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TREATMENT OF RATS WITH THE JAK-2 INHIBITOR FEDRATINIB DOES NOT LEAD TO EXPERIMENTAL WERNICKE'S ENCEPHALOPATHY

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Highlights

- Chronic treatment of rats with fedratinib (Fed) does not lead to neurological changes associated with development of thiamine deficiency (TD), the underlying cause of Wernicke's encephalopathy (WE).
- Treatment of rats with Fed does not alter the progress of TD in a well-established model of WE.
- Administration of Fed to rats does not inhibit activity of the thiamine-dependent enzyme transketolase in erythrocytes or decrease thiamine status.
- Treatment of cultured astrocytes with Fed does not decrease thiamine uptake into these cells.
- We conclude Fed does not directly cause TD and WE.

Abstract

Recent clinical trials suggest that patients with myelofibrosis can develop Wernicke's encephalopathy (WE) when treated with fedratinib, a specific Janus kinase-2 (JAK-2) inhibitor. To investigate this issue, we have examined 1) if fedratinib can produce or alter the course of this disorder, 2) its effects on thiamine-dependent enzyme activity and thiamine status, and 3) its influence on the uptake of thiamine. Animals administered fedratinib for 28 days at a comparable dose used to treat human cases of myelofibrosis showed no evidence of clinical signs of thiamine deficiency (TD). Rats treated with a combination of fedratinib and made thiamine-deficient exhibited no neurological differences in their progress to the symptomatic stage when compared to thiamine-

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deficient animals only. Treatment with the JAK-2 inhibitor did not compromise erythrocyte transketolase activity in the absence or presence of thiamine diphosphate, and thus thiamine status was not affected in a major way when compared to animals with TD. In addition, treatment of cultured astrocytes with fedratinib did not diminish the uptake of thiamine into these cells. Our findings suggest that treatment with fedratinib does not lead to or alter the progress of TD and thus do not support the notion that administration of this JAK-2 inhibitor directly results in the development of WE due to inhibition of thiamine transport. Known adverse effects of fedratinib involving compromised gastrointestinal function may be an important indirect contributing factor to previously reported cases of WE in patients with myelofibrosis.

Keywords: Vitamin B1, thiamine deficiency, myelofibrosis, astrocyte, neurodegeneration, excitotoxicity

1. Introduction

Thiamine deficiency (TD), the cause of Wernicke's encephalopathy (WE), results in histologic lesions in focal areas of the brain that include the thalamus, inferior colliculus, and brainstem structures such as the vestibular nuclei and inferior olives, and in which neuronal loss is an important feature [1]. Previous studies have demonstrated the involvement of an excitotoxic event in the cerebral pathophysiology of both TD and WE [2-5]. A key characteristic of TD is the inhibition of oxidative decarboxylation of pyruvate and α-ketoglutarate, leading to decreased ATP production in focal regions of the brain [6]. During the development of TD, both neuronal and astrocytic intermediary metabolism are impaired and protective astrocytic functions such as glutamate uptake

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are compromised [7]

Previous studies have identified an important beneficial role for the janus kinase 2 (JAK-2)-selective inhibitor fedratinib, also known as SAR302503 or TG101348 (N-tert-butyl-3-{5-methyl-2-[4-(2-pyrrolidin-1-yl-ethoxy)-phenylamino]-pyrimidin-4-ylamino}-benzenesulfonamide) in clinical trials involving its use in the treatment of myelofibrosis [8-10], a rare neoplasm characterized by the development of fibrosis within the bone marrow [11]. Notably, a small number of cases of WE have been identified in patients receiving fedratinib [10,12]. These findings have led to the discontinuation of clinical trials of fedratinib, despite the robust clinical benefit of this drug in patients with myelofibrosis.

In the present study we have perfomed a series of experiments designed to examine the potential of fedratinib to produce WE. *In vivo* studies were performed involving animals chronically treated with this drug in the equivalent dose range administered to patients with myelofibrosis in recent clinical trials. We assessed if fedratinib can produce TD or interfere with its progress in a well-established rodent model of the disorder [1], and also examined its influence on thiamine uptake in primary cultures of astrocytes.

