

7-formyl-10-methylisoellipticine, a novel ellipticine derivative, induces mitochondrial reactive oxygen species (ROS) and shows anti-leukaemic activity in mice

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Summary Acute myeloid leukaemia (AML) is the most common type of leukaemia in adults and is associated with high relapse rates. Current treatment options have made significant progress but the 5 year survival for AML remains low and therefore, there is an urgent need to develop novel therapeutics. Ellipticines, a class of cancer chemotherapeutic agents, have had limited success clinically due to low solubility and toxic side effects. Isoellipticines, novel isomers of ellipticine, have been designed to overcome these limitations. One particular isoellipticine, 7-formyl-10-methylisoellipticine, has previously showed strong ability to inhibit the growth of leukaemia cell lines. In this study the anti-leukaemia effect of this compound was investigated in detail on an AML cell line, MV4-11. Over a period of 24 h 7-formyl-10-methyl isoellipticine at a concentration of 5 μ M can kill up to 40 % of MV4-11 cells. Our research suggests that the cytotoxicity of 7-formyl-10-methylisoellipticine is partially mediated by an induction of mitochondrial reactive

oxygen species (ROS). Furthermore, 7-formyl-10-methylisoellipticine demonstrated promising anti-tumour activity in an AML xenograft mouse model without causing toxicity, implying the potential of isoellipticines as novel chemotherapeutic agents in the treatment of leukaemia.

Keywords Isoellipticine · 7-formyl-10-methylisoellipticine · Leukaemia · AML

Introduction

Acute myeloid leukaemia (AML) is characterised by an accumulation of myeloblasts in the bone marrow with a reduced capacity to differentiate into mature myeloid cells, disrupting production of normal functional blood cells [1–3]. Following the standard induction chemotherapy with cytarabine and daunorubicin complete response (CR) is achieved in 60–80 % of younger adults and cure rates are in the range of 5–60 %; however, CR rates are much less in the elderly age group even with intensive chemotherapies [4]. This is particularly concerning as AML accounts for 80 % of acute leukaemias in the adult population [5]. In addition, relapse rates of AML patients are high as only 40 % of patients younger than 60 years and 10–20 % of older patients remain in remission 5 years after diagnosis [6]. Clearly the success of conventional chemotherapy has been limited and as such novel therapeutics are urgently needed to treat this disease.

Although ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole) is known to be highly efficient against a range of cancer cell types [7] the clinical application of this agent is underdeveloped due to low solubility and toxic effects. As a result, ellipticine derivatives, isoellipticines have been designed with the intention of overcoming the limitations. Despite the historic validity of ellipticine derivatives in solid

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tumour models [8, 9], it was leukaemia cell lines in which isoellipticines demonstrated the greatest level of inhibition against in a NCI 60-cell line screen [10].

Ellipticines function by a number of mechanisms such as DNA intercalation, bio-oxidation and adduct formation [11–13]. Emerging results show that ellipticines are capable of producing mitochondrial damage [14], inducing endoplasmic reticulum stress [15] and inhibiting kinases such as c-Kit and AKT [16, 17]; however, ellipticine-induced inhibition of DNA topoisomerase II activity is still the most documented mechanism of action. It is therefore curious to note that topoisomerase II appears not the most important biological target with respect to anti-cancer activity of isoellipticines [10]. Thus, it is likely that this new class of compounds function by a unique mechanism of action, which has yet been fully explained.

Reactive oxygen species (ROS) refers to all highly reactive oxygen derivatives. ROS levels in malignant cells are frequently higher than in their normal cellular counterparts. It is therefore possible that ROS production by malignant cells may represent a potential therapeutic target. There are two possible approaches to manipulating ROS in malignant cells therapeutically. The first approach to targeting ROS in cancer is to use antioxidant molecules suppressing the high levels of ROS observed in some cancer cells [18]. Unfortunately, this approach has a number of limitations. Drummond et al. suggest that the reaction of the targeted ROS with an antioxidant may produce another type of ROS that does not react with the original antioxidant [19]. The second approach is a pro-oxidant approach. By increasing oxidant levels this treatment aims to cause catastrophic chemical damage only in those cells where oxidative stress was preexistent [18].

We previously reported that the isoellipticine derivative, 7-hydroxyisoellipticine induces apoptotic cell death by increasing ROS [20]. However, the origin of this ROS was unknown. In this study, we demonstrate that a more potent ellipticine derivative, 7-formyl-10-methylisoellipticine can induce mitochondrial ROS and cytotoxicity in AML cells and significantly slow down tumour growth in an AML xenograft mouse model without any evidence of systemic toxicity.

