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In-vitro investigation of the impact of methadone on 5-ALA-PDT

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ABSTRACT

Background: 5-Aminolevulinic acid-based photodynamic therapy (5-ALA-PDT) is an effective treatment for superficial skin tumors. However, its effectiveness for malignant squamous cell carcinoma (SCC) and glioblastoma (GBM) remain to be improved. In addition, the pain during PDT especially in skin tumors is an important limitation for using 5-ALA-PDT in dermatology. Recent investigations hypothesize that D, L-methadone (MTD) as an analgesic might also suppress the growth of various cancers by increasing their susceptibility to apoptosis. The aims of the presented two studies were to investigate whether MTD could impact the efficacy of 5-ALA-PDT for SCC and GBM *in vitro* and to explore the mechanism of MTD on 5-ALA-PDT.

Methods: The cell lines FADU (SCC) and A172 (GBM) were treated by 5-ALA-PDT with 0.01 mg/mL MTD for three days. The irradiation was carried out by means of a diode laser emitting at 635 nm at an irradiance of 100 mW/cm^2 for various light doses (irradiations). After defining the specific 5-ALA concentration and light dose to induce around 25% tumor cell death, protoporphyrin IX (PpIX) fluorescence, survival rates, apoptosis rates, and cell cycle phase were investigated to evaluate the effect with and without the presence of MTD. To explore the mechanism, flow cytometry was used to determine the expression of μ-opioid receptor (MOR), phosphorylation of c-Jun N-terminal kinase (JNK) and phosphorylation of B-cell lymphoma 2 (BCL-2). The naloxone, as an antagonist, was also used to investigate the role of MOR in this process.

Results: In the absence of MTD, FADU cells reached a 75% survival after incubation with 0.5 mM 5-ALA for twenty-four hours and illumination for 1 J/cm^2. For A172 cells, treatment with 1mM 5-ALA incubated for twenty-four hours and an illumination of 2 J/cm^2 resulted in a 75% survival. PpIX formation in both cell lines was only influenced marginally by MTD. The combination of MTD successfully enhanced the inhibition of 5-ALA-PDT for both tumor cell lines ($p < 0.05$). The effect of MTD was enhanced with the incubation time. MTD increased the apoptotic rate of FADU cells treated by 5-ALA-PDT from 20.6%±0.6% to 53.2%±1.6% and that of A172 cells from 22.8%±1.9% to 45.3%±2.2 % ($p<0.05$). The 5-ALA-PDT-induced cell cycle phase arrest in the G0/G1 phase on tumor cells was also improved by MTD ($p<0.05$). It was found that A172 cells expressed MOR on the cell membrane. Except for apoptosis, the activity of JNK and expression of phosphorylated BCL-2 induced by 5-ALA-PDT were also enhanced by the combination of MTD ($p<0.05$). The abovementioned MTD effects were reversed by the use of naloxone ($p<0.05$).

Conclusion: MTD significantly enhanced the inhibition of 5-ALA-PDT for tumor cells through the activation of the JNK pathway and phosphorylated BCL-2. These effects of MTD was MOR-

dependent. This study provides a theoretical basis using the MTD in the clinic to improve the efficacy of 5-ALA-PDT for SCC and GBM and to reduce the 5-ALA-PDT induced pain simultaneously. However, prior to translation into the clinic, clinical studies have to be performed to determine a suitable MTD concentration and protocol inducing an improved therapeutic effect and reduced possible side effects.

ABSTRACT (GERMAN)

Hintergrund: Die photodynamische Therapie auf Basis von 5-Aminolävulinsäure (5-ALA-PDT) ist eine wirksame Behandlung für oberflächliche Hauttumoren. Die Wirksamkeit bei malignen Plattenepithelkarzinomen (SCC) und Glioblastomen (GBM) könnte jedoch weiterhin verbessert werden. Darüber hinaus ist das Schmerzempfinden während der PDT, insbesondere bei Hauttumoren, eine starke Einschränkung für diese Behandlungsform. Jüngste Untersuchungen zeigen, dass D, L-Methadon (MTD) als Analgetikum auch das Wachstum verschiedener Krebsarten unterdrücken könnte, indem es deren Anfälligkeit für Apoptose erhöht. Ziel der vorliegenden beiden Studien war es zu untersuchen, ob MTD die Wirksamkeit von 5-ALA-PDT bei SCC und GBM in vitro beeinflussen kann, und den Mechanismus von MTD bei 5-ALA-PDT zu untersuchen.

Methode: Die Zelllinien FADU (SCC) und A172 (GBM) wurden drei Tage lang mit 5-ALA-PDT mit 0,01 mg / ml MTD behandelt. Die Bestrahlung wurde mit einem Diodenlaser bei 635 nm bei einer Bestrahlungsstärke von 100 mW / cm² für verschiedene Lichtdosen (Bestrahlungen) durchgeführt. Nach Definition der spezifischen ALA-Konzentration und der Lichtdosis zur Induktion eines Tumorzelltods von etwa 25% wurden die Protoporphyrin IX (PpIX) -Fluoreszenz, die Überlebensraten, die Apoptoseraten und die Zellzyklusphase gemessen, um den Effekt mit und ohne MTD zu bewerten. Um den Mechanismus zu untersuchen, wurde die Expression des μ-Opioidrezeptors (MOR), die Phosphorylierung der c-Jun N-terminalen Kinase (JNK) und die Phosphorylierung des B-Zell-Lymphoms 2 (BCL-2) mittels Durchflusszytometrie bestimmt. Als Kontrolle wurde der MOR-Antagonist Naloxon eingesetzt.

