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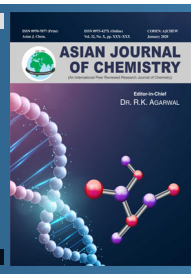

Design, Synthesis and *in vitro* Cytotoxicity Evaluation of New Fluorinated Ionic Salt (*S*)-(+)-2,3-Dihydro-1*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5,11(10H,11aH)-dione as Strategies for Improving Anticonvulsant Activity

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The reaction of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5,11(10H,11aH)-dione (**1**) with 4-(trifluoromethyl)benzoic acid (**2**, $C_8H_5F_3O_2$) in dimethylformamide leads to the formation of $C_8H_5F_3O_2$ (**1**) as a classical ionic salt **3**. The structure of new compound has been characterized by FTIR, ^{1}H NMR, ^{13}C NMR, HRMS spectroscopy. The new compound was tested for *in vitro* cytotoxicity evaluation by MTT assay against breast adenocarcinoma cell line of MCF-7 cells. A new compound **3** (IC_{50}:199 µM) emerged as minimal toxic when compared to clinical drugs carbamazepine, topiramate and benzodiazepine. A preliminary study of structure activity relationship revealed that the incorporation of fluoro or trifluoromethyl moiety into the compound, even through ionic bond formation, had a great effect on the biological activity and with less toxicity or side effects.

Keywords: Fluorinated benzodiazepine, Epilepsy, Cytotoxicity evaluation, Structure-activity relationship, Blood brain barrier.

INTRODUCTION

Epilepsy is a syndrome of different cerebral disorders of central nervous system (CNS) and characterized by paroxysmal cerebral dysrhythmia, manifested as brief episodes (seizures) of loss or disturbance of consciousness, frequently followed by convulsions [1,2]. About 1 % of the world populations have epilepsy, with almost 90 % of these people being in developing countries [3,4]. In recent years, several antiepileptic drugs (AEDs) like carbamazepine, phenytoin, benzodiazepine, topiramate and lamotrigine have been used as therapeutics agents against epilepsy [5]. Sisodiya *et al*. [6] reported that epilepsy is resistant to drug treatment in about one third of cases, but the mechanisms underlying this drug resistance are not understood.

There is a continuing demand for new anticonvulsant agents, as several of the currently available antiepileptic drugs have been associated with severe side effects, such as renal impairment, hyperkalaemia, drug cough and skin rashes. Patients with long term treatment could not tolerate these adverse side effects [7-10]. In some cases, cardiovascular and respiratory depression occurs due to denzodiazepine therapy [11]. Though the valid reasons for the abnormal behaviour of seizures, at any particular time, are still a mystery, there seems to be an in adequacy in the supply of oxygen content to both the seizures and lungs. Patients affected by such problems, tends to lose the nervous balance due to respiratory depression. Seizures at the time of abnormal vibrations, too lack in oxygen content and hence the abnormal vibrations prolongs for a longer period of time [12-14].

Fluorinated compounds are the focus of much interest in modern pharmaceutical chemistry. Incorporation of fluoro or fluoroalkyl substituents plays a significant role in development of drugs, including anticonvulsant active molecules [15,16]. They offer promising and amazing chemical diversity, there by inspiring the development of structurally diverse new molecules to play a vital role in drug discovery [17-19]. The properties of fluorine such as its small size, combined with the high electronegativity may modulate electronic, lipophilic and steric parameters crucial for biological activity [20,21]. Additionally, an excellent oxygen

carrying capacity, decrease of toxicity and side effects has been reported in many cases of fluorine containing derivatives [22,23].

The literature survey have prompted us to design and synthesized a new fluorinated ionic salt (*S*)-(+)-2, 3-dihydro-1*H*-pyrrolo [2,1-*c*][1,4]benzodiazepine-5,11-(10H,11aH)dione containing fluoro or trifluoromethyl substituents, in order to examine the influence of fluoro substituents on anticonvulsant activity. The new compound was evaluated for their *in vitro* cytotoxicity properties and correlated with known classical drugs such as carbamazepine, topiramate and benzodiazepine. Furthermore, new compound was investigated for anticonvulsant screening through the correlation of structure-activity relationships.