Materials and methods

7-formyl-10-methylisoellipticine synthesis

Isoellipticine was synthesised from indole using a synthesis developed by Gribble et al. [21]. 7-Formyl-10-methylisoellipticine was synthesised from isoellipticine in two steps *via* an aldehyde intermediate as described by Miller et al. [10]. The compound was of >95 % purity as assessed by high performance liquid chromatography–mass spectrometry.

Cell culture

The human AML cell line MV4-11 was purchased from DSMZ (Braunschweig, Germany) and was maintained in RPMI 1640 medium supplemented with 10 % foetal calf serum (#16000-044 Gibco), 2 mM L-glutamine and 1 % penicillin/streptomycin (both from Sigma Aldrich, Dublin, Ireland) in a humidified incubator at 37 °C with 5 % CO₂.

Analysis of cell number and cell viability

Cells were incubated in 2 ml of RPMI 1640 medium in six-well plates at 37 °C for the indicated times with 1, 2.5, 5, 10, 20 or 50 µM 7-formyl-10-methylisoellipticine, respectively or 0.5 % dimethyl sulfoxide (DMSO) control. A stock solution of 10 mM 7-formyl-10-methylisoellipticine, dissolved in DMSO was used. Viability of the cells was determined by counting with a haemocytometer after staining with trypan blue (#T8154 Sigma-Aldrich).

Cell cycle

Cells were incubated with 5 µM 7-formyl-10-methylisoellipticine, taken from a stock solution of 10 mM 7-formyl-10-methylisoellipticine, dissolved in DMSO, for 24 h, washed in phosphate-buffered saline (PBS) and fixed for 1 h in ice-cold 70 % ethanol. Fixed cells were incubated with RNaseA (100 µg/ml) (Boehringer, Ingelheim, Germany) for 40 min at 37 °C. Nuclei were stained with propidium iodide (20 µg/ml) and analysed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences Europe, Oxford, UK). Cell cycle distribution was analysed using this software.

Western blotting

Primary antibodies used for Western Blot were PARP (#9542), Caspase-3 (#9662), Cyclin D2 (#D52F9), Cyclin D3 (#2936), all from Cell Signalling Technology, Boston, MA, USA. All secondary antibodies for western blotting were infrared (LI-COR Biosciences UK Ltd, Cambridge, UK). All antibodies were used as per manufacturers' guidelines.

Cells were lysed with RIPA buffer [Tris–HCl (50 mM; pH 7.4), 1 % NP-40, 0.25 % sodium deoxycholate, NaCl (150 mM), EGTA (1 mM), sodium orthovanadate (1 mM), sodium fluoride (1 mM), cocktail protease inhibitors (Roche, Welwyn, Hertfordshire, UK) and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (200 mM)] for 20 min on ice followed by centrifugation at 14,000 g for 15 min to remove cell debris. Equivalent amounts of protein, as determined by the Bio-Rad Protein Assay (Bio-Rad, Hemel Hempstead, UK) were resuspended in loading dye with Dithiothreitol (DTT; Sigma) and boiled at 90 °C for 5 min. This

solution was then centrifuged at 16,000 g for 1 min and the total supernatant resolved using SDS–polyacrylamide gel electrophoresis and followed by transfer to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and incubated overnight with the appropriate antibodies. Antibody reactive bands were detected using a LI-COR Odyssey infra-red imaging system (LI-COR Biosciences UK Ltd, Cambridge, UK).

ROS measurement

Mitochondrial ROS were measured using MitoSOX probe (Life Technologies #m36008). The cells were incubated with 5 μ M of MitoSOX for 15 min in the dark. The incubation was followed by washing and analysis by flow cytometry. Mitochondrial ROS were inhibited by the mitochondrial hexokinase inhibitor Isonidamine (Sigma, #L4900) for 1 h followed by treatment with 5 μ M 7-formyl-10-methylisoellipticine for the indicated times.

In vivo toxicity of 7-formyl-10-methylisoellipticine

The *in vivo* toxicity of 7-formyl-10-methylisoellipticine was assessed using female BALB/c mice (6–8 weeks, Harlan Laboratories, UK). 7-Formyl-10-methylisoellipticine was first dissolved in DMSO (100 mg/ml) followed by dilution with a polyethylene glycol (PEG) 400 (PEG 400, Sigma)-water mixture (30 % PEG 400, volume/volume), which achieved the resultant solution with less than 1 % DMSO for injections. Animals (5 mice/group) were intraperitoneally (i.p.) injected with 7-formyl-10-methylisoellipticine at doses of 5, 10, 25 and 50 mg per kg body weight in a 0.2 ml injection volume at Day 1, 3, 5, 8, 10, 12, 15, 17, 19, 22 and 24 (the body weight were measured at same days prior injections). The mice were sacrificed on Day 25, major organs (the brain, heart, liver, spleen, lung and kidney) were stained using haematoxylin and eosin, and serum were collected for monitoring of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels using ALT and AST Activity Assay Kits (Sigma, #MAK052 and #MAK055).

