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Nilotinib treatment induces cognitive impairment by elevating hippocampal oxidative stress in rats

S.H. ALHARBI[1], A.H. ALHOWAIL[1], M.A. ALDUBAYAN[1], S.K. ALENEZI[2], M.J. ANWAR[2]

[1]Department of Pharmacology and Toxicology, College of Pharmacy, Qassim University, Qassim, Buraydah, Saudi Arabia
[2]Department of Pharmacology and Toxicology, Unaizah College of Pharmacy, Qassim University, Qassim, Unaizah, Saudi Arabia

Abstract. – OBJECTIVE: Protein tyrosine kinases (TKs) play a critical role in the regulation of various functions of a cell, including cellular proliferation, differentiation, and growth, and inhibitors of TKs have emerged as next-generation therapeutic agents in various types of cancer. Nilotinib, one of the TK inhibitors used to treat chronic myeloid leukemia, has been poorly investigated for its potential impact on memory function despite its ability to cross the blood-brain barrier (BBB). Thus, in this study, we investigated the effect of nilotinib on hippocampal-dependent cognitive functions and its potential mechanisms.

MATERIALS AND METHODS: Wistar albino male rats were divided into three groups of 10 each. The animals of group I (normal control) received drinking water only, while groups II and III were treated with nilotinib at doses of 15 mg/kg and 30 mg/kg, p.o. respectively, once daily for two weeks. The animals were subjected to behavioral tests after completion of drug treatment for the assessment of cognitive function using the Y-maze, novel object recognition (NOR) test, and elevated plus maze (EPM). The animals were euthanized after the estimation of blood glucose, and hippocampal tissues were dissected for the estimation of markers of oxidative stress.

RESULTS: Nilotinib produced impairment of memory function on the Y-maze, NOR test, and EPM. These results were also supported by a significant increase in glutathione (GSH), malondialdehyde (MDA), Akt, glycogen synthase kinase-3 beta (GSK3β), and total antioxidant capacity (TAC) in hippocampal tissue without altering the blood glucose level.

CONCLUSIONS: Nilotinib treatment produced significant impairment of cognitive function by inducing oxidative stress in the hippocampal tissue of rats.

Key Words:
Nilotinib, Y-maze, Novel object recognition, Elevated plus maze, Blood glucose, Oxidative stress, Antioxidants, Akt, GSK3β.

Introduction

Cancer patients frequently complain of a reduction in cognitive function and poor quality of life after beginning cancer therapy, as evidenced by a decrease in their work capacity and concentration ability in their daily lives. The development of protein kinase inhibitors (KIs) has been a breakthrough in targeted cancer therapy[1], due to their indispensable role in the cell signaling pathway through the phosphorylation of tyrosine residues of specific proteins[2], that regulate a variety of physiological functions[3]. However, the altered expression or function of protein kinases has not only been implicated in the pathogenesis of cancer, but also in several other non-oncological diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis[4-6]. The ubiquitous prevalence of tyrosine kinase throughout the brain, in particular the cerebellum and hippocampus, suggests that it is also involved in the regulation of neuronal function[7]. In addition, TKs also play a crucial role in the central nervous system[8,9]. Interestingly, protein tyrosine kinase has been reported in literature to play a key role in the induction of long-term potentiation, useful measures of neuronal function and integrity. Nonetheless, tyrosine kinase inhibitors have been reported[10] to inhibit the induction of long-term potentiation mechanisms of memory function regulation in the hippocampus. However, the use of TK inhibitors (TKI) during normal brain function can induce cognitive impairment[11,12]. In the present study, we investigated the ability of TK inhibitors to impair cognitive function and the potential mechanism.

Nilotinib is a TK inhibitor used to treat patients with chronic myeloid leukemia[13]. Regarding the mechanism of action, nilotinib inhibits

Corresponding Author: Ahmad Hamad Alhowail, Ph.D; e-mail: aalhowail@qu.edu.sa

the Philadelphia chromosome or Philadelphia translocation, a genetic abnormality in chromosome 22 of leukemia cells[14]. There are confounding reports[15-17] on the effect of nilotinib on memory function. Several lines of evidence[15,16] have shown that nilotinib affords protection against PD and AD, reducing neuronal loss in dopaminergic cells. Furthermore, nilotinib can improve the clearance of amyloid-beta in AD[17]. However, accumulating evidence suggests the contrary findings on memory function following treatment with TKIs. Multifaceted mechanisms have been proposed[11,18] to explain the TKI-induced memory impairment, including dysregulation of vascular endothelial growth factor receptor 2 (VEGFR2), affecting DNA demethylation patterns by alteration of specific genes[19], and increased reactive oxygen species in mitochondria[20]. In addition, nilotinib can also increase protein kinase B (PKB/Akt) activity, which increases the glucose transporter trafficking to the cell surface[21]. Moreover, Akt is considered a pro-survival pathway, and it can also inhibit GSK-3β, which enhances memory function[22]. Conversely, it has been reported[23] that nilotinib can induce cognitive impairment in patients with chronic myeloid leukemia; however, the mechanism underlying this cognitive impairment remains poorly clarified.