Ergebnisse: In Abwesenheit von MTD wurde für FADU-Zellen nach 24-stündiger Inkubation mit 0.5 mM 5-ALA und 1 J/cm² Beleuchtung ein Überleben von 75% ermittelt. A172-Zellen hingegen wurden für den gleichen Effekt mit 1 mM 5-ALA und 2 J/cm² behandelt. Die PpIX-Bildung in beiden Zelllinien wurde durch MTD nur geringfügig beeinflusst. Die Kombination mit MTD verstärkte erfolgreich die Hemmung von 5-ALA-PDT für beide Tumorzelllinien ($p < 0.05$). Die Wirkung von MTD wurde mit steigender Inkubationszeit verstärkt. MTD erhöhte die apoptotische Rate von mit 5-ALA-PDT behandeltem FADU von 20.6% ± 0.6% auf 53.2% ± 1.6% und die von A172-Zellen von 22.8% ± 1.9% auf 45.3% ± 2.2% ($p < 0.05$). Der 5-ALA-PDT-induzierte Zellzyklusstillstand in der G0 / G1-Phase der Tumorzellen wurde ebenfalls durch MTD verbessert ($p < 0.05$). A172-Zellen exprimieren MOR auf der Zellmembran. Mit Ausnahme der Apoptose wurden die Aktivität von JNK und die durch 5-ALA-PDT induzierte Expression von

phosphoryliertem BCL-2 auch durch die Kombination von MTD erhöht ($p < 0.05$). Die obigen MTD-Effekte wurden durch Verwendung von Naloxon gehemmt ($p < 0.05$).

Schlussfolgerung: Die Methadongabe erhöhte signifikant den Prozentsatz der durch 5-ALA-PDT induzierten Apoptoserate und verbesserte die Wirkung von 5-ALA-PDT auf den Zellzyklusstillstand in der G0/G1-Phase. Ein sicherer Hinweis, dass Methadon zu einer erhöhten Akkumulation von PpIX führt, ergab sich nicht. Der Effekt von Methadon auf die Wirksamkeit von 5-ALA-PDT in A172-Zellen war MOR-abhängig. MTD verstärkte die Aktivierung des JNK-Signalwegs und von phosphoryliertem BCL-2 signifikant in Abhängigkeit von MOR. Die vorliegenden Studien bieten eine theoretische Grundlage für die Verwendung der MTD in der Klinik, um die Wirksamkeit von 5-ALA-PDT bei SCC und GBM zu verbessern und gleichzeitig die durch 5-ALA-PDT verursachtes Schmerzempfinden zu reduzieren. Vor der Übertragung in die Klinik müssen jedoch klinische Studien durchgeführt werden, um eine geeignete MTD-Konzentration und ein geeignetes MTD-Protokoll zu bestimmen, die eine verbesserte therapeutische Wirkung und eine Verringerung möglicher Nebenwirkungen hervorrufen.

INTRODUCTION

This work is presented as a cumulative dissertation and consists of two published original manuscripts [1, 2]. 5-Aminolevulinic acid-mediated photodynamic therapy (5-ALA-PDT) is a relatively novel and minimally invasive modality for the treatment of tumors combining the actions of a photosensitizer, light, and oxygen [3]. In 1990, 5-ALA-PDT was firstly used for the treatment of cutaneous basal cell carcinoma (BCC) in the clinic [4]. In 1999, 5-ALA was developed as the medicine named Levulan® by DUSA Pharmaceuticals, Inc with an indication of actinic keratosis (AK) [5]. In 2007, Shanghai Fudan-zhangjiang Bio-Pharmaceutical Co.,Ltd. in China developed another 5-ALA medicine called AILA® for the treatment of condyloma acuminate [3]. In 2017, American FDA approved Gleolan®, another 5-ALA medicine developed by Photonamic GmbH and Co. KG for the intraoperative optical imaging of high-grade gliomas (HGGs) in the brain [6].

The mechanism of 5-ALA-PDT

5-ALA is an endogenous molecule in the body participating in haem biosynthesis [7]. The 5-ALA synthase is a rate-limiting enzyme for 5-ALA synthesis. 5-ALA was synthesized into coproporphyrinogen III firstly. Thereafter, coproporphyrinogen III is converted into protoporphyrin IX (PpIX). PpIX further generates haem under the rate-limiting activity of the enzyme protoporphyrin ferrocheletase. Normally, a haem feedback pathway regulates the enzyme 5-ALA synthase to control the amount of PpIX production. As 5-ALA can be administered topically or orally as a medicine, such exogenous 5-ALA can be taken up by the cells and bypasses the regulatory metabolic steps, resulting in saturation of the ferrochelatase activity and in an increase of PpIX generation [8].

After uptake of excess exogenous 5-ALA, most cells can convert it into PpIX, but this capacity was varying in different tissues and cells [9]. Generally, tumor cells show a more efficient capacity in PpIX production in comparison to non-tumor cells. This is because the activity of porphobilinogen deaminase is up-regulated in most malignant cells [10]. In addition, the activity of ferrochelatase in most malignant cells is inhibited resulting in a low effectiveness of haem formation [11]. It was proposed that the PpIX generation was mainly related to the ratio of porphobilinogen deaminase to ferrochelatase activity thus related to cell photosensitivity [12]. In addition, tumor cells usually accumulate more PpIX than normal cells partly because of the increased cellular metabolism, promoted 5-ALA uptake and attenuated PpIX elimination [11-13]. PpIX is the real substance that works as the photosensitizer in the 5-ALA-PDT. Under illumination the energy of photons is absorbed by the PpIX molecule and transferred to surrounding oxygen. The oxygen accepts the energy and changes into singlet oxygen and reactive oxygen species (ROS) which itself

can oxidize and damage the adjacent biomolecules, mitochondria, cell membrane and other organelles [14-16]. The above oxidative damage is the main mechanism of 5-ALA-PDT that further induces cell deaths. Except the direct killing for the tumor cells, 5-ALA-PDT exerts the anti-tumor effect also through the upregulation of various cytokines which related to anti-tumor immune responses, such as C-C motif chemokine ligand 8 (CCL-8), interleukin (IL)-1-β, chemokine C-X-C motif ligand 13 (CXCL13) and so on [17-20].

The application and efficacy of 5-ALA-PDT for squamous cell carcinoma and glioblastoma

Clinically, 5-ALA-PDT shows a lot of advantageous characters, such as allowing repetitive treatments to the same lesion, one-time treatment to the multiple lesions, minimally-invasive, the good restoring of structures and functions [21]. It is a common treatment for some premalignant and malignant diseases, such as AK, BCC, Barrett's esophagus, and bladder cancer in the clinic [16, 22-25]. 5-ALA-PDT is effective for the treatment of shallow cutaneous tumors while its efficacy for some malignant tumors remains to be improved, such as cutaneous squamous cell carcinoma (SCC) and glioblastoma (GBM) [26-28].