In vivo anti-tumour effect of 7-formyl-10-methylisoellipticine

Female CB17 severe combined immunodeficient (SCID) mice (6 weeks, Harlan Laboratories, UK) were used for *in vivo* anti-tumour study. The tumour-bearing model was established by subcutaneous injection of MV4-11 cells [1×10^7 in 50 % BD Matrigel™, BD Biosciences (#354234) into the right flank of mice] [22]. When the average tumour volume reached approximately 200 mm³ (Day 1) animals (8 mice/group) were i.p. injected with 7-formyl-10-methylisoellipticine (prepared as described in *In vivo* toxicity

of 7-formyl-10-methylisoellipticine section) at a dose of 25 mg per kg body weight in a 0.2 ml injection volume at Day 1, 3, 5, 8, 10, 12, 15, 17, 19, 22 and 25 (body weight and tumour growth were recorded at same days prior to injections). Tumour volume was calculated using the formula $a^2b(\pi/6)$, where a is the minor diameter of the tumour and b is the major diameter perpendicular to diameter a .

Statistical analysis

For the *in vitro* data values are mean \pm standard deviation (SD) and are representative of at least three individual experiments. For the *in vivo* data values are mean \pm standard error of the mean (SEM). The difference between two mean values was analysed using Student's t test. One-way analysis of variance (ANOVA) followed by Bonferroni's Post Hoc test was carried out to determine statistical significant differences in ALT and AST among all doses of 7-formyl-10-methylisoellipticine. Two-way ANOVA followed by Bonferroni's post hoc test was used to test the significance of differences in measured tumour growth and body weight. In all cases, $p < 0.05$ was considered statistically significant.

Results

7-formyl-10-methylisoellipticine synthesis

Isoellipticine differs from ellipticine due to the position of the nitrogen in the pyridine ring. The compound, 7-formyl-10-methylisoellipticine further deviates from this basic isoellipticine structure at position 7 where a formyl group is added and at position 10 where there is an additional methyl group (Fig. 1). It was synthesised using appropriately N10 substituted isoellipticine derivatives as starting materials [20].

7-formyl-10-methylisoellipticine induced apoptosis is dose and time dependent

MV4-11 is a well-established AML cell line used in this study. MV4-11 cells were treated with a range of concentrations of 7-formyl-10-methylisoellipticine for 24 h to evaluate the minimum concentration causing significant cytotoxicity (Fig. 2a). Using the trypan blue exclusion assay this concentration was determined as 5 μ M (Fig. 2a). Subsequently, the cytotoxic potential of 5 μ M 7-formyl-10-methylisoellipticine on the cells over a 96 h time period was examined (Fig. 2b). Results revealed that the *in vitro* anti-cancer effect of this compound is time-dependent, achieving more than 90 % cell death at 96 h (Fig. 2b). The cytotoxicity of 7-formyl-10-methylisoellipticine was confirmed by western blot. Figure 2c examines the protein expression of Poly (ADP-ribose) polymerase (PARP) and pro-caspase 3 following

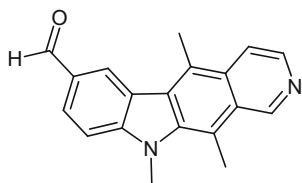


Fig. 1 The structure of 7-formyl-10-methylisoellipticine showing the formyl and methyl modifications

treatment with 7-formyl-10-methylisoellipticine. PARP is mainly involved in DNA repair and programmed cell death [23]. By 16 h post treatment the treated cells showed a

decrease in total PARP and an increase in the cleaved 89-kDa fragment of PARP. Cleavage of the precursor PARP marks the final commitment of apoptosis. In addition, caspase 3 plays an essential role in the execution of apoptosis [24]. Similar to PARP cleavage, procaspase 3 levels were decreased by 16 h. Taken together these results strongly suggest that 7-formyl-10-methylisoellipticine induces apoptotic cell death.

5 μM 7-formyl-10-methylisoellipticine increases the sub-G1 phase of MV4-11 cell cycle

From a clinical perspective, the ability of a drug to arrest the cell cycle is an attractive characteristic as most of the signaling pathways that result in uncontrolled cell proliferation converge in a signal that activates the cell cycle. The cell cycle consists of a series of unidirectional sequence of events from G1 to M. Theoretically blocking one phase of the cell cycle should interrupt the remaining phases and halt proliferation [25]. The capacity ellipticine [26] and of certain isoellipticines [20] to arrest cell cycle was previously described. The cell cycle arresting effects of 7-formyl-10-methylisoellipticine were assessed in Fig. 3a. It is shown that 5 μM 7-formyl-10-methylisoellipticine arrested up to 40 % of cells in the sub G1 phase after 24 h, which is similar with data recorded in [20]. This response was confirmed by western blot in Fig. 3b. Cyclin D is involved in regulating cell cycle progression [27]. It interacts with four cyclin dependent kinases (Cdks): Cdk2, 4, 5, and 6. Our results show that both cyclin D2 and cyclin D3 levels were inhibited by 16 h and levels remained low at 24 h. Inhibition of cyclin D induces down regulation of Cdks. These processes lead to an arrest of the cell in G0/G1 stage [28].

