

Intravenous Anesthetic Diazepam Does Not Induce Amyloid- β Peptide Oligomerization but Diazepam Co-administered with Halothane Oligomerizes Amyloid- β Peptide: An NMR Study

Pravat K. Mandal^{a,*}, Virgil Simplaceanu^b and Vincenzo Fodale^c

^a*Neurospectroscopy and Neuroimaging Laboratory, National Brain Research Center, Manesar, Gurgaon, India*

^b*Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA*

^c*Department of Neurosciences, Psychiatric and Anesthesiological Sciences, University of Messina, Policlinico G. Martino, Messina, Italy*

Accepted 4 December 2009

Abstract. Amyloid- β peptide ($A\beta$) oligomerization has a profound role in Alzheimer's disease pathophysiology. Biophysical studies have shown that smaller sized inhaled anesthetics promote oligomerization by inducing perturbation of three critical amino acid residues (G29, A30, and I31) located in the helix-loop-helix domain of $A\beta$. In this present experimental study, using state-of-the-art nuclear magnetic resonance, we have monitored the influence of a larger sized intravenous anesthetic, diazepam, as well as diazepam co-administered with halothane, on $A\beta$. It was concluded that diazepam (in isolation) does not interact with the G29, A30, and I31 residues, and no $A\beta$ oligomerization occurs in the presence of 0.101 mM diazepam, even after 63 days. However, when diazepam was co-administered with halothane, profound $A\beta$ oligomerization is observed. These results strengthen the hypothesis that the presence of smaller molecular sized anesthetic is instrumental in promoting $A\beta$ oligomerization even when co-administered with a larger sized anesthetic, namely diazepam.

Keywords: Amyloid- β , amyloid- β oligomerization, aqueous halothane, diazepam, inhaled anesthetics, intravenous anesthetics, NMR

INTRODUCTION

Alzheimer's disease (AD) is a major neurodegenerative disorder affecting millions of people worldwide. The cause of AD is unknown but it has been hypothesized that oligomeric amyloid- β peptides ($A\beta$) play an important role in AD pathology. Recent findings

support the hypothesis that several drugs of anesthesia could be a risk factor [1], through involvement in the neurodegeneration process, by promoting "toxic" $A\beta$ oligomerization. Since oligomeric $A\beta$ derivatives play a key role in the development of AD, the impact of all chemical agents used in the peri-operative period should be examined [2]. In this context, we have undertaken a systematic biophysical study to investigate the effect of anesthetics on $A\beta$.

Using state-of-the-art nuclear magnetic resonance (NMR) techniques [3–5], we have previously demonstrated the molecular interactions of $A\beta$ with isoflurane,

*Correspondence to: Dr. Pravat K Mandal, Neurospectroscopy and Neuroimaging Laboratory, National Brain Research Centre, Manesar, Gurgaon, India. E-mail: Pravat.mandal@gmail.com.

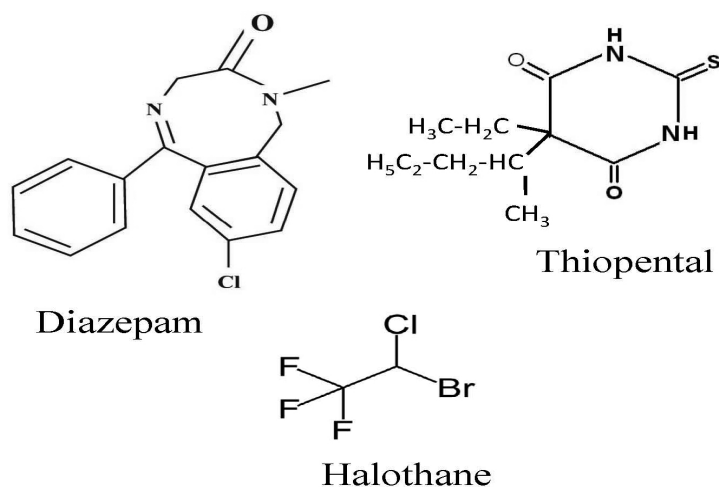


Fig. 1. Molecular structure of diazepam and halothane. The molecular volume of the intravenous anesthetic, diazepam, is much larger than that of the inhaled anesthetic, halothane. The chemical structure of thiopental is added for comparative illustration of molecular sizes.