2. Materials and methods

2.1. Materials

Pyrithiamine hydrobromide and dibutyryl cyclic AMP (dBcAMP) were purchased from Sigma-Aldrich (Oakville, ON). [^{3}H]-thiamine (50 Ci/mmol) was from American Radiolabeled Chemicals, Inc (St. Louis, MO). Fedratinib (TG101348) was purchased from ChemieTek (Indianapolis, IN). All other chemicals, unless otherwise stated, were purchased from GE Healthcare Life Sciences (Mississauga, ON).

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2.2. Rat model of TD

Male Sprague-Dawley rats (225 g) (Charles River, Montreal, QC)) were placed in five different groups: **A) TD group (n = 4).** Rats were fed a TD diet (Ralston Purina Inc., Richmond, IN) and injected daily with pyrithiamine hydrobromide (0.5 mg/kg body weight, i.p) as previously described [3]. **B) Fedratinib group (n = 6).** Rats were treated with fedratinib (2.27 mg per day, i.p.) for 28 consecutive days. **C) Fedratinib + TD group (n = 4).** Rats were treated with TD and fedratinib (2.27 mg per day, i.p.) in combination which lasted for the duration of TD, i.e. approximately 14 days. **D) Pair-fed control (PFC) group (n = 4).** Rats were placed on a TD diet limited in quantity to that consumed by their TD counterparts and injected daily with thiamine (100 μg in 0.2 ml saline, i.p.). Rats were placed on an identical diet to that of group A limited in quantity to that consumed by their TD counterparts and injected daily with thiamine (100 μg in 0.2 ml saline, i.p.). **E) Normal control group (n = 4).** Rats were allowed unrestricted access to the same thiamine containing diet as that fed to group D. Animals were assessed daily for symptoms of TD that include rotational and backward movements, ataxia, opisthotonus, nystagmus, and loss of righting reflex, and were sacrificed by decapitation at the appropriate time. All animal procedures were conducted in accordance with guidelines set out by the Canadian Council on Animal Care in Science and were approved by CIPA (Comité Institutionnel de Protection des Animaux) of the University of Montreal.

2.3. Erythrocyte transketolase activity and thiamine status

Transketolase activity in blood from rats (each group, n = 4) was measured using the technique of Massod et al [13] with modifications according to Hazell and colleagues [3]. Briefly, whole blood was collected into heparinized tubes from the heart at the time of sacrifice of the animal

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and frozen. Samples were later thawed, and refrozen and thawed two further times to ensure complete lysis and the hemolysed blood centrifuged at 3,000 rpm for 10 min. The hemolysate was then removed, taking care to avoid the intermediate buffy coat. Aliquots were then added to 50 mM potassium phosphate buffer (pH 7.6), and the mixture combined with ribose-5-phosphate (9 mM) then incubated for 30 min at 37°C. The reaction was stopped by the addition of TCA and the samples centrifuged at 2,500 rpm for 15 min. The supernatant was added to concentrated H_2SO_4 followed by boiling for 4 min. Addition of cysteine (3%) was followed by measurement of sedoheptulose-7-phosphate values 15 h later at 25°C from the difference in absorbance at 510 and 540 nm using a standard curve. Enzyme activity was measured as milliUnits per litre per min. For determination of thiamine status, measurement of the so called "TPP effect", or erythrocyte transketolase activity in the absence and presence of thiamine pyrophosphate (TPP) (9 mM) was performed. The percent change in enzyme activity when TPP was added to the assay system was used as an index of thiamine status as previously described [13].

