytoplasmic as well as membranous staining; d) Negative control

Correlation of disease parameters with *P-gp* and *MRP-1*

There may be a possibility of a relationship between drug-efflux transporters and the disease parameters of epilepsy. We tried to find any relation between increased expression of drug-efflux transporters and different disease parameters. We correlated the expression pattern of *P-gp* and *MRP-1* with the duration of epilepsy, duration of drug resistance, and frequency of seizures using Spearman's rank correlation. *P-gp* or *MRP-1* showed no correlation with any of the described parameters (Table 4).

4. Discussion

Drug efflux transporters are crucial for the maintenance of homeostasis in the brain in a physiological state. Pathological conditions, such as epilepsy seem to increase the activity of xenobiotic efflux manyfold. Thus, efflux transporters become a critical determinant of drug distribution within the central nervous system (CNS). We have investigated the expression pattern of drug efflux transporters *P-gp* and *MRP-1* in the tissue resected from multidrug-resistant MTLE patients and compared it with non-epileptic controls. We have found statistically increased gene expression profiles for both the efflux transporters in patients. Similar results were obtained in the protein analysis. Immunohistochemical and immunofluorescent studies have provided histological as well as cellular localization of these increased efflux transporters in hippocampal tissue in drug-resistant MTLE.

Drug resistance has been observed in about 1/3rd of the total patients treated with different AEDs. One of the most resistant forms of epilepsy in humans is MTLE with HS (Pohlen et al., 2017). Therefore, we only incorporated patients with confirmed HS in the present study. About 30%–50% of epileptic patients are resistant to the present treatments, which is associated with a five-fold death rate compared to the population (Sharma et al., 2015). The removal of epileptic tissue with surgery remains the only solution for drug-resistant patients (Bergey et al., 2017), but even then seizure freedom was
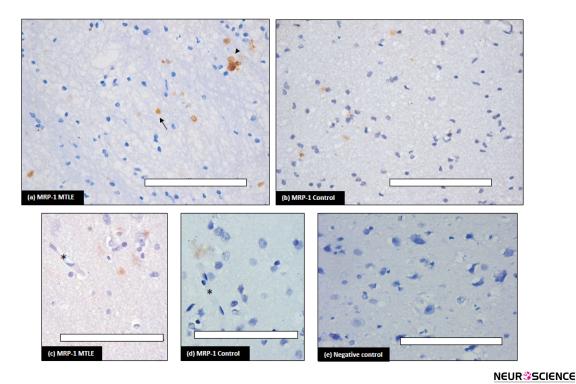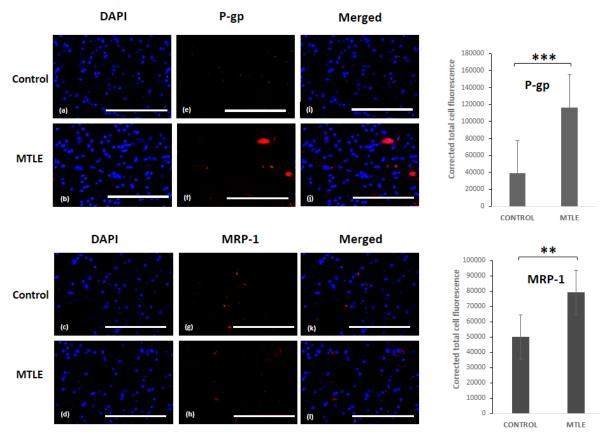
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Figure 3. Representative images of IR to *MRP-1* monoclonal antibody in the hilar region of the dentate gyrus (all scale bars 100 µm)

Note: a) Increased cytoplasmic IR of *MRP-1* can be seen in the neurons (arrowheads) and glial cells (arrows) of patients compared to the controls. b, c & d) *MRP-1* was not observed in the endothelial cells (asterisks) of capillaries in both patients and controls. e) Negative control.