desflurane, thiopental, and thiopental co-administered with halothane. Our studies lead us to the conclusion that the inhaled anesthetics, isoflurane and desflurane, at clinically relevant concentrations, interact with a specific region of $A\beta$ and induce $A\beta$ oligomerization [3]. Thiopental, an intravenous anesthetic, alone, does not oligomerize $A\beta$, however, when co-administered with halothane, $A\beta$ oligomerization was observed, leading to the conclusion that halothane causes $A\beta$ oligomerization and that the presence of thiopental does not prevent halothane-induced $A\beta$ oligomerization [5]. It was concluded that no $A\beta$ oligomerization was observed with thiopental due to its bulkier state. Hence, it is important to test whether molecular size of anesthetics has any role in $A\beta$ oligomerization [4] using another, larger sized anesthetic. To continue this systematic study of anesthetics, we chose the intravenous anesthetic, diazepam, (Fig. 1). We also extended our studies to investigate $A\beta$ interactions when two anesthetics, halothane (smaller sized anesthetic, 90–140 Å) and diazepam (larger sized anesthetic > 140 Å), are administered in combination.

The growing literature based on biophysical (*in vitro*), as well as animal model studies, indicates that certain anesthetics promote $A\beta$ oligomerization. In spite of the widespread use of diazepam in subjects with increased amounts of $A\beta$, there is no information available regarding molecular interaction of diazepam with $A\beta$. Hence, it is important to investigate $A\beta$ interactions with diazepam (alone) and co-administered with halothane. In doing so, we would like to accomplish three objectives:

1. To test if the widely used diazepam causes any $A\beta$ oligomerization;
2. To test if diazepam, co-administered with halothane, causes any $A\beta$ oligomerization;
3. To add yet another anesthetic study to test our hypothesis that the molecular size of anesthetic plays an important role in $A\beta$ oligomerization.

MATERIALS AND METHODS

To address the three, above mentioned objectives, NMR experiments were designed to investigate $A\beta$ interaction with diazepam and diazepam co-administered with halothane, in time-dependent studies.

Materials

^{15}N -labeled $A\beta_{40}$ (Recombinant Peptide Technologies, (Atlanta, GA, USA) Diazepam (MaynePharma), Halothane (Sigma), and Deuterated SDS D_{25} (Cambridge Isotope Laboratories) have been used in this study. NMR experiments were performed on a Bruker DRX 600 MHz spectrometer using a 5 mm triple resonance inverse probe with triple axis gradients.

Preparation of $A\beta$ peptide solution in SDS

$A\beta_{40}$ uniformly ^{15}N labeled lyophilized powder (0.2 mg) was added to SDS D_{25} solution and gently mixed. For our NMR studies, pH of the $A\beta$ solution was adjusted to 7.2 before addition of diazepam