SCC is the second most common skin cancer [29]. Importantly, it may spread, or metastasize, to other parts of body and ultimately lead to death [30]. Surgery is the first-line treatment for SCC, although various treatments like radiation are used in the clinic [31]. All conventional therapies have the significant challenges when treating large, metastatic, and invasive SCC [32, 33]. The efficacy of 5-ALA-PDT for SCC is not as good as for the 5-ALA-treatment of AK. A few clinical case reports showed 5-ALA-PDT is effective for the superficial SCC. Complete response of 5-ALA-PDT for superficial SCC was reported as 54% with a recurrence rate of 69% [34]. Xiuli Wang et al. successfully used 5-ALA-PDT to cure seven low grade of superficial SCC with a good cosmetic result [28]. However, for the Grade III~IV of SCC the effect of 5-ALA-PDT was poor. To improve the efficacy for SCC, surgery was sometimes performed before 5-ALA-PDT. After the incision, 5-ALA-PDT might play a role in enhancing the efficacy and improving the cosmetic results [35]. Compared to 5-ALA-PDT alone, surgery combined with 5-ALA-PDT improve the efficacy for SCC. However, the recurrence rate of SCC after this combination therapy is still very high [36]. In the mouse experiment, the result also shows 5-ALA-PDT has a poor effect for the large SCC [37]. The insufficient effectiveness for invasive growing or large SCC limits the application of 5-ALA-PDT [38, 39].

GBM is one of the most frequent and aggressive brain cancers. A brain glioma can cause headaches, vomiting, seizures, memory loss and other cranial nerve disorders [40]. The standard treatment for glioma includes the neurosurgical resection and radiotherapy followed by adjuvant chemotherapy. However, GBM usually has a poor prognosis because of its strong aggressiveness

and resistance to the treatments [41, 42]. 5-ALA is initially used as an imaging agent for fluorescence-guided surgical resection for GBM based on its high tumor selectivity [6]. Nevertheless, despite the application of fluorescence guided surgery by 5-ALA, the tumors will inevitably recur. To further prolong the survival of the patient, 5-ALA-PDT is presently under investigation for the treatment of GBM [43]. A few *in vitro* studies indicated that 5-ALA-PDT is an effective modality for the inhibition of the proliferation of GBM cells and GBM stem-like cells [44-48]. The study show PDT-induced necrosis is the main pathway for the inhibition. The receptor-interacting protein 3 (RIP3) is a key mediator of this 5-ALA-PDT induced glioma cell necrosis activated by singlet oxygen [49]. 5-ALA-PDT also can induce the tumor cells to upregulate proinflammatory cytokines and activating anti-tumor immune responses [50].

In the clinic, interstitial PDT (iPDT) is developed to treat GBM in the brain. IPDT is performed by using a stereotactic approach and by insertion of the diffusors of optical fibres into the treatment volume in order to achieve whole tumor volume illumination after oral administration of 5-ALA [51]. The therapeutic effect of 5-ALA-PDT is restrained by the depth of light penetration [26, 52, 53]. After careful treatment planning up to eight diffusors are simultaneously activated to cover the tumor volume with suitable amount of light. Recent studies suggest a relatively advantage of 5-ALA-PDT in improving the survival of GBM patients in comparison to the surgery [54]. It was demonstrated that after 5-ALA-PDT, the median survival of newly diagnosed GBM was 16.1 months when that of the recurrent GBM was 10.3 months [55]. Tumor cells out of the effective therapeutic volume of 5-ALA-PDT are illuminated with less light and may survive after the treatment of 5-ALA-PDT, leading to a poor result for large GBM.

In general, the efficacy of 5-ALA-PDT for deep or large tumors is limited. The treatment of large volume of SCC or GBM using current 5-ALA-PDT only is challenging [27, 56]. As a result, treatments by means of 5-ALA-PDT needs to be improved to enhance its efficacy for SCC and GBM [16, 57].

Methods to improve the efficacy of 5-ALA-PDT

To improve the efficacy of 5-ALA-PDT for SCC, calcitriol was used to pretreat the tumor before 5-ALA-PDT. Cell and mouse experiments showed that calcitriol can enhance the anti-tumor effect of 5-ALA-PDT via promoting the tumor-specific accumulation of 5-ALA induced PpIX [58, 59]. Calcitriol increases the PpIX production by increasing the porphyrin synthesis enzymes coproporphyrinogen oxidase [58]. Gefitinib, an EGFR inhibitor is also found to enhance the efficacy of 5-ALA-PDT for GBM through increasing PpIX production. The increase of PpIX production induced by Gefitinib is mediated by decreasing the expression of ATP Binding Cassette Subfamily G Member 2 (ABCG2) [60]. Other sensitizing medicines, such as imiquimod the iron

chelator deferoxamine, and metformin also show the abilities to enhance the efficacy of 5-ALA-PDT for some specific cancers [57, 60-65]. The nanoparticle technique demonstrates the effect in the enhancement of the accumulation of 5-ALA in GBM and SCC cells, leading to a high level of PpIX production and reinforced effectiveness [15, 24, 66]. However, the sensitizing effect of the above methods has not been proven clinically.

Pain limits the application of 5-ALA-PDT

The pain sensation as the main adverse response limits greatly the application of 5-ALA-PDT in the clinic, especially in dermatology [38, 67]. During the illumination, patients suffer from varying degrees of pain sensations [68]. Usually, if the pain may reach to an intolerable degree during the illumination this results in the interruption of the 5-ALA-PDT [69, 70] Even, the patient may refuse the forthcoming treatment of this excruciating pain. Although, the exact mechanism of the pain induced by irradiation of 5-ALA-PDT remains unclear, it is widely accepted that ROS generated during irradiation is the main contributor for the pain [38].