attained for at least 1 year in only 50%-80% of cases (Spencer & Huh, 2008). Overexpression of the drug-efflux transporters was first described for multi-drug resistant cancer cells. Overexpression of transporters was also found in capillary endothelial cells of brain tissue obtained from patients with resistant epilepsy. Various studies explain the involvement of ABC efflux transporters in drug-resistance MTLE; the vital transporters are *P-gp* and *MRP-1* (Aronica et al., 2004; Dombrowski et al., 2001; Kubota et al., 2006; Sisodiya et al., 2002; Sisodiya et al., 2006; Tishler et al., 1995; Weidner et al., 2018). Both efflux transporters maintain the stability of the internal environment by extruding the xenobiotics in physiological conditions. The expression pattern of these transporters changes from physiological to pathological states. In pathological conditions, drug efflux leads to suboptimal concentrations of drugs at the target site, causing multidrug resistance.

P-gp or MDR-1 is an ATP-binding cassette subfamily B member 1 (ABCB1). It is encoded by the *MDR1* (*ABCB1*) gene in humans located at chromosomal region 7q21 (Potschka et al., 2001). This is a single-stranded transmembrane glycoprotein composed of 1 280 amino acids. Various barrier and excretory tissues express *P-gp*. *P-gp* delivers hydrophobic and amphipathic molecules of cells or membranes by active transport from inside to outside. This physiological function of expelling xenobiotics of extensive molecular variability is considered the critical defense mechanism that protects the internal brain milieu. Under physiological conditions, *P-gp* is expressed in the luminal membrane of the brain capillary endothelial cells and in the astrocyte foot processes surrounding the capillaries that constitute the BBB. Its expression is slightly evident in neurons or glial cells. In pathological conditions, such as epilepsy, stroke, etc. biochemical cues from the variations in the internal environment of the brain lead to increased expression of *P-gp* (Miller et al., 2008). In the diseased state, *P-gp* is not only highly expressed in endothelial cells but also the brain parenchyma (D'GIANO, 1997). In our results, we have found over-expression of *P-gp* in the endothelial cells of the blood vessels as well as in the hippocampal neurons (Figure 2b and 2c). In animal tissue, it has been found that under physiological conditions, the *MDR1* gene expression is limited to capillary endothelial cells and astrocytes around capillaries. However, after epileptic seizures, *P-gp* is also expressed in the astrocytes and neurons in the parenchyma. Therefore, there is not only an increase in the expression level of the *P-gp* but also the number of sites that express these transporters (Volk et al., 2005). According to our results, the mRNA

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Figure 4. Immunofluorescence of *P-gp* and *MRP-1* using confocal fluorescence microscopy in the controls and MTLE cases (all scale bars 100 µm)

a, b, c, & d) DAPI-stained neuronal nuclei (blue); e & f) Red immunofluorescence, for *P-gp*; g) *MRP-1*; h) Panels, respectively; i & j) DAPI and *P-gp*; k & l) DAPI and *MRP-1*

Note: The area depicted in the images is the dentate hilar region of the hippocampus. Increased immunofluorescence of both efflux transporters is seen in the MTLE cases compared to the controls. The densitometric graphs with CTCF show a statistically significant increase of *P-gp* (P=0.0002) and *MRP-1* (P=0.006) proteins in MTLE.

**P<0.01, ***P<0.001 represents significant difference among the study groups (unpaired student t-tests), error bars show the Mean±SE.

expression of the gene (*MDR-1*) increased five folds in MTLE patients compared to the non-epileptic controls in the present study. Similarly, on WB, *P-gp* levels were also significantly raised in the patient group.