7-formyl-10-methylisoellipticine functions partially through generating mitochondrial derived ROS

Ellipticine has been shown to elevate intracellular reactive oxygen species (ROS) levels [14]. As previously described, one isoellipticine derivative, 7-hydroxyisoellipticine increased ROS [20]; however, the source of this ROS was unidentified. Mitosox has been used extensively *in vitro* to evaluate ROS production from the mitochondria [29]. We used Mitosox to determine if 7-formyl-10-methylisoellipticine increased mitochondrial ROS (Fig. 4a). The data demonstrate that 5 μM 7-formyl-10-methylisoellipticine increased mitochondrial ROS more than five folds relative to the control. To further confirm this increase in mitochondrial ROS, we inhibited mitochondrial respiration using lonidamine, a compound that functions through inhibition of mitochondrial bound hexokinase, particularly in cancer cells [30]. Cells that are pretreated with 10 μM lonidamine for 1 h before incubation with 5 μM 7-formyl-10-methylisoellipticine for another hour, produce less than half mitochondrial ROS levels relative to cells treated with 5 μM 7-formyl-10-methylisoellipticine

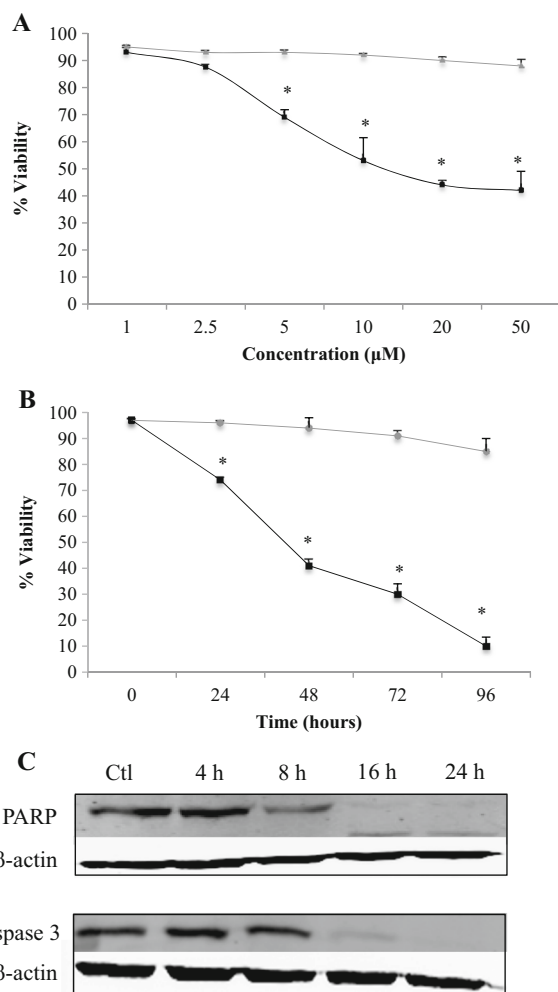


Fig. 2 7-Formyl-10-methylisoellipticine induced apoptosis is dose and time dependent. **a** The effects of a range of 7-formyl-10-methylisoellipticine on MV4-11 cells treated for 24 h, measured by trypan blue exclusion. *Black line* = 7-formyl-10-methylisoellipticine, *grey* = 0.5 % DMSO control. **b** The effects of 5 μM 7-formyl-10-methylisoellipticine on MV4-11 cells treated for 24 to 96 h, measured by trypan blue exclusion. *Black line* = 5 μM 7-formyl-10-methylisoellipticine, *grey* = 0.5 % DMSO control. **c** Western blot of PARP and pro-caspase 3 protein expression in MV4-11 cell treated with 0.5 % DMSO control (Ctl) and with 5 μM 7-formyl-10-methylisoellipticine at 4, 8, 16 and 24 h respectively using β -actin as a loading control. * = p -value < 0.05. The error bars represent \pm SD. $n=3$