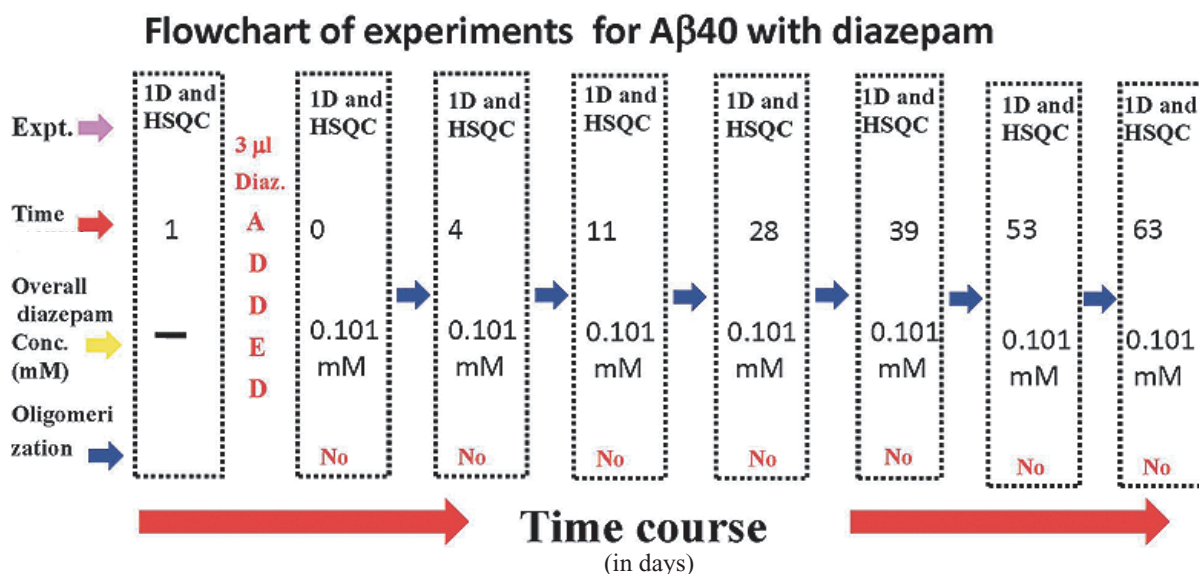


Fig. 2. Flowchart of the NMR experiment of diazepam with A β at seven time points. NMR experiments were followed for 63 days. Initially NMR experiments were performed on A β solution only, and NMR experiments followed after the addition of a 3 μ l diazepam solution.

(Fig. 1). In this study, A β interactions of one of the larger sized anesthetics, diazepam, have been investigated using NMR (Fig. 1). For comparison of molecular size, another anesthetic, thiopental, is also shown in Fig. 1.

The final volume of the A β solution for NMR studies was 500 μ l (control). After addition of a 3 μ l diazepam solution (concentration 10 mg/2 ml) to the A β solution, the final concentration of A β was 0.1 mM while the diazepam concentration was 0.101 mM and final volume of the A β solution was 503 μ l.

Preparation of aqueous solution of halothane

As described in our previous work, in order to generate a clinically relevant halothane concentration [6], 200 μ l neat anesthetic was added to 2000 μ l H₂O, shaken and allowed to equilibrate for less than 1 min; 75 μ l of the aqueous halothane solution was then added to freshly prepared 503 μ l A β + diazepam solution. The final A β concentration in this set was 0.2 mM, while diazepam concentration was 0.088 mM, and final volume of the A β solution was 578 μ l.

NMR experimental setup

A one dimensional ¹H NMR spectrum of A β ₄₀ was first recorded, followed by heteronuclear single quantum coherence (HSQC) ¹⁵N/¹H experiments [7]. To study A β -diazepam interaction, a 3 μ l solution of di-

azepam (neat) was added to the 5 mm tube containing 500 μ l A β ₄₀ solution. After diazepam + A β equilibrium was reached, one dimensional ¹H spectra were recorded, followed by HSQC (¹⁵N/¹H) experiments at several time points (up to a total of 63 days). In the presence of diazepam, no A β oligomerization [8] was observed up to 63 days. The experimental flowchart is presented in Fig. 2.

To study A β interactions with diazepam combined with halothane, a 3 μ l solution of diazepam (neat) was added to the 5 mm tube containing the 500 μ l A β ₄₀ solution. One dimensional ¹H and HSQC (¹⁵N/¹H) experiments were performed and then, a 75 μ l halothane solution was added. After halothane + diazepam-A β equilibrium was reached, one dimensional ¹H spectra and HSQC (¹⁵N/¹H) spectra were recorded at various time points.

NMR experiment details and data analysis

Both one dimensional proton (¹H) spectra and two dimensional HSQC (¹⁵N/¹H) experiments were performed at different time points. Details of procedure for the NMR studies are described in our previous work [5] and are not repeated here. NMR data were processed and analyzed using nmrPipe [9], PIPP [10], and SPARKY [11] software on an Octane2 Silicon Graphics computer. Assignments of the amide peaks of A β were taken from our earlier work [5] performed in a similar environment.