MRP-1 is a specific organic anion transporter. This is composed of 1 531 amino acids and is encoded by the *ABCC* gene located at chromosomal region 16p31.1. It is distributed in the kidneys, liver, lungs, testes, and peripheral blood mononuclear cells. In the brain, *MRP-1* is distributed in the choroid plexus epithelium and ependymal epithelium cells of BCB. These cells prevent the entry of harmful substances or drugs into brain tissue (Sodani et al., 2012). Sisodiya et al. found increased expression of *MRP-1* in samples collected from epileptic patients

(Sisodiya et al., 2002). In the present study, we have also found that the *MRP-1* gene was over-expressed in the epileptic brains and also translated into much higher MRP proteins. Over-expression of *MRP-1* has been reported but conflicting reports exist on its location in the nervous system. In the normal brain, the expression of *MRP-1* in the neurons and glial cells has not been constantly stated in the literature (Ashraf et al., 2014). However, in the present study, *MRP-1* IR was detected in both neurons and glial cells in control tissue, though it was mild compared to the IR seen in the epileptic tissue. Vliet et al have reported *MRP-1* expression in endothelial cells of the capillaries in hippocampal tissue from chronic epilepsy (Van Vliet et al., 2005). Contrary to this, in the present study, *MRP-1* immuno-positivity was

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Table 2. Clinical characteristics of 15 non-epileptic controls, age, sex, etiology diagnosis, post-mortem interval (pmi) and pathology

Line	Age (y)	Sex	Etiology Diagnosis	PMI (h)	Pathology
1	23	Male	Trauma	2	None
2	24	Male	Trauma	4	None
3	52	Male	Trauma	4	None
4	45	Female	Trauma	4	None
5	26	Male	Trauma	4	None
6	28	Male	Trauma	4	None
7	50	Male	Trauma	2	None
8	60	Male	Trauma	2	None
9	45	Male	Trauma	2	None
10	59	Male	Trauma	3	None
11	26	Male	Trauma	2	None
12	45	Female	Trauma	4	None
13	52	Male	Trauma	4	None
14	48	Male	Trauma	2	None
15	24	Male	Trauma	3	None

PMI: Post-mortem interval.

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not observed in endothelial cells of capillaries in either control or diseased tissues (Figure 3 a-d). Similar to our results, a consistent *MRP-1* overexpression in the parenchyma (neurons and astrocytes) not in endothelial cells in epileptic tissue has been reported by various studies (Chen et al., 2013; Kubota et al., 2006; Yi, 2012). Decleves et al. have also reported higher expression of *MRP-1* in cultured astrocytes compared to cultured endothelial cells (Decleves et al., 2000). This suggests that the efflux mechanism is also at work within the brain parenchyma in neurons and glial cells, and this becomes overactive in the resistant cases, preventing the AEDs from achieving clinically effective concentration at the target site.

Various animal trials have shown genetic as well as protein overexpression of both efflux transporter i.e. *P-gp* and *MRP-1*, in the epileptic brain tissue (Kuteykin-Teplyakov et al., 2009; Rizzi et al., 2002; Seegers et al ., 2002; Volk et al., 2005; Volk et al., 2004). In our study, we have observed similar up-regulation at genetic as well as protein levels for both the transporters. Jinming et al. investigated the expression levels of *P-gp* and *MRP-1* in the peripheral blood of patients with epilepsy and found them increased in the drug-resistant patients compared to the patients responding to AEDs (Jinming et al., 2018). A positron emission tomography (PET) study reported that increased *P-gp* function in the temporal lobe of patients with drug-resis-

Table 3. Semi-quantitative analysis of IHC of *P-gp* and *MRP-1* using H-score in MTLE patients and non-epileptic controls

Drug-efflux Transporters	TC/SC in 10 HPFs at 40x		H-score		P
	Control	Diseased	Control	Diseased	
P-gp	10722/293	15181/1175	57	268	<0.0001
MRP-1	16104/374	14487/701	49	183	0.0004

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Abbreviations: TC: Total cells; SC: Stained cells; HPFs: High power fields.

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Table 4. Spearman's rank correlation coefficient, ρ, and P calculated to find out the correlation of *P-gp* and *MRP-1* with duration of epilepsy, duration of drug-resistance and seizure frequency of MTLE patients

Drug-efflux Transporter		Duration of Epilepsy	Duration of Drug-resistance	Frequency of Seizures
MDR-1	ρ	0.03	-0.30	-0.27
	p	0.90	0.27	0.31
MRP-1	ρ	0.02	-0.03	-0.39
	p	0.92	0.90	0.14

MDR-1: Multidrug-resistance 1; *MRP-1*: Multidrug-resistance-associated protein-1.