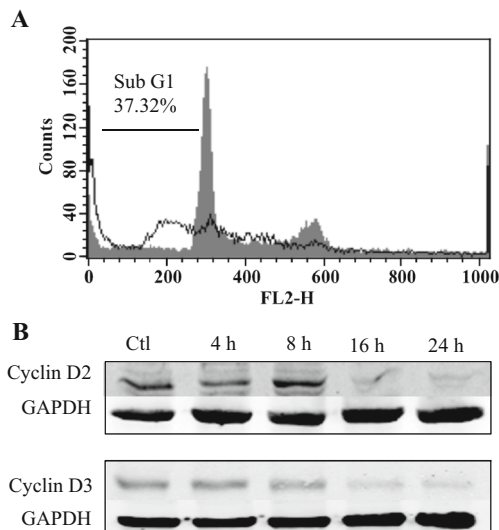


Fig. 3 5 μ M 7-Formyl-10-methylisoellipticine increases the sub-G1 phase of MV4-11 cell cycle. **a** The cell cycle of MV4-11 cells incubated with 5 μ M 7-formyl-10-methylisoellipticine for 24 h was analysed by propidium iodide staining. A representative profile is shown. Values represent the percentage (%) of cells in sub G1 phase of cell cycle following treatment with 5 μ M 7-formyl-10-methylisoellipticine. *Black line* = 5 μ M 7-formyl-10-methylisoellipticine, *grey* = 0.5 % DMSO control. **b** Western blot of cyclin D2 and cyclin D3 protein expression in MV4-11 cell treated with 0.5 % DMSO control (Ctl) and with 5 μ M 7-formyl-10-methylisoellipticine at 4, 8, 16 and 24 h respectively using β -actin as a loading control. * = *p*-value < 0.05. The *error bars* represent \pm SD. *n* = 3

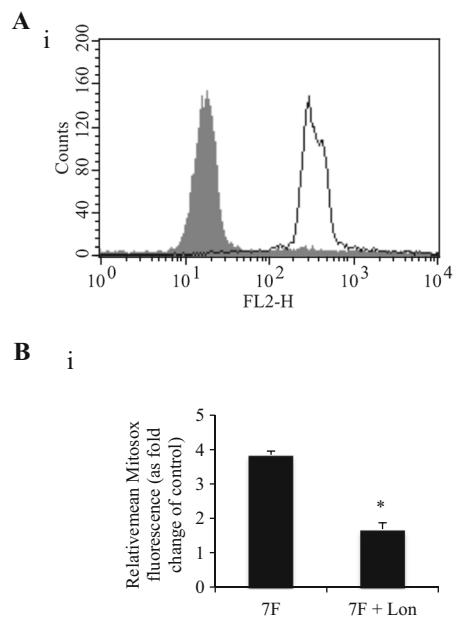
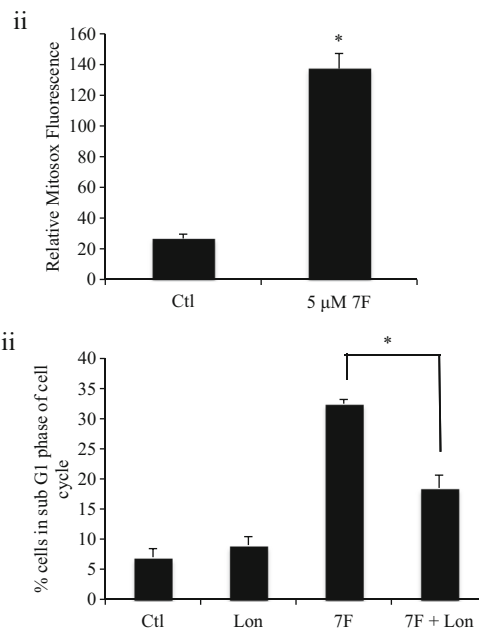


Fig. 4 7-Formyl-10-methylisoellipticine functions partially through generating mitochondrial derived ROS. **a i.** ROS levels of MV4-11 cells treated for 24 h with the control or 5 μ M 7-formyl-10-methylisoellipticine were measured by flow cytometry using Mitosox. **ii.** Quantification of ROS levels. Values are corrected for background fluorescence of 7-formyl-10-methylisoellipticine. **b i.** Quantification of ROS levels measured by flow cytometry, using Mitosox, of MV4-11 cells

alone for 1 h (Fig. 4bi). This decrease confirms that lonidamine is capable of reducing mitochondrial ROS induced by our compound. It is further confirmed that this inhibition of mitochondrial ROS significantly impaired the cytotoxic effect of 7-formyl-10-methylisoellipticine (Fig. 4bii). Cell cycle analysis showed that 5 μ M 7-formyl-10-methylisoellipticine achieved up to 35 % of cell death when leukaemia cells were treated for 24 h (Fig. 4bii). However, when cells are pretreated with 10 μ M lonidamine for 1 h 7-formyl-10-methylisoellipticine can only kill less than 20 % of the cells (Fig. 4bii). This reduction in the cytotoxicity of 7-formyl-10-methylisoellipticine by inhibition of mitochondrial ROS suggests that this isoellipticine functions at least partially through increasing mitochondrial ROS.