2.4. Cell culture preparation

Primary cultures of astrocytes from newborn rats (Charles River, Montreal, QC) were prepared according to Hazell et al [14]. Cells were grown in 5% CO_2 /95% air at 37°C. At confluency, astrocytes were maintained in DMEM (Invitrogen, Burlington, ON) containing 10% horse serum and treated with 0.1 mM dBcAMP continuously thereafter. To induce TD, cells were exposed to a custom-designed DMEM media lacking in thiamine (Invitrogen, Burlington, ON) and containing 5% horse serum, in the presence of the thiamine antagonist pyrithiamine (10 μM) for 10 days as previously described [15]. Previous studies indicated that this concentration of horse serum in TD media was sufficient to provide levels of thiamine well below the K_m value for high-affinity

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thiamine uptake. Control astrocytes were treated with TD media in which normal levels of thiamine (4 mg/L) had been added. Cultures were grown for a total of 3-5 weeks, during which the media was changed twice a week. At least 95% of cells were determined to be astrocytes based on GFAP immunocytochemistry.

2.5. Thiamine uptake

Uptake of thiamine was performed in astrocyte cultures as previously described [16] with modifications. Cells were incubated in custom-designed DMEM lacking in thiamine, along with 0.4 μCi/ml of [^{3}H]-thiamine (8 nM final concentration, consistent with previously reported K_m values for high affinity thiamine uptake in neuroblastoma cells) [17] in 5% CO_2 /95% air at 37°C. All incubation media contained dimethyl sulfoxide (final concentration 0.1%). Uptake was examined over a period of 10 min and then stopped by aspiration of the media and rapid washing of cells three times with ice-cold PBS. Cells were then harvested in 0.5 ml of 1 M NaOH. Sample aliquots were measured for incorporated radioactivity by liquid scintillation counting and protein content determined according to Lowry and colleagues [18].

2.6. Statistical analysis

Statistical analysis was performed using either multiple t-tests, grouped analysis or one-way analysis of variance (ANOVA) with *post-hoc* testing for multiple comparisons. A probability of $p < 0.05$ was chosen to establish significance between groups. Data were analyzed using Prism 7.02 (GraphPad Software Inc., La Jolla, CA). All results are expressed as mean ± SEM.

3. Results

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3.1. Rats exposed to TD

Figure 1 shows the results of rats made thiamine-deficient or treated with fedratinib, separately or in combination, in terms of changes in body weight compared to control animals. Rats exposed to TD showed a decrease in weight gain starting at about day 5 of TD, which became more dramatic at day 10, at which time body weight started to decrease precipitously. Animals with TD also developed clinical signs that included the appearance of ataxia, opisthotonus, nystagmus and a loss of righting reflexes at day 13-14 of TD. Control rats pair-fed to their counterparts with TD also showed a parallel loss of weight compared to animals treated with TD though less profound in magnitude, and did not develop clinical signs of TD.

3.2. Ability of fedratinib to produce or alter the course of TD

Administration of fedratinib (2.27 mg per day, i.p.), approximately equivalent to a human dose of 500 mg daily, to rats for 28 days produced a reduction in weight during days 5-7 of treatment by about 30-50 g compared to normal control animals, and which was maintained for the remainder of drug treatment. However, there was no significant effect of fedratinib treatment on this control group ($p = 0.4247$). No overt neurological changes were observed in these animals during the 28 day period of treatment with fedratinib. Rats treated with the JAK-2 inhibitor in combination with TD did not alter progress of the disorder ($p = 0.6585$), with a similar loss of body weight and onset of neurological changes occurring at the same time as that encountered by animals with TD alone.

3.3. Effects on erythrocyte transketolase activity and thiamine status

Following treatment with fedratinib for 28 days, animals showed no change in blood transketolase activity compared to control values (42.4 ± 3.6 mU/L) (Figure 2). However, thiamine-

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deficient rats showed a 55% reduction in enzyme activity ($F_{2,11} = 25.68$, $p < .0001$). Determination of thiamine status in fedratinib-treated animals showed a 12% decrease based on enhanced transketolase activity due to TPP addition, representative of a low risk of TD and which was not significant (Figure 3) ($p > 0.05$); on the other hand, thiamine-deficient animals that displayed clinical signs of the disorder developed a 48% reduction in thiamine status, indicative of severe TD ($F_{2,11} = 10.08$, $p = 0.0033$).