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tant TLE reverted to normal after epilepsy surgery; it was observed only in patients who responded to the surgery but not in patients having non-satisfactory surgical outcome (Bauer et al., 2014). It has been suggested that overexpression and increased activity of active efflux mechanisms in epileptic brains play a significant role in the therapeutic failure of pharmacological treatment of AEDs. Our results are consistent with these studies. In the present study, the quantum of increase was more for *P-gp* than *MRP-1*, which may indicate that *P-gp* plays a greater role in causing drug resistance in MTLE.

Conflicting reports exist in the literature regarding the status of *P-gp* and *MDR-1* after acute epilepsy. Few studies have reported increased mRNA expression for *P-gp* after 3-48 hours of seizure episode, returning to normal within 72 hours to 2 weeks (Rizzi et al., 2002). On the other hand, Kuteykin-Teplyakov et al found that after 6-24 hours of status epilepticus in rats, *P-gp* and *MRP-1* mRNA expression was decreased in the hippocampus, followed by increased expression after two days (Kuteykin-Teplyakov et al., 2009). The results of these studies show that up-regulation of *P-gp* and *MRP-1* is a temporary phenomenon in the early stage of epilepsy. We have used the hippocampal tissues from long-standing drug-resistant MTLE patients and have consistently found higher levels of both efflux transporters at genetic and protein levels. Therefore, long-term recurrent seizures may lead to permanent genetic up-regulation of the efflux transporters. We correlated *P-gp* and *MRP1* expression profiles with duration of epilepsy, duration of drug-resistance, and frequency of seizures of all the patients, to detect any trend describing the drug-resistance pattern. We found no correlation between any of these parameters with *P-gp* or *MRP-1*.

5. Conclusion

Drug resistance is common in MTLE and results in severe morbidity and mortality. We studied the expression pattern of *P-gp* and *MRP-1* in MTLE cases compared to age-matched non-epileptic controls. The tissue location for these transporters was found using monoclonal antibodies. These results show that *P-gp* and *MRP-1* are crucial determinants of bioavailability and tissue distribution of AEDs in the brain. According to our study, *P-gp* and *MRP-1* are overexpressed in most cases of the MTLE. This was confirmed at both genetic as well as protein levels. In MTLE patients, overexpression of *P-gp* was more pronounced than that of *MRP-1*. Drug efflux transporters act at brain barriers as well as within the brain parenchyma. The increase in efflux transporters levels can be pharmacologically inhibited to achieve optimal drug penetration to target sites in refractory MTLE and avoid surgery for seizure control.

Limitations

It is still not clear whether overexpression of efflux transporters is intrinsic (present before the onset of epilepsy) or acquired i.e. the result of epilepsy, uncontrolled epileptic seizures, treatment with different AEDs, or a combination of these factors. Moreover, only *P-gp* and *MRP-1* were studied in resistance mechanisms; other drug efflux transporters should also be studied. We only focused on the theory of multidrug resistance due to over-expression of transporters; transporter and target mechanisms may overlap in some patients.

Ethical Considerations

Compliance with ethical guidelines

Ethical approval for the study was taken before tissue collection from the Institutional Ethic Committee, Post Graduate Institute of Medical Education and Research. Informed consent was obtained from all individuals and from the relatives of deceased included in the study.

Authors' contributions

Conceptualization: Mandeep Kaur and Tulika Gupta; Methodology: Mandeep Kaur and Mili Gupta; Investigation: Navneet Singla, Yogender S Bansal, Bishan Das Radotra and Parampreet S Kharbanda; Writing original draft: Mandeep Kaur; Review & editing: Tulika Gupta; Funding acquisition: Tulika Gupta; Resources: Tulika Gupta and Daisy Sahni; Supervision: Daisy Sahni and Sunil Kumar Gupta.

Conflict of interest

The authors declared no conflict of interest.

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