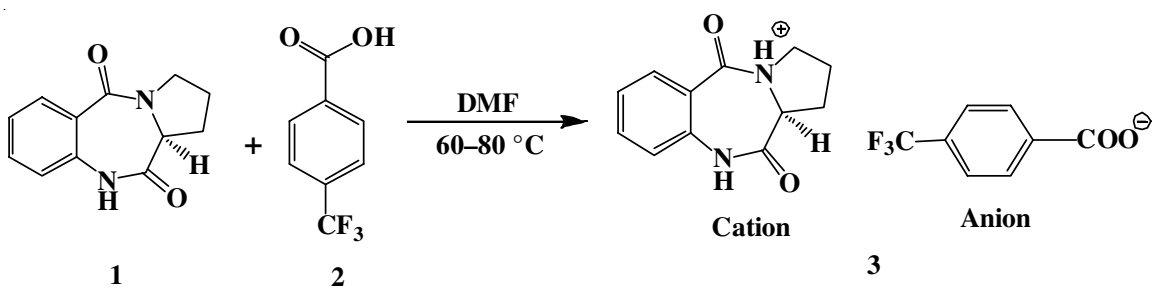
EXPERIMENTAL

Benzodiazepine (BDZ), carbamazepine (CBZ), topiramate (TPM) and 4-trifluoromethyl)benzoic acid used were generic 99 % pure and procured from Sigma-Aldrich (Steinheim, USA). Dimethylformamide (DMF) was purchased from Merck Scientific Inc. (Darmstadt, Germany) and DMF was used as an effective solvent. All the chemicals and solvents were used without further purification.

The IR spectrum of synthesized compound was recorded on Shimadzu 8400-S FT-IR spectrophotometer using KBr and absorptions are reported in cm^{-1}. The ^1H NMR spectra were recorded on 300 MHz (Bruker) spectrometer in appropriate solvents using tetramethylsilane (TMS) as an internal standard or the solvent signals as secondary standards and the chemical shifts are reported in δ values (ppm). ^{13}C NMR spectra were recorded on 75 MHz spectrometers. High resolution mass spectra were obtained by using ESI-QTOF mass spectrometry. To monitor the reactions, purity of the reactants and products was confirmed by analytical thin-layer chromatography (TLC) performed on silica gel GF_{254} pre-coated plates. After elution, plate was visualized under UV illumination at 254 nm for UV active materials. Melting points were measured in open capillaries on Nessler digital Auto melting point apparatus and are uncorrected.

General procedure for synthesis of ionic salt of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5,11 (10H, 11aH)-dione with 4-(trifluoromethyl)benzoic acid (3): (*S*)-(+)-2,3-Dihydro-1*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5, 11(10H,11aH)-dione (**1**) (0.4 g , 2 mmol) was dissolved in DMF at 60-80 ºC for 30 min. 4-(Trifluoromethyl)benzoic acid (**2**) (2.4 mmol) was added gradually to the above solution and stirred for 6 h. The completeness of reaction was confirmed by TLC. The mixture was cooled at ambient temperature and separated in aqueous phase medium. Solid precipitate thus formed was filtered, washed with water and dried to give an ionic salt of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo[2,1-*c*][1,4] benzodiazepine-5, 11(10H,11aH)-dione with 4-(trifluoromethyl)benzoic acid (**3**) in 85 % yield as pure white salt (**Scheme-I**). m.p.: 150-153 ºC; IR (KBr, ν_{max}, cm^{-1}): 3326, 3073, 2929, 2852, 2553, 2359, 1698, 1648, 1588, 1428, 1322, 1288, 1241, 1062, 941, 857, 761, 645, 539. ^1H NMR (300 MHz, DMSO-d_6): δ 10.65 (s, 1H), 8.10 (d, *J* = 31.1 Hz, 2H), 7.80 (d, *J* = 7.4 Hz, 2H), 7.63 (dd, *J* = 43.2, 7.4 Hz, 1H), 7.54 (t, *J* = 8.12 Hz, 1H), 7.23 (d, *J* = 8.30 Hz, 1H), 7.17 (d, *J* = 7.93 Hz, 1H), 4.13 (t, *J* = 6.0 Hz, 1H), 3.59 (dd, *J* = 4.34 Hz, *J* = 7.93 Hz, 1H), 3.46-3.57 (m, 2H), 1.82-1.94 (m, 2H), 1.22 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 166.26, 156.85, 153.26, 133.78, 132.71, 129.35, 126.90, 124.80, 124.47, 124.42, 124.37, 124.16, 121.20, 48.88, 47.50, 33.13, 30.83, 29.88, 25.23. HR-MS (ESI): calcd. *m/z* for positive ions: 217.2438 ($C_{12}H_{12}NO_2NH^+$), found: 217.1428; calcd. *m/z* for negative ions: 189.1114 ($C_8H_4OF_3O^-$), found: 189.0159.