3.4. Effect of fedratinib on thiamine uptake

In order to examine if fedratinib specifically inhibits thiamine transport, and in so doing might produce TD, we studied [^{3}H]-thiamine uptake into normal astrocytes and in cultures exposed to the drug for 12 hr in the absence or presence of TD. Figure 4 shows the effect of treatment of cells with fedratinib (17 μM, approximately equivalent to a human dose of 500 mg). Uptake of thiamine appeared to be unaffected by fedratinib or TD on its own compared to control values (0.8 pmol/min/mg protein). However a combination of fedratinib and TD significantly decreased thiamine uptake by 24% ($F_{3,20} = 6.491$, $p = 0.0030$).

4. Discussion

In recent clinical trials involving the chronic administration of fedratinib to patients with myelofibrosis, a small subset of these cases developed clinical signs consistent with the development of WE [12,19] An *in vitro* study has also suggested this drug may directly cause WE by inhibiting thiamine uptake into cells [20]. Our findings indicate that chronic treatment of rats with fedratinib at a dose approximating that administered in cases of myelofibrosis does not lead to neurologic changes consistent with the development of TD. In addition, fedratinib did not interfere with the progress of

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TD in a well-established model of experimental WE. Overall, our findings do not support the notion that this JAK-2 inhibitor directly produces WE.

In this study we examined the ability of fedratinib to reduce thiamine status in rats using the erythrocyte transketolase assay and TPP effect. Our results showed that following treatment with this JAK-2 inhibitor for 28 days (roughly equivalent to 3 years in humans), animals showed no decrease in transketolase activity such as would occur during the development of TD. Furthermore, assessment of thiamine status based on the response of transketolase to the addition of TPP indicated that while fedratinib mildly decreased thiamine status by 12%, this was considered from a clinical standpoint to be low risk and not statistically significant. On the other hand, thiamine-deficient animals developed a considerable reduction in thiamine status by approximately 50%.

During treatment with fedratinib, animal weight was found to be decreased by approximately 30-50 g in a consistent fashion over the 28 day course of treatment, starting at day 5 and maintaining this effect for the remainder of treatment. These results suggest the drug may interfere with food intake, e,g. by making the animals feel unwell and in so doing negatively influence the satiety centre of the brain, it may negatively influence gastrointestinal (GI) tract absorptive function, or may otherwise prevent the absorption of nutrients over time, e.g. by producing nausea or diarrhoea. Although rats are unable to vomit, evidence of diarrhoea was detected on a few occasions in animals treated with fedratinib. It is possible, therefore, that this JAK-2 inhibitor may have produced GI problems in the animals during treatment, and that administration of this drug for longer periods could lead to TD as a consequence of non-specific nutritional deficits induced as a result of the GI irritation. Indeed, use of fedratinib to treat patients with myelofibrosis is reported to produce adverse effects that include GI irritability when administered acutely [21], and diarrhoea, nausea and vomiting when treated chronically [9,10,12]. Patients treated with fedratinib were administered the

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drug using doses that were not dependent on body weight, increasing the risk of WE in those cases with e.g. smaller body mass and more elderly individuals in which the uptake of thiamine or the metabolism/elimination of fedratinib might be more readily compromised due to GI-tract dysfunction. It is also important to note that a major limitation of the present study is that the effect of fedratinib on thiamine integrity in myelofibrosis was not examined. Since loss of appetite and weight loss are typically observed in this disease, GI dysfunction due to treatment with this JAK-2 inhibitor may be more likely to lead to the development of TD in e.g. experimental myelofibrosis than in normal rats. Previous studies have established that compromised GI function such as occurs in hyperemesis gravidarum or following gastric surgery can lead to TD and WE [22-25]. In addition, other circumstances that reduce nutritional (and thus thiamine) intake also increase the risk of developing WE, as in human immunodeficiency virus/acquired immunodeficiency syndrome [26-28]. Thus it is possible fedratinib might indirectly lead to TD due to its negative influence on nutrition, resulting in WE in those few cases of myelofibrosis which may also have been nutritionally compromised towards development of this vitamin disorder.