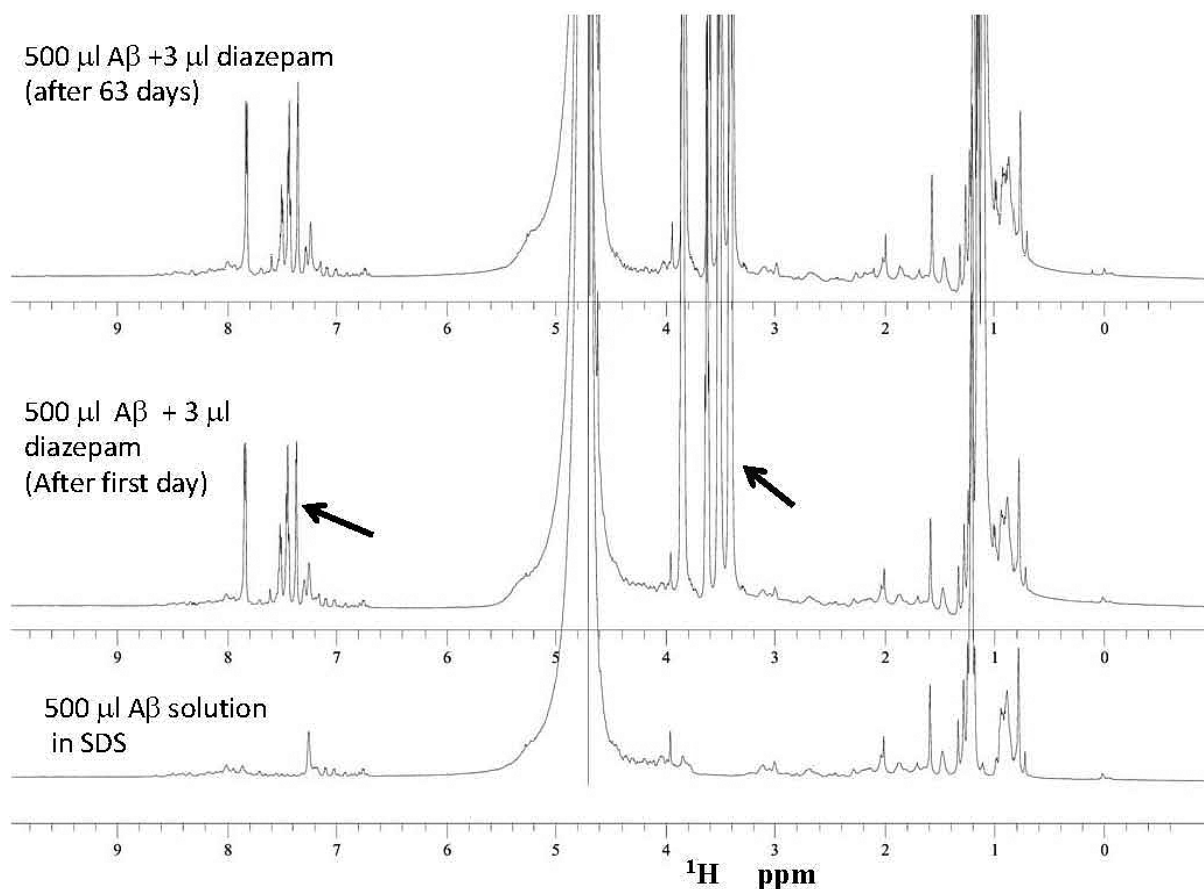


Fig. 3. 600 MHz NMR one dimensional ^1H spectra of $\text{A}\beta$ without diazepam and with diazepam at day one and after 63 days at room temperature. One dimensional spectra were collected at each time point before the HSQC spectra were recorded.

RESULTS AND DISCUSSIONS

A β interactions with diazepam

Figure 3 shows the one dimensional ^1H spectra of $\text{A}\beta$ and $\text{A}\beta$ with diazepam at different time points. The additional peaks in the one dimensional ^1H spectra are marked by arrows to indicate the presence of diazepam. Notably, the intensities and/or chemical shift of the ^1H peaks of diazepam remain unaltered. Figure 4 shows the overlay of $^{15}\text{N}/^1\text{H}$ HSQC spectra of $\text{A}\beta$ without diazepam (blue), and with diazepam at different time points: day one (green) and 63 days later (red). In the presence of diazepam, no chemical shift was observed for the crucial residues (G29, A30 and I31) involved in $\text{A}\beta$ oligomerization, based on other anesthetic studies with halothane, isoflurane and desflurane and propofol [3]. Even in the presence of 0.101 mM diazepam (the calculated cerebral concentration of intravenous anesthetics are approximately in the range of $0.100 \pm$

0.025 mM [5]), we did not find any $\text{A}\beta$ oligomerization after 63 days.