***in vitro* 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay:** Cytotoxicity activity of carbamazepine, topiramate and benzodiazepine and newly synthesized compound **3** were studied by means of a colorimetric microculture assay (MTT) [24,25]. Cell viability assay was performed on breast adenocarcinoma cell line, MCF-7 on a 96-well microtitre plate (Corning, USA) at a cell density of 10^4 cells per well. Viability was measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase enzyme to insoluble coloured formazan crystals. The cells were cultured in Dulbecco′s modified Eagle′s medium (DMEM, Sigma, USA) supplemented with 10 % FBS and incubated in 37 ºC under 5 % of CO_2. After 24 h, a culture medium was changed and the cells were exposed to serial dilutions of antiepileptic drugs such as carbamazepine, topiramate and benzodiazepine and compound **3**. DMSO was used as the vehicle for drug treatment with appropriate controls. The desired concentrations of compounds/complexes were added to the wells with respective vehicle control. Cell viability was assessed after 48 h by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using XPERT MTT assay kit (Himedia, India). The cells were then incubated for 3 h with MTT solution (5 mg/mL). Then, the supernatant from each well was carefully removed and the formazan crystals formed as a product were then dissolved in 100 µL of DMSO and the absorbance at 540 nm wavelength was recorded in Robonic ELISA Plate Reader. IC_{50} (concentration of drug necessary to induce 50 % inhibition) was determined by comparing the absorbance value of treated cells with that in the control cells by probit analysis.



Scheme-I: Synthesis of ionic salt of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5,11(10H,11aH)-dione with 4-(trifluoromethyl) benzoic acid (**3**)

RESULTS AND DISCUSSION

Synthetic approach for the synthesis of ionic salt of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5,11-(10H,11aH)dione with 4-(trifluoromethyl)benzoic acid (**3**) is presented in **Scheme-I**. The newly synthesized compound **3** was fully characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, HRMS with electron spray ionization (ESI) analysis.

IR spectra exhibited N-H stretching vibrations at 3326 cm^{-1} and the three amide carbonyl C=O functional group stretching was observed at 1698, 1648 and 1588 cm^{-1}. A peak C-N stretch was seen at 1288 cm^{-1} and C-O at 1062 cm^{-1}. NMR spectra revealed a peak by singlet of secondary amide proton at 10.65 ppm. The aromatic protons showed peaks at 7.0-8.5 ppm as multiplet. Compound **3** was also characterized from ^{13}C NMR spectrum by the appearance of amide carbonyl carbons at δ 166.26, 156.85 and 153.26 ppm. In HRMS (ESI) with positive and negative ionization, MS spectrum peak appeared at *m/z* 217.1428 and 189.0159 as molecular ions $C_{12}H_{12}NO_2NH^+$ (cation) and $C_8H_4OF_3O^-$ (anion), respectively, confirmed that compound **3** as ionic salt of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5,11-(10H,11aH)dione with 4-(trifluoromethyl)benzoic acid.

Pharmacology: The toxicity evaluation of standard anti-epileptic drugs carbamazepine, topiramate, benzodiazepine and compound **3** was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [26] against MCF-7 (breast adenocarcinoma) cell line. The inhibitory activities (IC_{50}) are summarized in Table-1. As seen, compound **3** exhibited lower values with IC_{50} of 199 µM at a concentration lower than comparable with benzodiazepine, carbamazepine and topiramate, whose IC_{50} values were 722, 325 and 315 µM, respectively and considered as less toxic.

TABLE-1
MTT ASSAY INHIBITION ACTIVITIES (IC_{50}) IN µM

Compound	Breast adenocarcinoma cell line (MCF-7)
Carbamazepine (CBZ)	325
Topiramate (TPM)	315
Benzodiazepine (BDZ)	722
New compound (**3**)	199

Fig. 1 represents the dose response curves of carbamazepine, topiramate, benzodiazepine and compound **3** in breast adenocarcinoma cell line of MCF-7 cells. The toxicity of carbamazepine coincides with compound **3** up to 25 µg/mL. The toxicity of compound **3** was very low when compared with benzodiazepine, carbamazepine and topiramate at a concentration of 50 µg/mL (Fig. 1).