7-formyl-10-methylisoellipticine is not toxic to BALB/c mice at the given doses

After determining the efficacy of 7-formyl-10-methylisoellipticine *in vitro*, the next step was to determine whether 7-formyl-10-methylisoellipticine has anti-tumour activity *in vivo*. Although DMSO is routinely used in the laboratory as a solvent or vehicle for poorly aqueous soluble drugs, it has presented unexpected side effects in laboratory animals and humans [31]. As an alternative, PEG with various



treated for 1 h with 5 μ M 7-formyl-10-methylisoellipticine (7F) or 5 μ M 7-formyl-10-methylisoellipticine in combination with 10 μ M lonidamine (7F+Lon). **ii.** Percentage of MV4-11 cells in the sub G1 phase of the cell cycle following treatment with control (Ctl), 10 μ M lonidamine (Lon), 5 μ M 7-formyl-10-methylisoellipticine (7F), or 5 μ M 7-formyl-10-methylisoellipticine preceded by 1 h treatment with 10 μ M lonidamine (7F+Lon) The *error bars* represent \pm SD. * = *p*-value < 0.05. *n* = 9

molecular weights (i.e. PEG 400) has presented less *in vivo* toxicity and is substantially used as the pharmaceutically accepted solvent for chemotherapeutics [32]. The compound prepared using a PEG 400-water cosolvent (30 % PEG 400, volume/volume) demonstrated a similar *in vitro* anti-proliferative effect as observed by the compound dissolved in DMSO (Supplementary Figure 1), indicating that this cosolvent is suitable for administration of 7-formyl-10-methylisoellipticine and will be used in the following *in vivo* studies.

To evaluate *in vivo* toxicity mice were i.p. injected with 7-formyl-10-methylisoellipticine at a range of doses (Fig. 5). Results in Fig. 5a demonstrate that 7-formyl-10-methylisoellipticine does not cause a change in body weight at any of the given doses. In addition, the treatment of 7-formyl-10-methylisoellipticine did not significantly ($P > 0.05$) increase levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) relative to negative control (Fig. 5b). Furthermore, no significant difference in terms of cell morphology and tissue structure was observed in specified major organs in mice with or without treatment of compound (Supplementary Figure 2). All of this evidence suggests that 7-formyl-10-methylisoellipticine does not cause systemic toxicity.

7-formyl-10-methylisoellipticine has anti-tumour activity in SCID mice bearing xenograft tumour

An AML xenograft mouse model was used to determine the therapeutic effect of 7-formyl-10-methylisoellipticine (Fig. 6). Based on the results from our toxicity experiments, we proceeded to use 25 mg/kg in the therapeutic model. Our results display that 7-formyl-10-methylisoellipticine significantly slowed down the tumour growth (a 4-time reduction compared to the control group at the end point of the experiment) (Fig. 6a). In addition, Fig. 6b reiterates this finding by showing that 7-formyl-10-methylisoellipticine significantly reduces tumour mass, up to 7-time reduction relative to the control.

Discussion

In this study, we have reported the anti-leukaemia effect of a novel ellipticine derivative, 7-formyl-10-methylisoellipticine, *in vitro* and *in vivo* and have studied the mechanism by which it exerts its activity. This compound demonstrates a profound cytotoxicity in MV4-11 cells achieving a significant anti-proliferative effect (up to 40 % cell death) for 24 h (Fig. 2a) and inhibiting more than 90 % cell proliferation at 96 h (Fig. 2b). The cytotoxicity results from apoptosis (Fig. 2c); more importantly, 5 μ M 7-formyl-10-methylisoellipticine induced a greater apoptotic effect achieving an increase in PARP

and procaspase 3 cleavages at 16 h (Fig. 2c) when compared to previously reported 7-hydroxyisoellipticine that caused apoptosis at 48 h at same conditions [20]. These results suggest that 7-formyl-10-methylisoellipticine may have a more potent clinical application over its counterparts.

The apoptotic cell death is further confirmed by cell cycle analysis (Fig. 3). Figure 3a shows that 5 μ M 7-formyl-10-methylisoellipticine led to an accumulation of cells in the Sub G1 phase, which is accompanied with inhibition of Cyclin D2 and D3 (subunits of Cyclin D required for activation of cell cycle entry [26]) (Fig. 3b). The machinery controlling the mammalian cell cycle consists of Cdks, which are activated by association with Cyclin regulatory subunits. As targeted cancer therapies aim to interrupt a specific component of the complex signalling pathways that result in uncontrolled cell proliferation [25], the knowledge that 7-formyl-10-methylisoellipticine suppresses this component of the cell cycle apparatus may be beneficial for designing a more targeted strategy for AML therapy.