A β interactions with diazepam co-administered with halothane

Figure 5 shows the overlay of $^{15}\text{N}/^1\text{H}$ HSQC spectra of $\text{A}\beta$ with diazepam (blue), and $\text{A}\beta$ with diazepam and halothane after 15 hours (in red). In the presence of halothane co-administered with diazepam, a chemical shift change was observed for the crucial residues (G29, A30, and I31) involved in $\text{A}\beta$ oligomerization. There is no further chemical shift change of G29 over time. However, in the presence of halothane co-administered with diazepam, we observed $\text{A}\beta$ oligomerization after 15 hours at 27°C , as evidenced by the drastically decreased signal intensity. The G29 shifts in the presence of halothane, and in light of previous studies [3] with other anesthetics, when the G29 position shifted in a dose-dependent fashion as well as a function of the na-

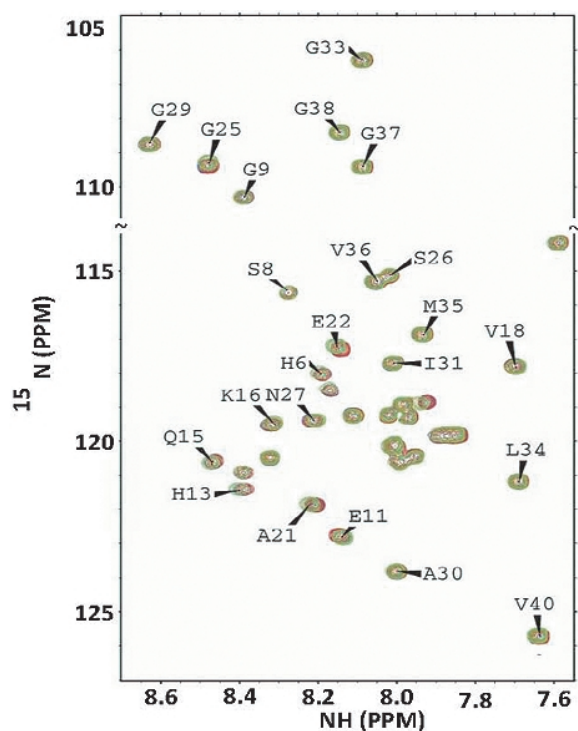


Fig. 4. HSQC spectra of $A\beta_{40}$ in the presence of 0.101 mM diazepam. The HSQC spectrum (control, $A\beta_{40}$ without diazepam) is shown in blue, and the HSQC spectra in the presence of diazepam after day one and day 63 are shown in green and red, respectively. It is important to note that G29, A30, and I31 do not show any chemical shift perturbation due to the addition of diazepam; no $A\beta$ oligomerization was observed.

ture of the anesthetic, the magnitude of the chemical shift correlates with the propensity of the anesthetic to induce oligomerization, as presently observed.

Importance of the chemical shift of G29 and I31

In the presence of diazepam, halothane induces chemical shifts of G29 and I31 signals over an 18–20 Hz range and $A\beta$ oligomerization was observed within 15 hours. In another study with isoflurane at clinically relevant concentrations, NH peak of G29 shows a 9 Hz chemical shift change and $A\beta$ oligomerization was observed within 9 days [4]. It appears that the higher the chemical shift change of G29 NH, the faster $A\beta$ oligomerization occurs and this chemical shift change can serve as a marker for $A\beta$ oligomerization propensity. In contrast, $A\beta$ did not oligomerize in the presence of diazepam alone. Similar observations were found in our earlier work where $A\beta$ did not oligomerize in the presence of thiopental, even at a higher concentration, and G29, A30, and I31 amide peaks did not show