SAR analysis: Incorporation of high order fluorinated functional groups (CF_3) into compound **3**, induces a high electronegativity and hence increases the lipophilicity of compound and decrease metabolism, there by enhance the absorption of molecule. Generally, lipophilic drugs could pass through blood brain barrier (BBB) and reach to its receptors in the central nervous system (CNS) with relative ease [27]. Currently, clinically used anticonvulsant agents possess cyclic amide or acyclic amide or hetero atom present in the ring. Several investigations indicate that the presence of at least one aryl group, one or

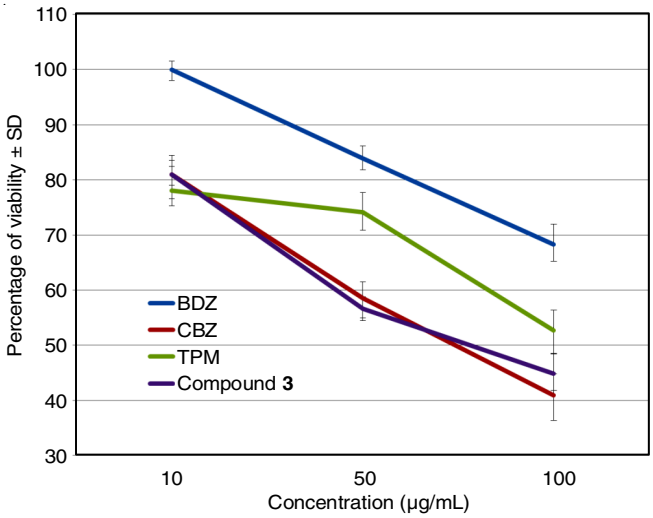


Fig. 1. Cytotoxicity effect of CBZ, TPM, BDZ and compound **3** on MCF-7 cell lines

two electron donor atoms and/or an NH group in a special spatial arrangement is necessary for anticonvulsant activity.

With regard to ionic salt of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5,11-(10H,11aH)dione with 4-(trifluoromethyl)benzoic acid (Fig. 2), it possesses two -NH functional groups, which are capable of improving anticonvulsant activities represented in zone 1 and zone 2. 4-Trifluoromethyl benzamide ring can be substituted with a variety of functional groups but fluorinated functional groups (zone 3) tend to give grater potency compared to other heteroatoms (like S, N, O) possessed by conventional anti-epileptic drugs.

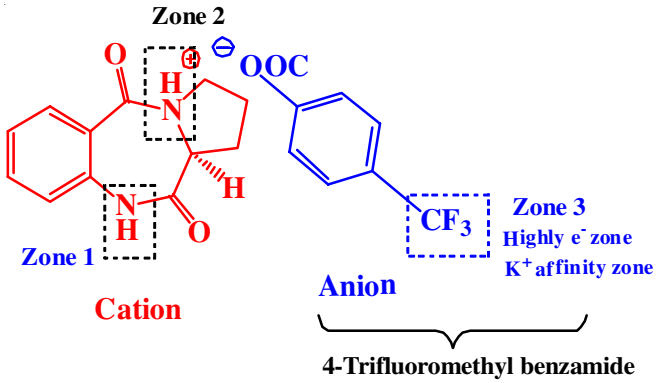


Fig. 2. Structure-activity-relationship of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5,11(10H,11aH)-dione with 4-(trifluoromethyl) benzoic acid with zones 1, 2 and 3

Fluorine has been commonly employed as a bioisosteric replacement for hydrogen atom due to its chemical inertness, ability to modulate pharmacokinetic parameters like pKa and lipophilicity; and increased metabolic stability relative to hydrogen [28]. Higher order fluorinated and bulky functional groups like trifluoromethyl (CF_3) and pentafluorothio (SF_5) groups have also been used.

Furthermore, compound **3** was investigated for anticonvulsant screening through the correlation of structure-activity relationships of clinically used conventional antiepileptic drugs drugs compares with number of carbon, hydrogen, oxygen, nitrogen and fluorine atoms present in the molecule (Table-2).

None of the conventional AEDs possess fluorine in their structure except retigabine and fluorofelbamate (Table-2). Fluorofelbamate seems to be highly toxic and produce severe side effects [29,30]. Retigabine possess only one fluorine atom with which it is capable of inhibiting the K^+ channel of blood brain barrier (BBB), which no other drugs are capable of retigabine with just one fluorine atom in its structure is responsible for first-in-class K^+ channel inhibitor for the treatment epilepsy [31,32]. Compound **3** that possesses three fluorine atoms is necessarily expected to inhibit K^+ channel with relative ease. Further, oxygen carrying capacity of the new drug should necessarily be much higher than that of retigabine. We presume that the presence of three fluorine atoms in our compound too is capable of inhibiting the K^+ channel.