Oxidative stress occurs due to changes in the non-enzymatic and/or enzymatic mechanisms that counteract reactive oxygen species (ROS) overproduction[24,25]. This excessive ROS production can cause many neurotoxic effects, such as lipid peroxidation, mitochondrial dysfunction, enhanced inflammation[26], damage to the cellular structure, and apoptosis[27-29]. Several lines of evidence have illustrated[30,31] the association between oxidative stress and cognitive impairment. Further, it has been demonstrated[32,33] that increased lipid peroxidation and tissue MDA levels, depletion of reduced glutathione (GSH), and TAC contribute to the pathogenesis of nilotinib-induced memory dysfunction.

Despite the substantial literature on TKI-induced oxidative stress and cardiovascular events, it is plausible that TKIs with significant BBB permeability may also affect cognitive function. In addition, cancer patients with cognitive dysfunction have a sixfold higher mortality rate compared to those with normal memory functionality. Therefore, it is quite intriguing to investigate the effect of nilotinib treatment on memory function and on markers of cellular oxidative stress, which could enhance the current understanding of neurocognitive dysfunction and elucidate the role of oxidative stress in the nilotinib-induced decline of memory function in rodent models.

Materials and Methods

Animals and Treatment

Thirty Wistar albino male rats (aged 10-12 weeks; weighing, 200-250 g) were housed in polypropylene cages (n = 3 per cage) under a 12-h light/dark cycle and maintained at 25±2°C. Rats were allowed free access to food and drinking water. Nilotinib (procured from Novartis Pharmaceuticals, Wood Lane, London, UK) was dissolved in the drinking water and administered daily at low and high doses (15 and 30 mg/kg, respectively) through oral gavage once daily for two weeks[34]; the control group received only drinking water. Body weight was measured every two days, and mortality was recorded daily. Behavioral tests were performed during the light cycle.

Behavioral Paradigm

Y-maze test of short-term working memory

The wooden Y-maze (dimensions: 50 × 10 × 18 cm), comprised of three arms (painted brown for ease of visualization) separated by 120° intervals, was illuminated from above to ensure consistent light distribution. The test for short-term memory function was performed in two sessions comprising training and testing. During the training run, one arm of the Y-maze (novel arm) was blocked, and rats were allowed access to the other arms with different shape cues located at the end for 15 min. The test run was initiated 3 hours later, and exploration of all arms was allowed for 5 min. The number of entries and time spent in the novel arm were recorded[35].

Novel objective recognition (NOR) test of memory function

The NOR test apparatus comprised an open wooden box (40 × 40 × 40 cm) containing familiarization objects consisting of two white cups, and a black box of equal size as the novel object. During the 15-min training session, the rats explored the familiarization objects. After 3 h, the 5-min test session was conducted with one of the familiarization objects replaced by the novel object, and the time spent exploring the novel object was recorded[36].

Elevated plus maze (EPM) test of learning and memory processes

The EPM test apparatus consisted of pairs of opposing arms (open and closed; each 50 × 10 × 30 cm) with a central platform (10 cm^2). In the training session, the rat was placed at the end of an open arm and allowed to explore the apparatus for 10 min. After 3 hours, this process was repeated in the test session, and time spent in the closed arm and the transfer latency time (i.e., the time taken to move from the open arm to either of the closed arms) were recorded[37].

Biochemical Estimations

Blood glucose estimation

Glucose levels in tail vein blood samples were analyzed using an Accu-Chek glucometer (Roche Diabetes Care, Inc., Indiana, USA) according to the manufacturer's instructions.

Antioxidant content

The animals were euthanized, and the entire brain of each rat was carefully extracted and placed over an ice-cold Petri dish. Their hippocampi were immediately removed and homogenized in phosphate-buffered saline. Samples were homogenized individually using a glass Teflon homogenizer and then centrifuged at 14,000 × g for 5 minutes. The supernatant was collected into Eppendorf tubes (1.5 ml) and stored at -80°C for analysis of antioxidant content. The total antioxidant capacity (TAC), GSH, MDA, Akt, and GSK3β levels were analyzed using ELISA kits (Cloud-Clone Corp., Houston, TX, USA) according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean and analyzed using one-way analysis of variance (ANOVA). The data were subjected to Tukey's test to evaluate parameters, and statistical significance was set at $p \leq 0.05$. Survival data were assessed using Kaplan-Meier survival curves (n = 10 experiments) (Figure 1).

Results

The Effect of Nilotinib on Survival Rate

As shown in Figure 2, chronic treatment with nilotinib at doses 15 mg/kg and 30 mg/kg did not affect the survival rate as there was no significant change in percentage survival compared to control animals.