To reduce the pain during 5-ALA-PDT, various pain management modalities are clinically under investigation. Topical anesthesia was used for pain relief during 5-ALA-PDT, but a clinical trial showed it was insufficient to control the 5-ALA-PDT pain [71]. Anecdotal evidence suggests that local infiltration anesthesia and cold-air analgesia can reduce the 5-ALA-PDT pain to a certain extent [72, 73]. However, the analgesic effects of the above two methods are limited and are appropriate for mild or moderate pain relief [74]. The nerve block is demonstrated to be an effective pain management during 5-ALA-PDT, especially for patients with extensive lesions [69, 70, 75]. The inhalation analgesia with a nitrous oxide/oxygen mixture is also demonstrated to be an effective and well-tolerated method for achieving significant pain reduction during 5-ALA-PDT [76, 77]. However, neither the nerve block nor the inhalation analgesia is an easy option that can be performed for every patient. Daylight-PDT, as a novel PDT protocol, showed to be less painful than conventional PDT [78, 79]. It successfully reduces the 5-ALA-PDT pain by shortening the drug-light interval and using daylight instead of technical red light. In recent years, it is found that using a low power density, a short drug-light interval or a two-step irradiance protocol contributes to reduction of the 5-ALA-PDT related pain [80-83]. The short-term efficacy PDT-treatment outcomes of above painless modality are similar to those of conventional PDT. However, additional studies are required to clarify the long-term efficacy of those novel treatment concepts [82]. Oral analgesia is another potential effective method for the relief of 5-ALA-PDT induced pain. The three-step analgesic ladder established by the World Health Organization is used to relieve the cancer pain in the clinic, including the pain developed by the treatment [84]. However, the non-opioids analgesics and weak opioids, such as acetaminophen, oxycodone, and tramadol

used in clinics for the alleviation of 5-ALA-PDT related pain does not achieve a satisfied effect [68, 69, 80]. A more potent analgesic is required for the alleviation of 5-ALA-PDT induced pain.

MTD is a strong analgesic and a potentiating agent

MTD is a strong long-acting opioid receptor agonist. It belongs to the level III opioid medicine which owns a high affinity binding to the mu opioid receptor (MOR) [85]. It is a racemic mixture of 2 enantiomers including R-MTD and S-MTD. After the administration, MTD is widely distributed throughout the body and shows a long half-life of 24 hours [86]. In most cases, MTD can reduce cancer-related pain from moderate or severe to mild or no pain [87]. MTD and its metabolites are finally excreted from the body by liver and kidney. The main adverse events with MTD includes sleepiness, nausea, antidiuresis, constipation, hypotension, and exacerbation of asthma [87]. Compared to other level III opioid drugs, MTD dosn't have to rotate to other opioids frequently because of reduced drug resistance phenomenon [88]. In addition, withdrawals and deaths with MTD are also less than with other level III opioid drugs [89]. Nowadays, MTD is extensively used as a safe and efficient analgesic for the cancer pain [90, 91].

Recently, it is claimed that MTD shows the potency to enhance the efficacy of doxorubicin or cisplatin for the inhibition of GBM, leukemia, prostate cancer, bladder cancer and so on [89, 92-97]. Friesen et al. reported that MTD sensitize the inhibition of doxorubicin for leukemia tumor cells and GBM A172 cells significantly [96, 98]. Stadlbauer et al. also found a synergistic inhibition of MTD with doxorubicin for GBM and prostate carcinoma cell lines [95]. It is found that the potentiating effect of MTD for the chemotherapeutic drugs is possibly dependent on the drug and on the cell line used [99]. The reported mechanisms of potentiating effect of MTD for the chemotherapy of tumor include:

1 After binding to MOR, MTD inhibits adenylyl cyclase resulting in down regulation of cyclic adenosine monophosphate (cAMP) [100]. The down-regulation of cAMP, which is a second messenger, participates in the potentiating effect of MTD for the apoptosis of tumor cells treated by doxorubicin [93, 96].

2 MTD can also promote the tumor cell apoptosis via up-regulating 9 caspase-3 and caspase, down-regulating of X chromosome–linked inhibitor and B-cell lymphoma-extra large (BCLxL) [101].

The abovementioned two mechanisms further support the conclusion that MTD can sensitize the inhibition of chemotherapy for tumor cells. However, so far it was only proved that the potentiating effect of MTD exists in chemotherapy induced intrinsic apoptosis [97, 101].

The impact of MTD on 5-ALA-PDT

5-ALA-PDT mainly induces tumor cells death by the apoptosis pathway [50]. Considering its good analgesic and sensitizing effect for chemotherapy, MTD might be used as a potential potentiating agent of 5-ALA-PDT for tumors. An improved 5-ALA-PDT modality with more efficient and less painful is expected to be established based on the combination with MTD.

The question arose whether MTD may have an impact on or can promote the effectiveness of 5-ALA-PDT or not? To answer this question, an *in vitro* study was carried out using SCC and GBM cell lines [1]. The impact of MTD on 5-ALA-PDT for SCC (FADU cell line) and GBM (A172 cell line) was studied by the measurement of PpIX fluorescence (accumulation of PPIX in the cell), cytotoxicity, apoptosis rates, and cell cycle. The results showed the MTD only influenced marginally on the PpIX formation in both cell lines. The cytotoxicity of 5-ALA-PDT for both cell lines were significantly increased in the presence of MTD ($p< 0.05$). This sensitizing effect of MTD on 5-ALA-PDT for tumor cells was mainly caused by increasing the apoptosis of tumor cells. The 5-ALA-PDT-induced cell cycle phase arrest in the G0/G1 phase on both cell lines was also improved by applying MTD ($p<0.05$).

Potential mechanism for promoting the 5-ALA-PDT-induced apoptosis

The above mentioned study demonstrates the ability of MTD to promote the 5-ALA-PDT-induced apoptosis of both SCC and GBM cell lines. However, the mechanism of MTD remains unclear. Because the apoptosis was the main death route, it was speculated that MTD might act on a conjunct signal pathway that promotes the apoptosis affected by both MTD application and 5-ALA-PDT. The JNK activity pathway, also known as stress-activated protein kinase pathway (SAPK) is the main cellular response to various stress, such as ROS, γ-irradiation, ultraviolet, mechanical stress, anticancer chemotherapy drugs and so on [102, 103]. It was proved that 5-ALA-PDT can activate JNK pathway participating in the apoptosis of tumor cells [104-107]. Meanwhile, the activated MOR was also reported to show the ability to initiate JNK pathway [108, 109]. MTD is a strong agonist of MOR. Therefore, it is deserved to explore whether JNK pathway contributes to the potentiating effect of the MTD on 5-ALA-PDT for tumor cells.