It has been proposed that some current mainstay cancer therapies, such as daunorubicin may rely on their ability to increase ROS to exert their full cytotoxic effect [33]. This is possible as the highly stressed state of malignant cells may partly explain the selectivity of these cytotoxic drugs [34]. In the present work, it is shown that 7-formyl-10-methylisoellipticine produced ROS via the mitochondria (Fig. 4a). In addition, lonidamine was employed to further confirm the mitochondria as the main source of 7-formyl-10-methylisoellipticine induced ROS production (Fig. 4b). The detailed knowledge on the source of ROS may be advantageous when designing adjuvant therapy.

It is interesting to note that the combination of lonidamine with 7-formyl-10-methylisoellipticine did not completely impair the anti-leukaemia effect of the drug (Fig. 4bii). One possibility is that the compound functions through an additional mechanism of action as even in combination with lonidamine, 7-formyl-10-methylisoellipticine still manages to bring about approximately 20 % cell death (Fig. 4bii). Assuming that 7-formyl-10-methylisoellipticine functions by multiple mechanisms of action seems reasonable as it demonstrates an efficient induction of apoptotic cell death (Figs. 2 and 3). However, it is also possible that the cell death still observed is simply because the two compounds in combination are too toxic for the cells or because the lonidamine could not sufficiently reduce the 7-formyl-10-methylisoellipticine induced mitochondrial ROS to basal levels (Fig. 4bi).

Although ellipticines display a lack of hematological toxicity [35], they still caused unexpected toxic side effects [36]. In this study, the *in vivo* toxicity analysis of 7-formyl-10-methylisoellipticine indicates that the compound did not cause significant signs of toxicity in terms of animal body weights, the liver enzymes and the histology of major organs (Fig. 5 and Supplementary Figure 2). Although we proceeded to the

Fig. 5 7-Formyl-10-methylisoellipticine is not toxic to BALB/C mice at the given doses. **a** Graph shows the mean mouse body weight derived from five individual mice in response to treatment with control or the indicated dose of 7-formyl-10-methylisoellipticine. **b** Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activity in the serum of mice in response to treatment with control or the indicated dose of 7-formyl-10-methylisoellipticine. Results are expressed as means. Error bars show the SEM