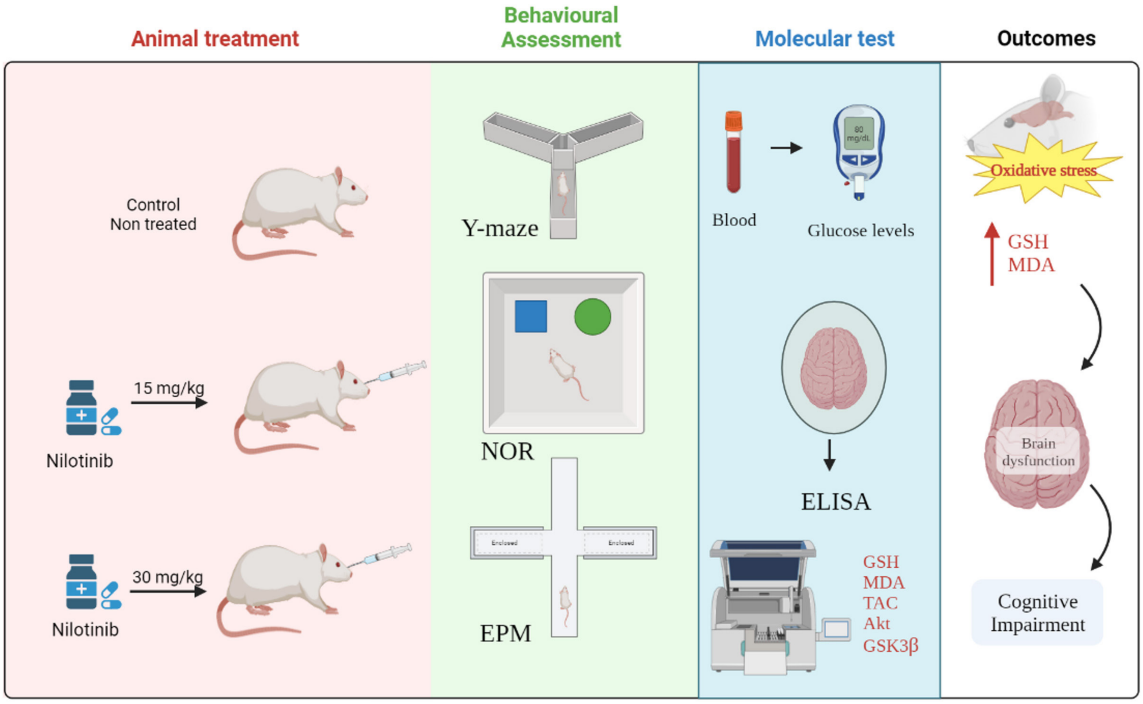


Figure 1. Conceptual diagram of the design and result of the study.

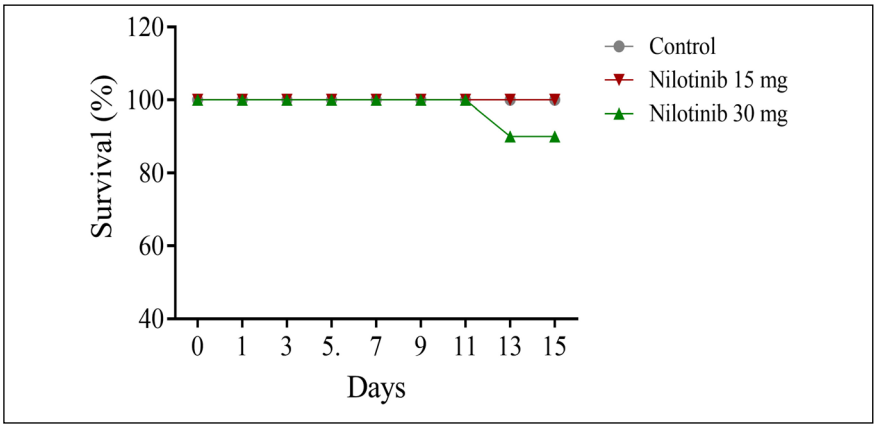


Figure 2. Effects of nilotinib (15 and 30 mg/kg, p.o.) for 2 weeks on the percentage survival of rats.

The Effect of Nilotinib on Body Weight

Chronic treatment with nilotinib at doses of 15 mg/kg and 30 mg/kg for two weeks did not produce a significant change in normalized body weight (Figure 3A). There were no significant differences in body weight of the nilotinib-treated (15 and 30 mg/kg) treated groups, as compared to control groups (Figure 3B).

The Effect of Nilotinib on the Behavior of Rats on the Y-maze Test

Figures 4A and 4B summarize the behavior of animals on the Y-maze test following chronic treatment with nilotinib at doses of 15 mg/kg and 30 mg/kg, respectively. There was a reduction in the number of entries into the novel arm (Figure 4A) and time spent on the novel arm (Figure 4B) as compared to the control group. Nilotinib at a higher dose (30 mg/kg) produced a greater reduction in the number of entries into the novel arm and time spent in the novel arm, though statistically insignificant if compared to the control.

The Effect of Nilotinib on the Behavior of Rats on Novel Object Recognition (NOR) Test

Administration of nilotinib (15 mg/kg and 30 mg/kg) for two weeks produced an alteration in memory function on the novel object recognition (NOR) test, evidenced by a dose-dependent reduction in time spent exploring the novel object ($p<0.05$ and $p<0.01$, respectively), as compared to the control group (Figure 5).