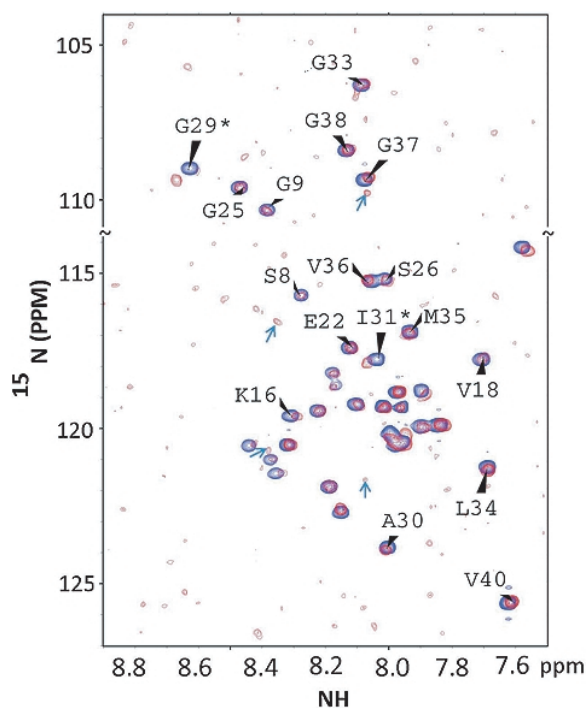


Fig. 5. HSQC spectra of $A\beta_{40}$ in the presence of 0.101 mM diazepam and halothane. It is important to note that G29 and I31 show chemical shift change due to presence of halothane co-administered with diazepam. 2D HSQC spectra were continually collected over time. The HSQC spectrum of $A\beta$ + diazepam is shown in blue, and the HSQC spectrum in the presence of diazepam + halothane after 15 hours, in red; $A\beta$ oligomerization was observed.

any chemical shift change in the presence of thiopental. However, in the presence of thiopental, other residues such as Q15 did show chemical shift changes, indicating interaction of thiopental with $A\beta$. In the present study, it was found that diazepam interacts with $A\beta$ in a similar manner to thiopental and no oligomerization of $A\beta$ is observed due to diazepam alone.

Model for diazepam-anesthetic interaction and role of anesthetic size for $A\beta$ oligomerization

The topological coexistence of anesthetics, as well as $A\beta$, facilitates interaction of $A\beta$ with anesthetics [5]. Figure 6 represents a plausible mechanism of diazepam- $A\beta$ interaction. $A\beta$ is generated by β - and γ -secretase on the amyloid- β protein precursor ($A\beta$ PP) in the normal process of aging and higher quantities of $A\beta$ are available in the aged brain. Thus, an excessive $A\beta$ load is available for anesthetic interaction in the elderly. Our earlier studies [3] have identified the crucial amino acid residues that are involved in the oligomerization process. Diazepam, as well as thiopen-

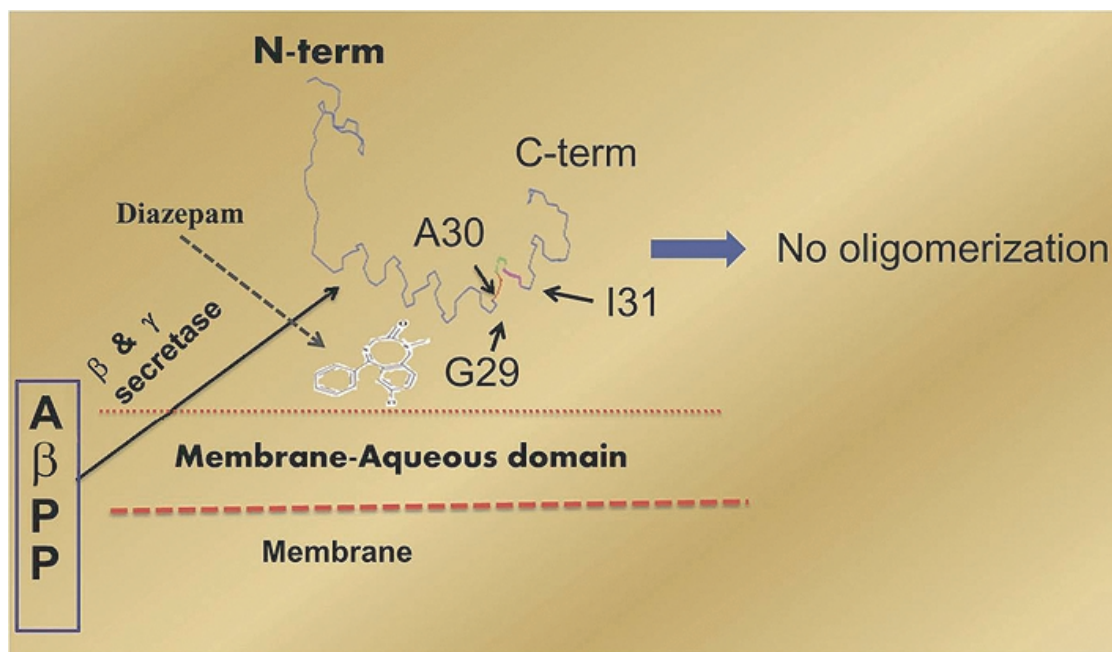


Fig. 6. Schematic presentation of the interaction between diazepam and A β . Due to its bulkier size, diazepam cannot access the critical residues G29, A30, and I31 located in the helix-loop-helix region. Hence, no A β oligomerization was observed. The Figure is adapted and modified from ref [5].

tal, due to its bulkier size, cannot be accommodated in the helix-loop-helix region and therefore no perturbation of the three residues was observed. We infer that no oligomerization of A β was found in the presence of diazepam (Fig. 4).