In the present study, conditions were considered relatively optimal for the development of TD in animals treated with fedratinib. For example, rats were treated with an approximately equivalent dose of fedratinib to that experienced by patients with myelofibrosis. In addition, blood-brain barrier transport of fedratinib is considered relatively high (Zhang et al., 2014), thus ensuring a fairly high degree of entry of the drug into brain. Furthermore, intraperitoneal administration of this JAK-2 inhibitor would be expected to lead to higher levels of the drug than following oral intake. Despite these conditions, no animals treated with fedratinib developed TD during the month long treatment period.

Recently, fedratinib was reported to inhibit thiamine uptake in Caco-2 cells, suggesting the

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drug might lead to WE due to this direct effect [20]. However, this report provides limited insight into whether fedratinib is capable of producing WE since i) normal cultured cells were not examined, ii) an ability of the drug to produce TD in these abnormal cells was not studied, iii) no *in vivo* studies were performed that might suggest the drug was capable of producing WE, and iv) no explanation was forwarded as to why fedratinib did not produce WE in the vast majority of myelofibrosis patients chronically treated with the drug.

In order to examine if fedratinib specifically inhibits thiamine transport, and in so doing might produce TD, we studied thiamine uptake into primary cultures of astrocytes in the absence or presence of TD and following exposure to this JAK-2 inhibitor. Previous studies have shown that K_m values for thiamine transport into cells vary considerably, ranging from the low nanomolar to low millimolar range, depending on cell type and external thiamine concentration [17,29-31]. A fedratinib concentration of 17 μM was chosen for use which is roughly comparable to that administered daily to a 50 kg individual treated with 500 mg of the drug, i.e. similar to the myelofibrosis patients that developed WE when treated with this drug. Previous studies have identified astrocytes to be a specfic cell type targeted in TD in histologically vulnerable areas of the brain [32]. The present findings indicate that uptake of thiamine is unaffected by treatment with fedratinib or TD on its own. Although it may seem surprising that cells made thiamine-deficient did not show a decrease in thiamine uptake, it is important to note these cells were made metabolically deficient by prior 10 day treatment with pyrithiamine and an absence of thiamine in the media using an *in vitro* astrocyte model of TD developed in our laboratory [15]. However, these treatment conditions were not present during incubation with fedratinib, i.e. pyrithiamine was not included, which would otherwise have artificially inhibited the uptake of thiamine by competing with the vitamin at the transporter binding site. Nevertheless, the cells remained metabolically impaired due

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to TD during these experiments because of the absence of thiamine in the media. On the other hand, a combination of fedratinib and TD produced a decrease in thiamine uptake. This may reflect an effect of this JAK-2 inhihitor on the functional integrity of these cells. Although acute exposure of astrocytes to fedratinib produced some microscopic evidence of toxicity in the 0-20 μM range, measurements of mitochondrial inner membrane potential (Δψm) in astrocytes treated with fedratinib using the membrane potential-dependent aggregate-forming lipophilic cation JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanide iodide) and flow cytometry suggest that short-term (1 hr) exposure to this JAK-2 inhibitor does not lead to depolarization of Δψm (unpublished observations). This is consistent with the notion that treatment with this drug in the low micromolar concentration range does not produce significant toxicity in these cells under acute conditions. Further studies are necessary to determine the underlying basis of this combined effect of fedratinib and TD.

In conclusion, results of the present study do not support the notion that chronic treatment of patients with fedratinib specifically targets thiamine uptake, directly leading to the development of WE.

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FIGURE LEGENDS

Figure 1. Weight of animals during exposure to the JAK-2 inhibitor fedratinib (Fed). After 28 days of treatment with the drug, no neurological evidence of TD was observed. In addition, the drug does not interfere with developing TD, in particular it does not accelerate progress of the illness. Fedratinib, however, was able to reduce the weight of the animals by about 30-50 g, starting at about day 5 of treatment and maintaining them at this smaller size for the rest of the duration of treatment.