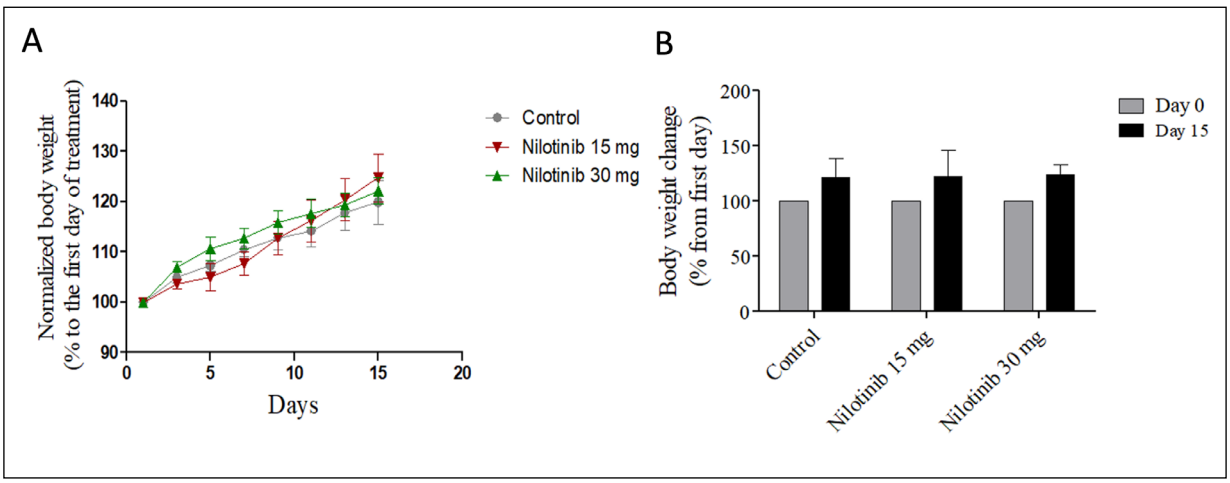


Figure 3. Effects of nilotinib treatment on rat body weight. **A**, Representative graph of the body weight from the first day of nilotinib treatment to the end of the study period. **B**, Representative graph of the change in body weight on the last day of nilotinib treatment compared with that on the first day. Data represent the mean ± standard error.

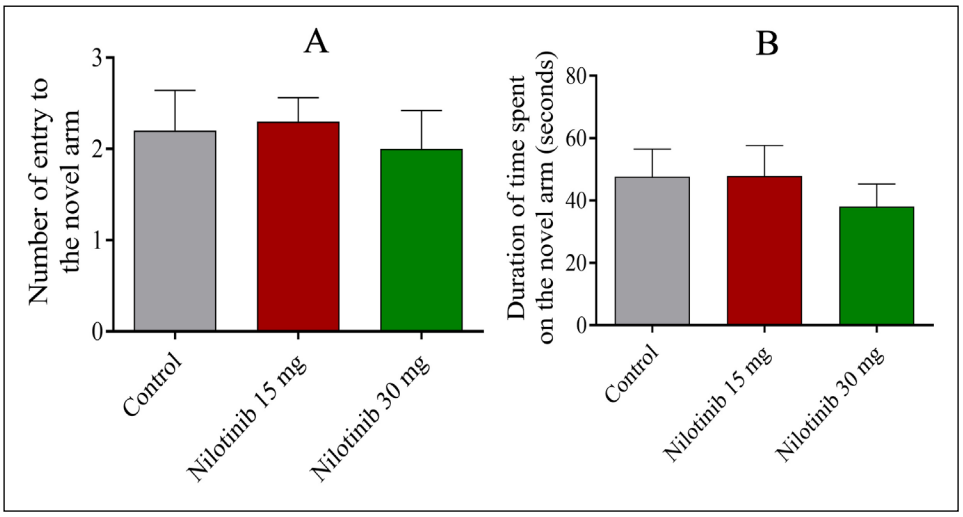


Figure 4. The effect of nilotinib on Y-maze parameters. **A**, Novel arm entry number. **B**, Total time spent in the novel arm. Data represent the mean ± standard error.

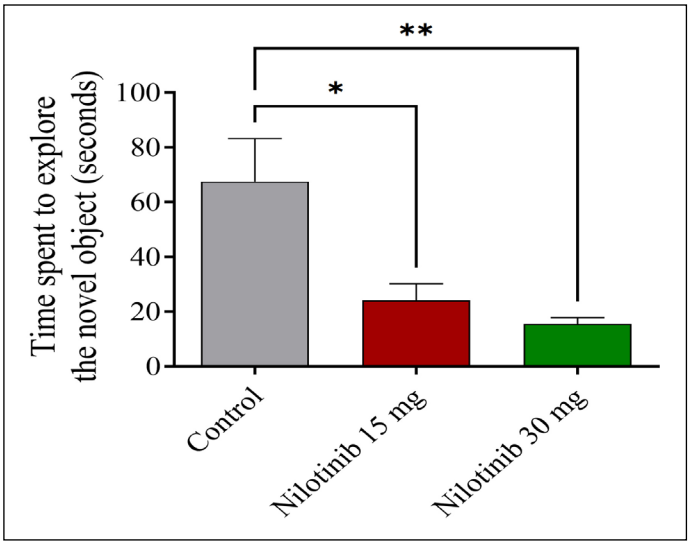


Figure 5. Effects of nilotinib on time spent to explore the novel object (seconds) of novel object recognition test. Data were represented as mean ± standard error of the mean. *p<0.05, **p<0.01 *vs.* control. Significant by one-way ANOVA followed by Tukey analysis (n = 10 rats per group).