Clinical significance of this research

The main effects of benzodiazepines are sedation, hypnosis, decreased anxiety, anterograde amnesia, centrally mediated muscle relaxation, and anti-convulsing activity [12]. Elderly patients are anxious before and during surgery, and the tranquillizing properties of diazepam injected intravenously provides both premedication and conscious systemic sedation for regional anesthesia [13]. Secondly, diazepam is widely used for induction and maintenance of anesthesia [14], and intravenous anesthesia based on diazepam is a method of anesthesia in geriatric cancer patients [15]. Thirdly, diazepam is administered during diagnostic procedures in patients with AD [16]. Apart from subjects with neurodegenerative diseases, A β is naturally present in the brain, with elevated levels in the elderly.

Finally, in the intensive care unit (ICU), diazepam has been recommended for sedation [17], in organophosphate poisoning [12], and in first-line treat-

ment of convulsive status epilepticus, particularly for elderly individuals [18]. Larger quantities of A β are also reported in the majority of ICU populations.

Obviously, in human subjects with high concentrations of A β , who are scheduled for prolonged sedation or narcosis (= anesthesia), direct administration of anesthetics that affect the rate at which A β bind together could increase risk, as there is more A β available to oligomerize if an oligomerization-inducer drug is administered.

In general clinical practice, we should be aware that diazepam is a commonly used benzodiazepine [19], also in the aged population [20]: one in four of the elderly uses one or more benzodiazepines [19]. Moreover, in elderly subjects, intrinsic metabolic drug clearance of diazepam is impaired by up to about 20–60%, increasing both drug blood concentrations and effect duration [21].

In our research, oligomerization does not occur even after prolonged interaction of diazepam with A β , and therefore this anesthetic should be considered relatively safe with respect to A β PP metabolism. This notable absence of the tendency of diazepam to promote “toxic aggregation” of A β should be underlined, keeping in mind the impaired intrinsic metabolic drug clearance, which was pointed out in earlier work [21].

Could data from our experiment have relevance in research for the development of therapies against AD? As intravenous agents propofol and thiopental, and here also diazepam, due to their molecular size, cannot be accommodated in the loop region containing the key residues (G29, A30, and I31), no perturbation of these critical residues is seen and A β oligomerization is not observed. Understanding how and why certain anesthetics promote A β aggregation must be considered an important objective. Based on these results, pharmaceutical and biotechnology companies should be encouraged to synthesize and produce new anesthetic agents with large sized molecules to avoid A β oligomerization and increase patient safety. On the other hand, a systematic, well designed research on the role of G29, A30, and I31 A β critical residues could provide a new pharmacological strategy of intervention aimed at delaying A β oligomerization.

CONCLUSIONS

Ongoing biophysical studies [5,6] using state-of-the-art NMR spectroscopy, have demonstrated that the molecular size of the anesthetic is a significant contributing factor in inducing A β oligomerization. This has been validated by animal model studies using inhaled anesthetics. Our biophysical studies with diazepam and thiopental indicated that, in this experimental model, these intravenous anesthetics do not induce A β oligomerization. Future clinical studies using intravenous anesthetics (e.g., propofol, diazepam and thiopental) and intravenous anesthetics co-administered with halothane are warranted.

ACKNOWLEDGMENTS

Dr. Subbulakshmy Natarajan and Prof. Partha Raghunathan, National Brain Research Centre are appreciated for comments. Research support (to PKM) from National Brain Research Centre, Dept. of Biotechnology, Government of India is appreciated. The research is also partly supported by a grant from the Italian Ministry for University and Research, Program for the Development of Research of National Interest (PRIN Grant #2007H84XNH – Scientific coordinator: V. Fodale).

Authors' disclosures available online (<http://www.j-alz.com/disclosures/view.php?id=224>).