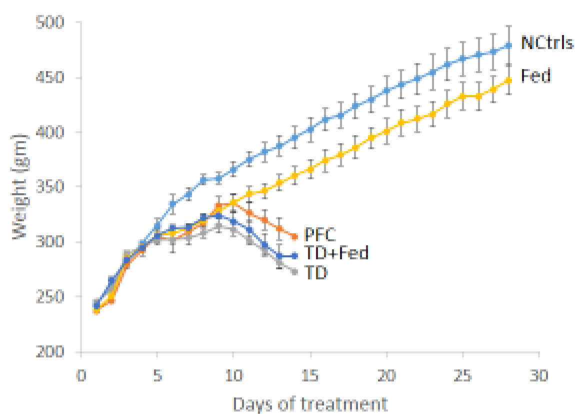

Figure 2. Erythrocyte transketolase assay. Treatment with the JAK-2 inhibitor fedratinib (Fed) showed no decrease in red cell transketolase activity, unlike animals with TD in which enzyme

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activity was greatly diminished. Data are mean ± SEM from different rats (each group, n = 4 or 6) performed in duplicate (*p < 0.05, one-way ANOVA with *post-hoc* Tukey test).

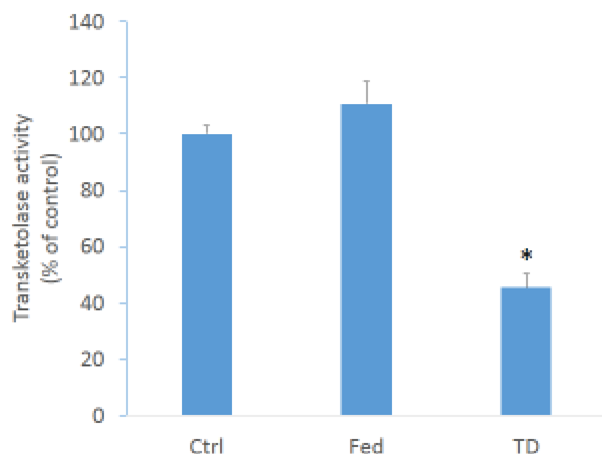

Figure 3. Thiamine status. While fedratinib mildly decreased thiamine status, this was considered to be low risk relative to normal rats and not significant. On the other hand, animals with TD developed a considerable reduction in thiamine status. Data are mean ± SEM from different rats (each group, n = 4 or 6), performed in duplicate. (*p < 0.05, one-way ANOVA with *post-hoc* Tukey test).

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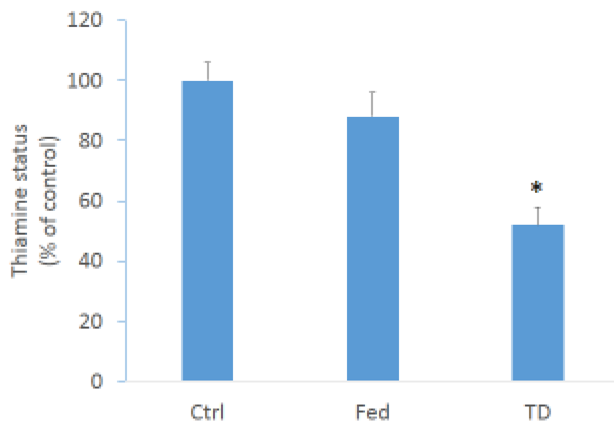

Figure 4. [^{3}H]-Thiamine uptake in cultured astrocytes. Uptake of thiamine was unaffected by treatment with fedratinib or TD on its own. However, a combination of fedratinib and TD produced a significant decrease in thiamine uptake compared with control values. Data are mean ± SEM of two separate experiments, each performed in triplicate. (*$p < 0.05$, one-way ANOVA with *post-hoc* Dunnett's test).

Thiamine uptake
(% of control)
120
100
80
60
40
20
0
*
Ctrl
Fed
TD
TD+Fed