The structure-activity relationship studies revealed that the introduction of fluoro- or trifluoromethyl substituents into the compound enhanced the anticonvulsant efficacy with less toxic and side effects. The lowest IC_{50} value (199 µM) was obtained with compound **3**, which appeared as the low toxicity against breast adenocarcinoma cell line of MCF-7 cells. The influence of fluoro- or trifluoromethyl substituents into the compound plays a key role in enhancing the efficacy of anticonvulsant activity [33]. It could be connected with unique properties of the fluorine such as high electronegativity, which together with significant steric effects of fluorine and trifluoromethyl groups may modulate electronic and steric parameters crucial for biological activity. Furthermore, fluorination always increases lipophilicity, which is fundamental physico-chemical property of compounds that plays a pivotal role in the transport of a molecule through cellular membranes and influences the localization of compound in the therapeutic site of action. As a result, combination of above factors yielded in compounds with increased anticonvulsant activity with decreases the toxic or side effects.

A thorough analysis of fluorinated benzene derivatives might reveal an electronic or steric requirement in the active site binding region of (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo-[2,1-*c*]-[1,4]benzodiazepine-5,11-(10H,11aH)dione with 4-(tri-fluoromethyl)benzoic acid. Given the location of the active site, (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5,11-(10H,11aH)dione with 4-(tri-fluoromethyl)benzoic acid likely does not alter the voltage sensing properties of channel instead stabilizing the open conformation of ion channel through the selective domains.

Analysis with other cyclic secondary amines (ionic salt): To make the new compound potentially valid and effective, Generic comparison has been made with two other compounds that possess cyclic secondary amines. One, we have synthesised an ionic salt of indole and 4-(trifluoromethyl)benzoic acid (TFMBA, Fig. 3). FTIR and ^1H NMR confirmed the formation of (secondary amine) ionic salt. Of course, some minor traces of impurities were reflected in the NMR.

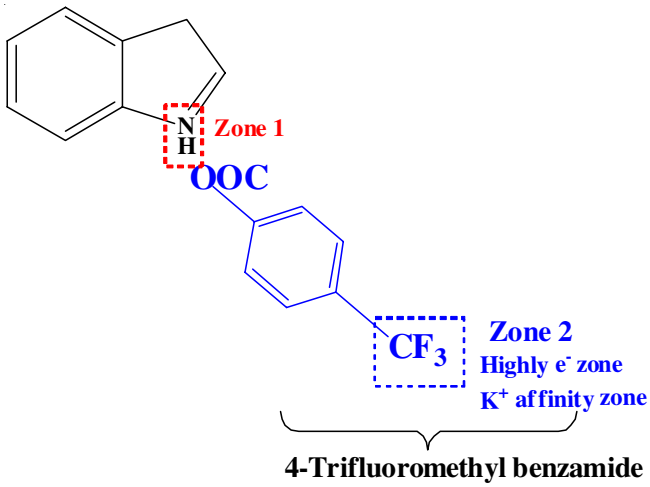


Fig. 3. Structure-activity-relationship of indole with 4-(trifluoromethyl)-benzoic acid with zones 1 and 2

As a second choice, we have assumed (not synthesized) ionic salt of retigabine + TFMBA. It could easily be conceived that a chance of formation of ionic salt (Fig. 4) was very high. Indole with 4-(trifluoromethyl)benzoic acid (Fig. 3) possesses -NH functional groups and fluorinated functional groups (represented in zone 1 and 2). In case of ionic salt of indole + TFMBA, NMR and FTIR studies confirmed the formation of a stable salt. The candidate molecule indole does not possess any antiepileptic activity, which is not conducive for the basics of this study. It may be a small molecule, may have a comfortable travel through transvascular route (TVR) and less toxic, but still will never support much needed antiepileptic activity. Zone 1 & Zone 2 may be active similar to the new drug. But non-antiepileptic activity of the candidate molecule indole over shadows all these advantages.