REFERENCES

- [1] Kuehn BM (2007) Anesthesia-Alzheimer disease link probed. *JAMA* **297**, 1760.
- [2] Kalman J, Palotas M, Pakaski M, Hügyecz M, Janka Z, Palotas A (2006) Unchanged rat brain amyloid precursor protein levels after exposure to benzodiazepines *in vivo*. *Eur J Anaesthesiol* **23**, 772-775.
- [3] Mandal PK, Fodale V (2009) Isoflurane and desflurane at clinically relevant concentrations induce amyloid beta-peptide oligomerization: an NMR study. *Biochem Biophys Res Commun* **379**, 716-720.
- [4] Mandal PK, Fodale V (2009) Smaller molecular-sized anesthetics oligomerize A β peptide simulating Alzheimer's disease: a relevant issue. *Eur J Anaesthesiol* **26**, 805-806.
- [5] Mandal PK, Pettegrew JW (2008) Abeta peptide interactions with isoflurane, propofol, thiopental and combined thiopental with halothane: A NMR study. *Biochim Biophys Acta* **1778**, 2633-2639.
- [6] Mandal PK, Pettegrew JW (2008) Clinically Relevant Concentration Determination of Inhaled Anesthetics (Halothane, Isoflurane, Sevoflurane, and Desflurane) by (19)F NMR. *Cell Biochem Biophys* **52**, 31-35.
- [7] Mandal PK, Majumdar A (2004) A comprehensive discussion of HSQC and HMQC pulse sequences. *Concepts Magn Resonance Part A* **20A**, 1-23.
- [8] Mandal PK, Pettegrew JW (2004) Alzheimer's disease: Soluble oligomeric A beta(1-40) peptide in membrane mimic environment from solution NMR and circular dichroism studies. *Neurochem Res* **29**, 2267-2272.
- [9] Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **6**, 277-293.
- [10] Garrett DS, Powers R, Gronenborn AM, Clore GM (1991) A common sense approach to peak picking two-, three- and four-dimensional spectra using automatic computer analysis of contour diagrams. *J Mag Reson* **95**, 214-220.
- [11] Goddard TD, Kneller DG (1994) SPARKY 3, University of California, San Francisco.
- [12] Peter JV, Moran JL, Pichamuthu K, Chacko B (2008) Adjuncts and alternatives to oxime therapy in organophosphate poisoning – is there evidence of benefit in human poisoning? A review. *Anaesth Intensive Care* **36**, 339-350.
- [13] Salonia A, Suardi N, Crescenti A, Colombo R, Rigatti P, Montorsi F (2006) General versus spinal anesthesia with different forms of sedation in patients undergoing radical retropubic prostatectomy: results of a prospective, randomized study. *Int J Urol* **13**, 1185-1190.
- [14] Olkkola KT, Ahonen J (2008) Midazolam and other benzodiazepines. *Handb Exp Pharmacol*, 335-360.
- [15] Author N (2009) Choice of components and a method of anesthesia in geriatric cancer patients at high cardiovascular risk. *Anesthesiol Reanimatol* **2**, 27-31.
- [16] Foster NL, VanDerSpek AF, Aldrich MS, Berent S, Hichwa RH, Sackellares JC, Gilman S, Agranoff BW (1987) The effect of diazepam sedation on cerebral glucose metabolism in Alzheimer's disease as measured using positron emission tomography. *J Cereb Blood Flow Metab* **7**, 415-420.
- [17] Liu LL, Gropper MA (2003) Postoperative analgesia and sedation in the adult intensive care unit: a guide to drug selection. *Drugs* **63**, 755-767.
- [18] Treiman DM (2007) Treatment of convulsive status epilepticus. *Int Rev Neurobiol* **81**, 273-285.

- [19] Johnell K, Fastbom J (2009) The use of benzodiazepines and related drugs amongst older people in Sweden: Associated factors and concomitant use of other psychotropics. *Int J Geriatr Psychiatry* **24**, 731-738.
- [20] Liu GG, Christensen DB (2002) The continuing challenge of inappropriate prescribing in the elderly: an update of the evidence. *J Am Pharm Assoc (Wash)* **42**, 847-857.
- [21] Butler JM, Begg EJ (2008) Free drug metabolic clearance in elderly people. *Clin Pharmacokinet* **47**, 297-321.