The Effect of Nilotinib on the Behavior of Rats on Elevated Plus Maze (EPM) Test

In the EPM test of learning and memory processes, chronic treatment with nilotinib produced a dose-dependent increase in transfer latency on the elevated plus maze test in rats. Nilotinib at both doses (15 mg/kg and 30 mg/kg, p.o.) produced an increase in transfer latency, though the effect produced by the higher dose (30 mg/kg) was statistically significant (p<0.01), as compared to the control group (Figure 6).

The Effect of Nilotinib on Blood Glucose Levels

Figure 7 shows the effect of chronic nilotinib treatment on blood glucose levels in rats. There was an insignificant change in blood glucose levels following nilotinib (15 mg/kg and 30 mg/kg) treatment as compared to the control group. The blood glucose lowering effect of the higher dose (30 mg/kg) is bigger, though statistically insignificant, as compared to control animals.

The Effect of Nilotinib on Hippocampal Akt and GSK-3β Levels

Administration of nilotinib at doses of 15 mg/kg and 30 mg/kg for two weeks produces statistically insignificant changes in hippocampal Akt and GSK-3β levels, as compared to the control group (Figure 8A and B).

The Effect of Nilotinib on Hippocampal GSH, MDA, and TAC Levels

Figure 9 summarizes the effect of nilotinib on markers of oxidative stress in hippocampal

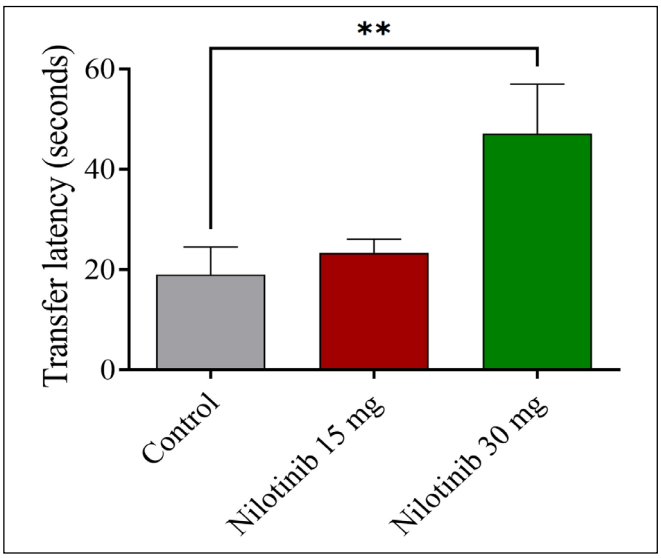
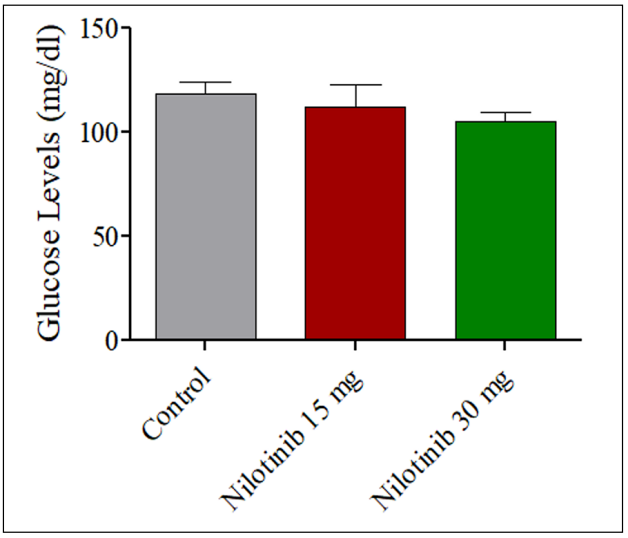


Figure 6. Effects of nilotinib on the transfer latency time in the elevated plus maze test. Data were represented as mean ± standard error of the mean. **$p<0.01$ *vs.* control. Significant by one-way ANOVA followed by Tukey analysis (n = 10 rats per group).

Figure 7. Effects of nilotinib on blood glucose levels in rats. Data represent mean ± standard error.