JNK pathway can further induce BCL-2 phosphorylation and inactivate the ability of BCL-2 for the suppression of apoptosis [110]. Dephosphorylated BCL-2 is an oncoprotein with an ability to inhibit tumor cells apoptosis [111]. Whereas, the impact of phosphorylated BCL-2 on the regulation of apoptosis is still uncertain and conflicting [112]. The phosphorylated BCL-2 activated by the growth factor or nicotine showed the ability to inhibit the tumor cells apoptosis and resist the chemotherapy [113-115]. BCL-2 phosphorylation was reported as an element for full BCL-2 death suppressor signaling activity [116]. However, phosphorylated BCL-2 activated by paclitaxel showed a converse ability to promote apoptosis in several tumor cell lines indicating

phosphorylation [117-121]. Importantly, the phosphorylated BCL-2 activated by hypericin mediated PDT was also found to exert the function to promote the tumor cells apoptosis [107].

In this thesis, the results show MTD as a MOR agonist has an ability to up-regulate the activated JNK and phosphorylated BCL-2 treated by 5-ALA-PDT on tumor cells ($p<0.05$). The phosphorylated BCL-2 in this thesis induced by MTD plus 5-ALA-PDT shows an ability to promote the apoptosis of tumor cells [2]. Naloxone, a MOR antagonist, mostly inhibits the above potentiating effects of MTD on 5-ALA-PDT-induced apoptosis and the phosphorylation of JNK and BCL-2 ($p<0.05$). The 5-ALA-PDT-induced apoptosis has a positive correlation with the expression of phosphorylated JNK and BCL-2. It implies that 5-ALA-PDT-induced apoptosis can be enhanced by the combination with MTD, mostly through the MOR-mediated upregulation of phosphorylated JNK and BCL-2.

Conclusion and outlook

The efficacy of 5-ALA-PDT for some malignant tumors needs to be enhanced. In addition, the pain sensation is the main side effect of 5-ALA-PDT. In this study, MTD as a strong analgesic was proven to simultaneously increase the cytotoxicity of 5-ALA-PDT to SCC and GBM tumor cells mainly through the apoptosis pathways [1]. The 5-ALA-PDT-induced apoptosis is enhanced by MTD mainly in a MOR-dependent manner through the phosphorylation of JNK and BCL-2 [2]. This study provides a theoretical basis using MTD to improve the effectiveness of 5-ALA-PDT for SCC and GBM and to reduce the 5-ALA-PDT induced pain sensation simultaneously in clinical application. This combination also shows a promising future for the treatment of SCC, glioblastoma, and possibly also basal cell carcinoma, prostate cancer and so on. Potentially, MTD may also improve the effectiveness of other photosensitizer-mediated PDT for tumors. If the combination therapy of MTD plus 5-ALA-PDT would be successfully translated to clinical application, it will solve the existing pain problem of 5-ALA-PDT and additionally enhance its efficacy. Prior to that, more animal and clinical studies need to be done to clarify the efficacy and safety of MTD. The suitable concentration and protocol for the application of MTD also need to be investigated for the patient in the clinic. The expression of MOR on tumor cells may impact the effectiveness of MTD, so that the effectiveness might be limited in tumors that express less MOR. Although 5-ALA-PDT induced apoptosis has a positive correlation with the up-regulation of phosphorylated JNK and BCL-2, further inhibition experiments should be carried out to investigate the importance of phosphorylated JNK and BCL-2 for the MTD related effect.

Contribution and declaration

Doctoral candidate Lei Shi undertook most of the work in this research under the guidance of Prof. Dr. Ronald Sroka, Dr. Herbert Stepp and PD Dr. Heike Pohla. The main contributions of Lei Shi include assisting in the design of experiments, conducting the experiments and experimental evaluation, incl. data acquisition, data analysis and statistics, and drafting the research papers. The above studies were published as two original articles. Doctoral candidate Lei Shi is the first author of the following two published articles. The co-authors signed the "Cumulative Thesis" form and agreed to PhD student Lei Shi to use the following publications to obtain the doctoral degree in human biology. All authors confirm that the publications will not be used as part of another dissertation.

- **Shi Lei**, Buchner Alexander, Pohla Heike, Pongratz Thoma, Rühm Adrian, Zimmermann Wolfgang, Gederaas A Odrun, Zhang Linglin, Wang Xiuli, Stepp Herbert, Sroka Ronald. Methadone enhances the effectiveness of 5-aminolevulinic acid-based photodynamic therapy for squamous cell carcinoma and glioblastoma in vitro. J Biophotonics. 2019, 29:e201800468. JIF: 3.768

- **Shi Lei**, Heike Pohla, Alexander Buchner, Linglin Zhang, Thomas Pongratz, Adrian Rühm, Wolfgang Zimmermann, Odrun Arna Gederaas, Xiuli Wang, Stepp Herbert, Sroka Ronald. MOP-dependent enhancement of methadone on the effectiveness of ALA-PDT for A172 cells by upregulating phosphorylated JNK and BCL2. Photodiagnosis Photodyn Ther. 2020 Jan 13:101657. doi: 10.1016/j.pdpdt.2020.101657. JIF: 2.589

PUBLICATION I

Methadone enhances the effectiveness of 5-aminolevulinic acid-based photodynamic therapy for squamous cell carcinoma and glioblastoma in vitro

Shi Lei, Buchner Alexander, Pohla Heike, Pongratz Thoma, Rühm Adrian, Zimmermann Wolfgang, Gederaas A Odrun, Zhang Linglin, Wang Xiuli, Stepp Herbert, Sroka Ronald. J Biophotonics. 2019, 29:e201800468. JIF: 3.768

Short version:

The efficacy of 5-aminolevulinic acid - photodynamic therapy (5-ALA-PDT) for squamous cell carcinoma (SCC) and glioblastoma remains to be improved. The analgesic drug methadone is able to sensitize various tumors to chemotherapy. In this in-vitro study, the influence of methadone to the effectiveness of ALA-PDT for SCC (FADU) and glioblastoma (A172) was investigated regarding protoporphyrin IX (PpIX) fluorescence (accumulation in cell), survival rates, apoptosis, and cell cycle phase, each with or without the presence of methadone. The production of PpIX was increased by methadone in FADU cells while it was decreased in A172 cells. The survival rates of both cell lines treated by 5-ALA-PDT were significantly reduced by the combination with methadone ($p<0.05$). The percentage of apoptotic cells of both cell lines treated by 5-ALA-PDT was significantly increased if treated with methadone ($p<0.05$). Methadone also improved the effect of 5-ALA-PDT on the cell cycle phase arrest in the G0/G1 phase ($p<0.05$). This study demonstrates the potential of methadone to influence the cytotoxic effect of 5-ALA-PDT for both SCC and glioblastoma cell lines.