An assumed structure of retigabine with 4-(trifluoromethyl)-benzoic acid (Fig. 4) also possesses two -NH functional groups

TABLE-2
COMPARES THE NUMBER OF C, H, O, N AND F ATOM PRESENT IN CONVENTIONAL AEDs WITH THAT OF THE NEW DRUG. THEIR ACTIVITY IS ALSO BEING COMPARED

Compound	Atoms number					Activity
	C	H	O	N	F	
Carbamazepine ($C_{15}H_{12}N_2O$)	15	12	1	2	x	Na^+ channel inhibition
Topiramte ($C_{12}H_{21}NO_8S$)	12	21	8	1	x	Na^+ channel inhibition
Lamotrigine ($C_9H_7Cl_2N_5$)	9	7	x	5	x	Decreases glutamate releases
Benzodiazepine ($C_{12}H_{12}N_2O_2$)	12	12	2	2	x	Cl^- channel inhibition
Fluorofelbamate ($C_{11}H_{13}FN_2O_4$)	11	13	4	2	1	NMDA receptor antagonist and increase GABA availability
Retigabine ($C_{16}H_{18}FN_3O_2$)	16	18	2	3	1	K^+ Channel inhibition
Retigabine with 4-(trifluoro methyl)benzoic acid ($C_{24}H_{22}F_4N_3O_4$)	24	22	4	3	4	Anticipate K^+ channel inhibition
New compound (**3**) ($C_{20}H_{17}F_3N_2O_4$)	20	17	4	2	3	Anticipate K^+ channel inhibition
Indole with 4-(tri fluoro methyl)benzoic acid ($C_{16}H_{11}NO_2F_3$)	16	11	2	1	3	Anti-epileptic activity will not be there

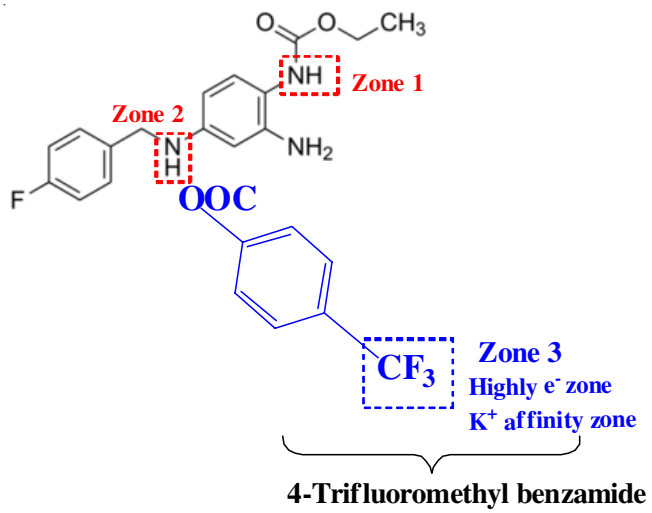


Fig. 4 Assumed structure-activity-relationship of retigabine with 4-(trifluoromethyl)benzoic acid with zones 1, 2 and 3

and fluorinated functional groups (represented in zone 1, 2 and 3). It leads to improved anticonvulsant activity and K^+ channel inhibition. But, structure is highly complex possessing 24 carbons & 22 hydrogen atom (hydrogen bonding) which is relatively huge in number (Table-2). Hence, toxicity of drug could steeply increase with the concentration. Further, drug may not be stable in structure, as per neuroanatomy studies, molecules with complex structure and high toxicity cannot easily diffusion through BBB and as well their travel through TVR will not be comfortable. This indeed is a considerable disadvantage over compound **3**.

Fluorinated ionic salt (compound **3**) which is the attached 4-(trifluoromethyl)benzoic acid and loosely bound indicating the non-complex structure, easy diffusion across BBB and comfortable travel through TVR itself gives potent cytotoxicity (Table-1).

Conclusion

In this present study, a new fluorinated ionic salt (*S*)-(+)-2,3-dihydro-1*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5,11-(10H,11aH)dione with 4-(tri-fluoromethyl)benzoic acid (**3**) was synthesized and evaluated for their *in vitro* cytotoxicity evaluation by MTT assay against breast adenocarcinoma cell line of MCF-7 cells. Compound **3** (IC_{50}:199 µM) emerged as minimal toxicity comparable to clinical drugs carbamazepine, topiramate and benzodiazepine. Compound **3** was also investigated for anticonvulsant screening through the correlation of structure-activity relationships of clinically used drugs, which indicated that compounds with fluoro- or trifluoromethyl moiety showed enhance anticonvulsant activities with less toxicity effects. Hence, it may be concluded that synthesized fluorinated ionic salt (**3**) holds a promise for the development as potential anticonvulsant agents after further optimization.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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