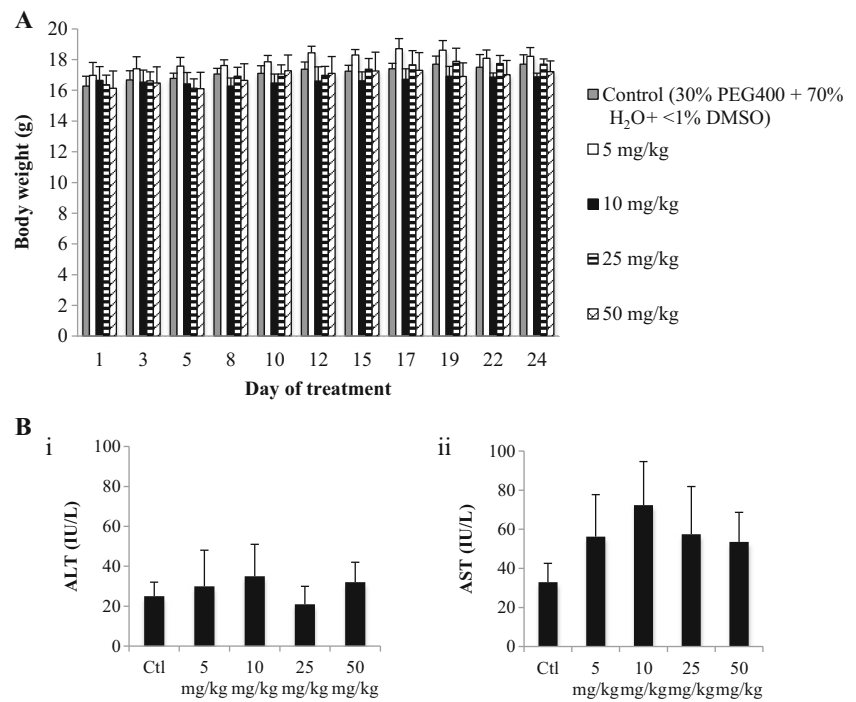
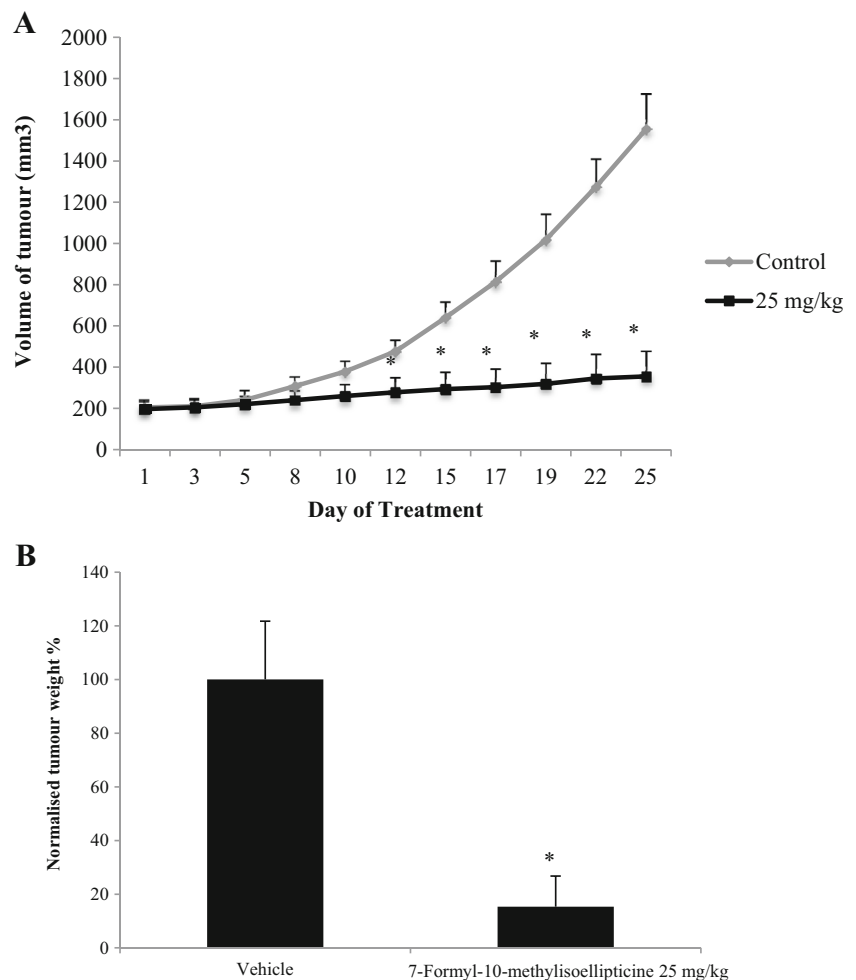


Fig. 6 7-Formyl-10-methylisoellipticine has anti-tumour activity in SCID mice bearing xenograft tumour. **a** Volume of tumour in SCID mice bearing xenograft tumour in response to treatment with control or the 25 mg/kg 7-formyl-10-methylisoellipticine. **b** Normalised tumour weight in SCID mice bearing xenograft tumour in response to treatment with control or 25 mg/kg 7-formyl-10-methylisoellipticine. Results are expressed as means derived from eight individual mice in each of the two groups on the final day of the experiment. Error bars show the SEM



therapeutic model with 25 mg/kg it is worth noting that 50 mg/kg was not toxic in BALB/c mice and therefore could potentially be used in future studies.

Consistent with *in vitro* anti-proliferative effects, 7-formyl-10-methylisoellipticine displayed promising anti-tumour activity in an AML xenograft model (Fig. 6). Although the given dose did not completely eradicate the tumour, it did significantly impede its growth throughout the experiment. It is known that AML is most often treated by combination therapy [37]. Therefore to completely eradicate the tumour it may be necessary to design a regimen that combines 7-formyl-10-methylisoellipticine with another drug.

We have showed that 7-formyl-10-methylisoellipticine has a potent cytotoxic effect on leukaemia cells, which is at least partially mediated by the induction of mitochondrial ROS. We aim to continue to investigate the mechanism of action of this novel compound class, which we believe has a clear potential clinical application. Furthermore, our study documents for the first time the therapeutic potential of an isoellipticine compound in a subcutaneous AML cell-derived xenograft (CDX) model, and future studies will evaluate therapeutic profiles of 7-formyl-10-methylisoellipticine in the orthotopic AML patient-derived xenograft (PDX) models [38]. We anticipate that the recent research on ellipticine derivatives, such as this study, will lead the development of an ellipticine analogue that may be employed clinically.

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Compliance with ethical standards

Ethical statement All animal experimental procedures were approved by the ethical committee at the University College Cork and performed in accordance with the European Union (Protection of Animals Used for Scientific Purposes) Regulations 2012 (S.I. No. 543 of 2012) and Directive 2010/63/EU for animals used for scientific purposes.

Conflict of interest The authors declare that they have no conflict of interest.

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