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FULL ARTICLE

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Methadone enhances the effectiveness of 5-aminolevulinic acid-based photodynamic therapy for squamous cell carcinoma and glioblastoma in vitro

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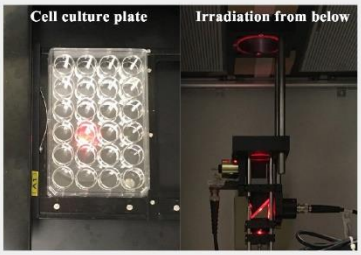
Abstract



Although having shown promising clinical outcomes, the effectiveness of 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT) for squamous cell carcinoma (SCC) and glioblastoma remains to be improved. The analgesic drug methadone is able to sensitize various tumors to chemotherapy. In this in vitro study, the influence of methadone to the effectiveness of ALA-PDT for SCC (FADU) and glioblastoma (A172) was investigated on the protoporphyrin IX (PpIX) fluorescence, survival rates, apoptosis, and cell cycle phase, each with or without the presence of methadone. The production of PpIX was increased by methadone in FADU cells while it was decreased in A172 cells. The survival rates of both cell lines treated by ALA-PDT were significantly reduced by the combination with methadone ($P < .05$). Methadone also significantly increased the percentage of apoptotic cells and improved the effect of ALA-PDT on the cell cycle phase arrest in the G0/G1 phase ($P < .05$). This study demonstrates the potential of methadone to influence the cytotoxic effect of ALA-PDT for both SCC and glioblastoma cell lines.

KEYWORDS

5-aminolevulinic acid-based photodynamic therapy (ALA-PDT), apoptosis, cell cycle phase, glioblastoma, methadone, squamous cell carcinoma (SCC)

1 | INTRODUCTION

5-Aminolevulinic acid-based photodynamic therapy (ALA-PDT) is well established for the treatment of early stage skin tumors and is under investigation for the treatment of other cancers [1–3]. After uptake of exogenous ALA, tumor cells synthesize large quantities of intracellular protoporphyrin IX (PpIX). PpIX as a photosensitizer can transfer the energy from

light to oxygen, generating reactive oxygen species, which damage or kill tumor cells [4, 5]. In the clinic, ALA-PDT has many advantages such as allowing repetitive application to multiple lesions, non-trauma, excellent cosmetic results and functional recovery at special skin sites [6]. It has been widely applied for the treatment of actinic keratosis, basal cell carcinoma, cutaneous squamous cell carcinoma (SCC), glioblastoma and other cancers [7, 8]. However, it is difficult to eliminate invasive SCC or large glioblastoma with ALA-PDT alone [2, 9]. Therefore, the effectiveness of ALA-PDT for SCC and glioblastoma remains to be improved [10, 11].

Pain is the most severe side effect of ALA-PDT on skin tumors. Nowadays, pain is the key limitation for the application of ALA-PDT in dermatology [12]. During PDT irradiation, patients always suffer different levels of pain [13]. Sometimes, due to the severe pain caused by the photodynamic reaction, the irradiation has to be interrupted until a nerve block anesthesia is implemented to relieve the pain [14]. Because of the pain during treatment, some patients frequently move the body to escape the light, resulting in the reduction of irradiance on tumors. Moreover, some patients refuse to accept the next round of PDT just because of pain.

Methadone is a long-acting opioid receptor agonist that has been used extensively as a highly effective and safe medication in the treatment of pain [15]. Some recent studies claim an ability of methadone to sensitize glioblastoma, prostate cancer, leukemia, bladder cancer and other tumors to the treatment of doxorubicin or cisplatin in vitro or in vivo [16–22]. So far, the sensitizing effect of methadone was mainly observed for chemotherapeutic agents where it promoted intrinsic apoptosis [17, 21–23]. Considering its good analgesic effect, combining methadone with ALA-PDT might be a promising treatment modality for tumors. However, the sensitizing effect of methadone on ALA-PDT for SCC or glioblastoma remains to be clarified.

This study was performed to investigate, whether the in vitro effectiveness of ALA-PDT for SCC and glioblastoma is affected when applied in combination with methadone.

2 | METHODS

2.1 | Cell incubation

FADU cells were originally obtained from the Deutsche Managementsystem Zertifizierungsgesellschaft. A172-cells were purchased from the American Type Culture Collection. Both cell lines were cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Germany) supplemented with 10% fetal bovine serum (Biochrom AG, Germany), penicillin (100 IU/mL), streptomycin (100 µg/mL) and 1% MEM non-essential amino acids solution (Gibco; Thermo Fisher Scientific).

2.2 | Photodynamic therapy

ALA (Fagron GmbH & Co. KG, Barsbüttel, Germany) stock solutions were freshly prepared in serum free medium each time. Cells were incubated in serum free medium containing ALA for 24 hours. Cells were irradiated with laser light delivered by a fiber (core diameter: 600 µm) with custom-made projection optics connected to a 635 nm Ceralas diode laser (CeramOptec GmbH, Bonn, Germany) at irradiation of 100 mW/cm^2 irradiance in a temperature stabilized ($T = 37°C$) light tight box [24].

2.3 | Measurement of cytotoxicity

The cytotoxicity was assessed based on survival rates as determined at 24 hours after irradiation by the Cell Counting Kit-8 (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) assay. Initially the measurement of cytotoxicity was used to determine a sublethal treatment parameter set of ALA-PDT alone, used to achieve around 25% killing of cells, by means of different ALA and light parameters. Then, to investigate the impact of methadone on ALA-PDT, the cytotoxicity of different treatment protocols as indicated in Table 1 was investigated. All experiments in this study were performed in triplicates and biologically repeated three times.