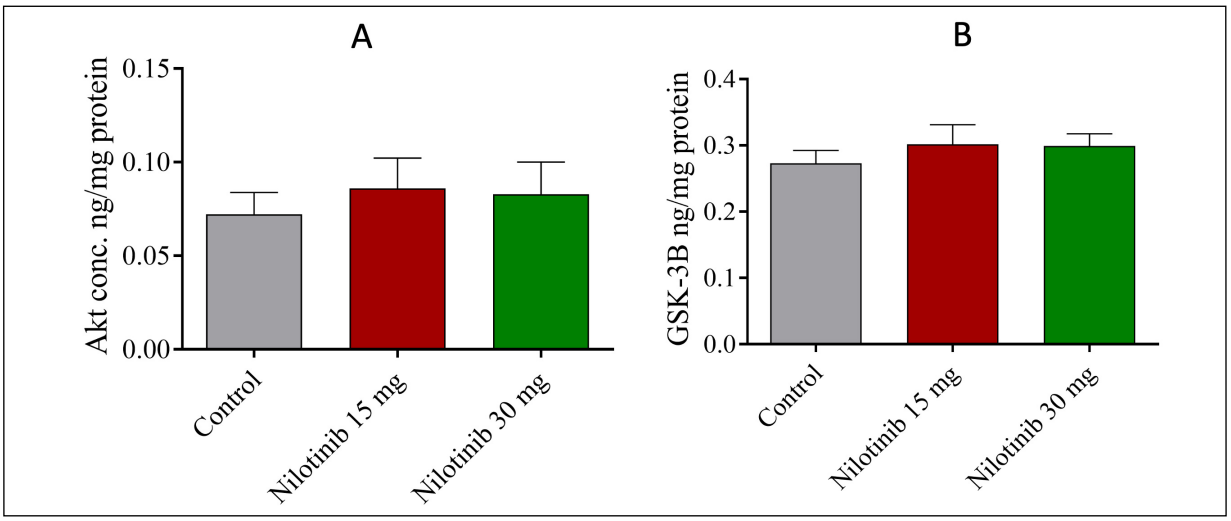


Figure 8. Effect of nilotinib treatments on the hippocampal expression of Akt (**A**) and GSK3β (**B**) in rats. Data represent mean ± standard error.