2.4 | Methadone incubation scheme

To investigate the effect of methadone on ALA-PDT-induced cytotoxicity, cells were treated with 10 µg/mL D, L-methadone (Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany) for 3 days (Figure 1). To find the optimal timing of methadone treatment, incubation with methadone for 3 days in the whole ALA-PDT period was further compared with incubation for 2 days before irradiation and/or for 1 day after irradiation. The detailed incubation and irradiation scheme is depicted in Figure 1.

2.5 | Uptake of ALA to induce PpIX fluorescence

The production of PpIX fluorescence in each group was measured using a flow cytometer (FACSCalibur, Becton Dickinson Heidelberg, Germany). PpIX fluorescence was induced by a $\lambda = 635$ nm red diode laser and measured in the FL3 photomultiplier tube (670-nm long pass filter) of the flow cytometer [25]. The mean fluorescence intensity (MFI) of PpIX fluorescence was calculated using a specified software (FlowJo v10 Workspace, FlowJo LLC, Oregon).

TABLE 1 Groups of in vitro experiments

Application	Control groups							Treatment group
	Untreated control	Methadone only	ALA only	ALA plus methadone	Light only	Methadone plus light	ALA-PDT	ALA-PDT plus methadone
ALA	−	−	+	+	−	−	+	+
Methadone	−	+	−	+	−	+	−	+
Light	−	−	−	−	+	+	+	+

Abbreviations: ALA, 5-aminolevulinic acid; ALA-PDT, 5-aminolevulinic acid-based photodynamic therapy.

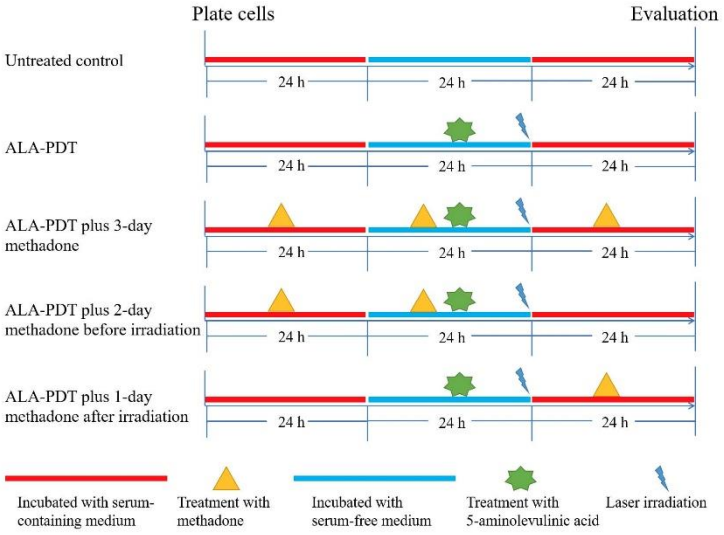


FIGURE 1 The flow chart of the experiment to find the optimal timing of methadone treatment

2.6 | Apoptosis rate

The tumor cells were collected from 24-well plate into flow cytometry tubes at 24 hours after irradiation. Then, cells were washed with 1 mL PBS twice and re-suspended in 200 μL of Annexin V buffer with 5 μL Annexin V-FITC and 5 μL 7-AAD (BD Biosciences, Heidelberg, Germany) for 20 minutes. The apoptotic rate was determined by flow cytometry. The Annexin V-positive cells were acknowledged as apoptotic cells.

2.7 | Determination of cell cycle phase

Tumor cells were harvested as mentioned in the Apoptosis Rate section. Cells were fixed in icecold 70% ethyl alcohol for 4 hours and washed with PBS twice. Then cells were treated with 100 μL of a 100 μg/mL ribonuclease solution (Abcam, Berlin, Germany) and 20 μL 7-AAD (BD Biosciences) for 20 minutes before measurement using flow cytometry [26]. The cycle phase was calculated using a cell cycle analysis software (ModFit LT v3.2; Verity Software House, Inc., Maine).

2.8 | Statistical methods

Statistical tests were performed using SPSS 13.0 software (IBM Corporation, Armonk, New York). Mann-Whitney *U* test was used to compare the survival rates, PpIX fluorescence signals, apoptosis rates and cell cycle phases between different groups. *P*-values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Parameters for sublethal photodynamic therapy

To find the appropriate treatment parameters for sublethal photodynamic therapy for each cell line separately, which causes approximately 25% cell death remaining sensitive for any enhancement of cytotoxicity by co-incubation with methadone, the survival rates of both cell lines after ALA-PDT using different ALA concentrations and light doses were determined. The results show ALA-PDT killed tumor cells in a dose-dependent manner. Generally, a higher ALA concentration or

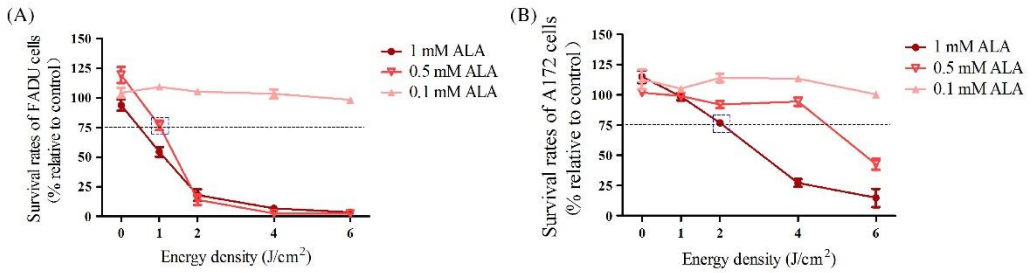


FIGURE 2 Determination of parameters for sublethal 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT). The survival rates (mean ± SEM, n = 9) of squamous cell carcinoma FADU cell line (A) and glioblastoma A172 cell line (B) following ALA-PDT

light dose led to a lower survival rate as shown in Figure 2. In case of FADU the sublethal therapy of 76.8% ± 0.8% (mean ± SEM) of survived FADU cells can be induced by incubation with 0.5 mM ALA and an irradiation of 1 J/cm^2. For A172 cells, a similar mean survival rate of 76.8% ± 0.5% was measured when incubated with 1 mM ALA and irradiated with 2 J/cm^2. Therefore, these parameters were used for further experiments to investigate the impact of methadone.