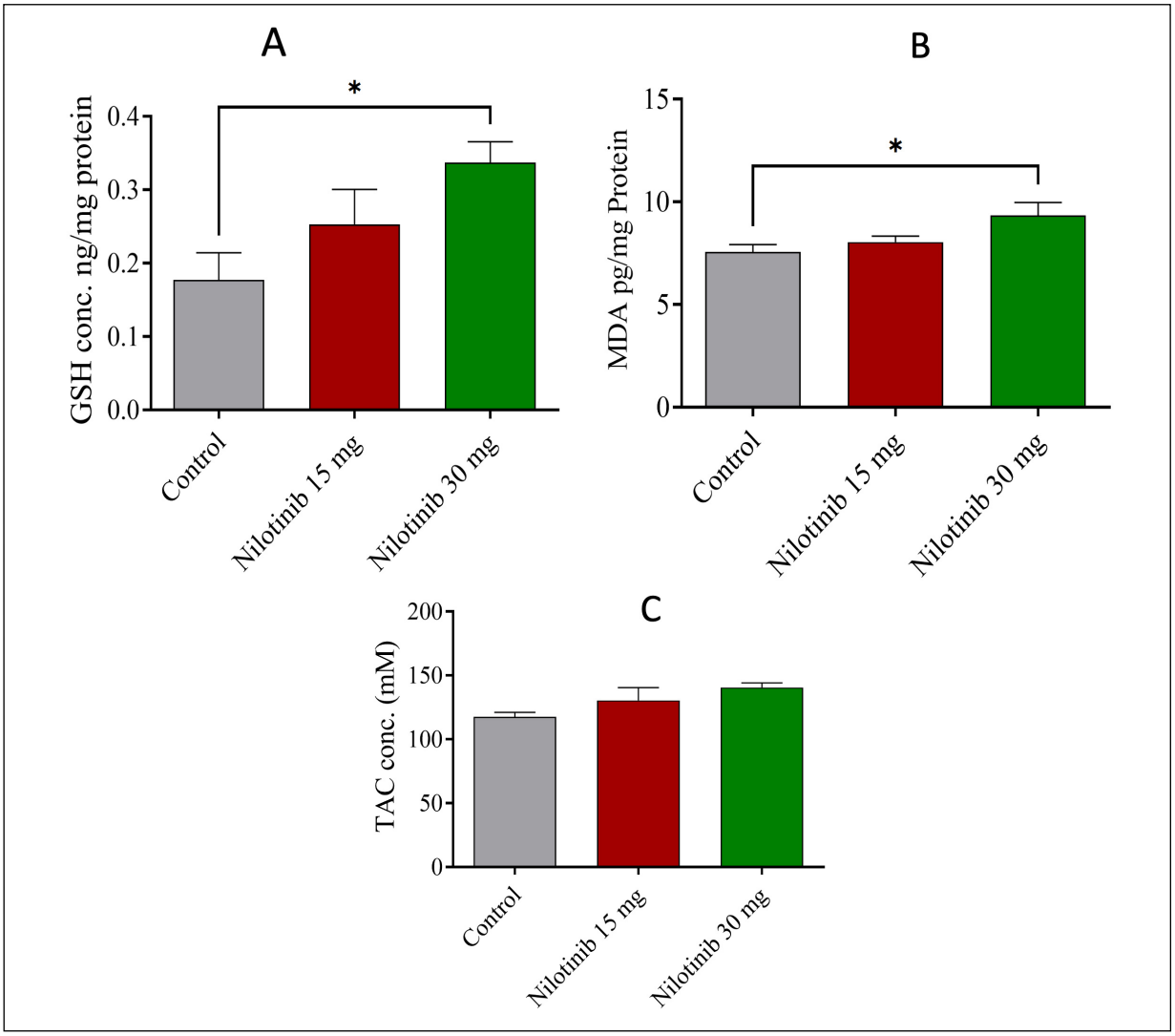


Figure 9. Effect of nilotinib on brains GSH (**A**), MDA (**B**) and TAC (**C**) levels in rats. Data represent mean ± standard error.

tissue. There was dose-dependent elevation of hippocampal GSH (Figure 9A), MDA (Figure 9B), and TAC (Figure 9C) levels, as compared to the control group. The hippocampal increase in GSH and MDA was statistically insignificant with the lower dose (15 mg/kg), whereas the higher dose (30 mg/kg) produced a significant (p<0.05) increase in GSH and MDA levels, as compared to the control group. However, the TAC levels in hippocampal tissue were also elevated in a dose-dependent manner following nilotinib treatment though statistically insignificant as compared to control.

Discussion

Clinical studies[23] have revealed that nilotinib can alter cognitive performance. Since TK is well known[38,39] for its crucial roles in regulating learning and memory processes, we postulated that nilotinib-mediated TK inhibition could induce cognitive impairment by over-activating the protein kinase B (PKB, or Akt) signaling pathway. To test this hypothesis, we employed the Y-maze, NOR test, and EPM tests to evaluate hippocampal-dependent tasks in nilotinib (15 mg/kg and 30 mg/kg) treated rats. Our results revealed that

daily treatment with nilotinib (15 mg/kg and 30 mg/kg, p.o.) for two weeks produced significant impairment of spatial memory function in rats. However, nilotinib has been reported[40-42] to alleviate memory dysfunction in AD and PD patients by preventing the degeneration of dopaminergic neurons. Contrary to this, there are some reports[23] of impaired memory function in patients receiving nilotinib treatment for leukemia. In the current study, we showed that chronic administration of nilotinib induced cognitive dysfunction using multiple behavioral tests, including Y-maze, NOR test, and EPM test in rats. The Y-maze paradigm is based on a hippocampal-dependent task and is usually used to assess spatial working memory in rodents[43,44]. Nilotinib (15 and 30 mg/kg) treatment produced an insignificant increase in the number of novel arm entries and time spent in the novel arm, compared with the control rats. Conversely, in the NOR tasks, nilotinib-treated rats spent less time exploring the novel object than the control ones. These findings indicate that nilotinib treatment resulted in the impairment of some cognitive functions. Furthermore, the memory impairment effect of nilotinib was dose-dependent, as there was a significant reduction in exploration time with 15 mg/kg followed by 30 mg/kg of nilotinib. Therefore, these data suggest that nilotinib treatment disrupts the brain regions to different degrees, causing impairment in memory function on the NOR test and no effect on Y-maze tasks. The transfer latency (TL) on an elevated plus maze is one of the most extensively used models for assessing rodent learning and memory processes[45]. Our result showed that rats treated with 30 mg/kg nilotinib exhibited a significant increase in transfer latency and freezing time during latency as compared to controls, whereas rats treated with 15 mg/kg nilotinib also exhibited a similar effect that was statistically insignificant. This finding indicates that nilotinib induces anxiety-like behavior, which is well known to be associated with cognitive impairment. Altogether, these findings suggest that nilotinib treatment can impair cognitive function to varying degrees, which is in agreement with studies[19] reporting impairment of learning and memory in rats following TKIs using Morris' water.

It is generally recognized that proper glucose metabolism is essential for the development and maintenance of learning and memory. The reduced blood glucose following nilotinib treatment could also contribute to reduced learning and memory, as observed in our study. This finding is in line with a previous study[46] that reported a reduction of dose-dependent reduction in blood glucose levels with nilotinib in rats. Although the blood glucose lowering by nilotinib in this study was statistically insignificant, it suggests that nilotinib potentially does impact the expression or trafficking of insulin receptors by activating the Akt pathway and the localization of glucose transporters[21].

Akt is downstream of several receptor-signaling pathways, such as insulin receptors, mediating cell survival[47]. Activation of Akt is involved in the trafficking of glucose transporters 1 and 4 to the cell surface, facilitating glucose uptake[48,49]. It plays a central role in learning and memory and synaptic plasticity *via* activation or inhibition of other protein kinases, such as GSK-3β[50], which regulates glycogen synthesis in response to insulin receptor activation. As a downstream regulator of the Akt pathway, a GSK-3β activity is inhibited by phosphorylation of Ser-9 residue mediated by active Akt.

Therefore, the assessment of hippocampal Akt and GSK-3β protein levels could decipher the plausible implication in increased oxidative stress and associated cognitive dysfunction following nilotinib treatment at doses of 15 mg/kg and 30 mg/kg once daily for two weeks in rats. The results revealed that glucose levels were slightly reduced in a dose-dependent manner in nilotinib-treated rats; however, it was not statistically significant. Similarly, our result showed that the expression of Akt and GSK-3β were not significantly altered following nilotinib treatment at either of the doses. Taken together, the cognitive impairment in nilotinib-treated rats can be attributed to oxidative stress. Our findings are in agreement with previous studies[51] that reported impairment of memory function through the alterations in insulin receptor signaling, Akt, and GSK-3β activities in hippocampal neurons. However, additional studies are required to investigate the molecular mechanisms.

Long-term oxidative stress, which can damage cellular function[52], is characterized by increased production of reactive oxygen species (ROS) and malondialdehyde (MDA), a marker of lipid peroxidation[24,53]. In response to the cellular oxidative stress, both enzymatic such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) and non-enzymatic, such as reduced glutathione (GSH) and metal-binding proteins (MBPs) are utilized by biological system as antioxidant defense mechanisms[54-56]. There is increasing evi-

dence[53,57,58] suggesting the involvement of oxidative stress in aging, neurodegenerative diseases, neurotoxicity, and neuronal dysfunction. Indeed, the brain's susceptibility to oxidative stress and its effects on metabolism and synaptic activities have been reported[58,59] in AD and PD. Studies[60] also suggest that oxidative stress alters or depletes endogenous antioxidant capacity. For instance, the level of GSH was reduced in neurodegenerative diseases, and it has been hypothesized in literature that increasing the levels of GSH was identified as a potential therapeutic target. In addition, MDA is one of the products that resulted from ROS oxidation, which is considered a biomarker of oxidative stress.

GSH is a tripeptide antioxidant that is composed of glutamate, cysteine, and glycine[61]. GSH is an antioxidant because of its capacity to neutralize oxidants by reacting with reactive oxygen species[62]. The current study result observed a consistent elevation of GSH in the brain of nilotinib-treated rats (30 mg/kg), which could represent a response of compensation for oxidative stress that caused poorer cognitive outcomes in nilotinib-treated rats. Similarly, the levels of MDA were significantly higher in nilotinib-treated rats (30 mg/kg), which showed oxidative stress occurred. This finding indicates that GSH activity was elevated as a response to neutralizing the increased levels of MDA and oxidative stress in nilotinib-treated rats.

In addition to the previous, total antioxidant capacity (TAC) was measured to investigate the effects of nilotinib treatment. TAC level is a commonly used biomarker for early detection of cellular oxidative stress induced by some drugs or diets[6,63]. The TAC level was not significantly altered, although a statistically insignificant increase in a dose-dependent manner was observed following nilotinib treatment. The resulting oxidative stress in hippocampal tissue following nilotinib treatment could also be at least partly contributed by modulation of the Akt/GSK-3β pathway. Intriguingly, our findings were also consistent with a previous study wherein inhibitors of GSK-3β have shown a protective effect against ischemic/reperfusion-induced cerebral damage in rats[64]. This resulted in an understanding that nilotinib treatment could potentially increase cellular oxidative stress. Our findings of cellular oxidative stress following nilotinib treatment are in line with previous studies[65] that reported increased oxidative stress-mediated apoptosis of leukemic cells.

In this study, we investigated the effects of nilotinib treatment on cognitive impairment using a dose that was equivalent to the one used to treat leukemia in patients. Furthermore, the results of the cognitive impairment are similar to those observed in cancer patients. In addition, the animal experiments were performed using rats of the same strain, age, and sex to minimize the effects of differences on the study outcomes. Moreover, the study was conducted in wild-type, cancer-free rats to evaluate the direct effects of nilotinib treatment and exclude possible interference by cancer effects.

Conclusions

The results of our study revealed that nilotinib treatment induced impairment in memory and learning. Although nilotinib treatment stimulated a slight decline in glucose levels, the precise mechanism underlying nilotinib-induced memory impairment remains to be comprehensively elucidated.

Authors' Contributions

Conceptualization, A.H.A.; methodology, A.H.A., S.K.A.; software, A.H.A.; validation, S.H.A., M.A.A. and S.K.A.; formal analysis, S.H.A.; investigation, S.H.A.; resources, A.H.A.; data curation, A.H.A.; writing—original draft preparation, S.H.A.; writing—review and editing, M.J.A.; visualization, S.H.A.; supervision, A.H.A.; project administration, A.H.A.

Conflicts of Interest

The authors declare that there is no conflicts of interest.

Funding

This research received no external funding.

Ethics Approval

The study was approved by the Institutional Animal Care and Use Committee in the Deanship for Scientific Research at Qassim University (under number 22-15-08).

Informed Consent

Not applicable.

Data Availability

Data are available upon request for reasonable requests.

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