3.2 | Influence of methadone on ALA-mediated PpIX accumulation

As sketched in Figure 1, cells were incubated with 10 µg/mL D, L-methadone for 2 days for the determination of PpIX fluorescence before irradiation. Based on the specific treatment parameters to achieve sublethal PDT, FADU cells were incubated with 0.5 mM ALA, while A172 cells were incubated with 1 mM ALA, for 24 hours each. In all cases, the PpIX fluorescence histograms showed a single, relatively narrow peak, justifying the use of MFI as a quantitative measure of cellular PpIX content. As shown in Figure 3, with the FADU cell line, the MFI of PpIX fluorescence ($MFI_{ALA + MET}$ = 172.1 ± 1.1 a.u.) in the ALA plus methadone group was slightly higher than that in the ALA only group (MFI_{ALA} = 145.4 ± 0.6 a.u.). However, with the A172 the PpIX fluorescence intensity in the ALA plus methadone group ($MFI_{ALA + MET}$ = 254.1 ± 3.8 a.u.) was slightly lower than in the ALA only group (MFI_{ALA} = 297.2 ± 2.7 a.u.).

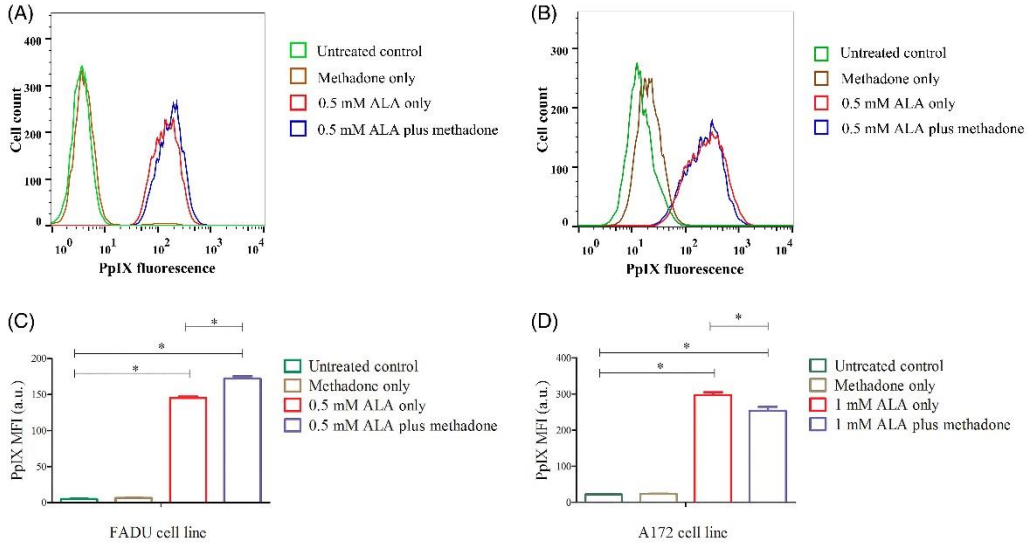


FIGURE 3 Methadone influences protoporphyrin IX (PpIX) formation only marginally. FADU cells (A and C) and A172 cells (B and D) were incubated for 24 h with 5-aminolevulinic acid (ALA) in the presence or absence of methadone. Representative histograms (A, B) and mean PpIX mean fluorescence intensity (± SEM) (C, D) are shown (n = 9). *P < .05

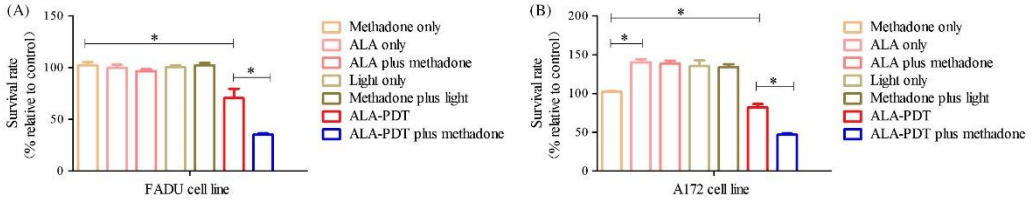


FIGURE 4 Methadone enhances 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT)-induced cytotoxicity. FADU (A) and A172 cells (B) were cultured in the presence or absence of methadone for 3 days. Survival rates (mean ± SEM) were determined 24 h later by the CCK-8 method (n = 9). *$P < .05$

3.3 | Methadone effect on ALA-PDT-induced cytotoxicity

FADU and A172 cells were treated as sketched in Table 1. The investigation of FADU cells showed survival rates of all non-PDT groups of around 100% as shown in Figure 4A. However, the survival rate of the ALA-PDT group (70.6% ± 1.7%) was significantly lower compared to the non-PDT groups ($P < .05$). Compared with the ALA-PDT group a further significant drop in the survival could be observed in the ALA-PDT plus methadone group (35.3% ± 1.4%; $P < .05$), indicating that the used methadone application protocol results in enhanced effectivity of ALA-PDT for FADU cells. A similar observation was made during investigation with the A172 cell line (Figure 4B). Of note, a significant increase in A172 cell number was observed in the ALA only (140.4% ± 2.0%), light only (135.3% ± 1.9%), ALA plus methadone (138.4% ± 1.5%) or methadone plus light (134.1% ± 0.9%) groups (Figure 4B).

The cytotoxicity of different methadone incubation scheme (Figure 1) is shown in Figure 5. It can be derived that methadone application in each time protocol leads to an enhancement of the ALA-PDT effect, with the strongest enhancement observed by incubation with methadone for all 3 days.

3.4 | Methadone-related ALA-PDT-induced apoptosis

The relative apoptosis rates by ALA-PDT, ALA-PDT plus methadone (for 3 days) and some control groups are shown in Figure 6. For both FADU cells and A172 cells, the percentage of apoptotic cells after ALA-PDT was remarkably higher compared to that obtained with the control groups. The percentage of apoptotic FADU (mean ± SEM: 53.2% ± 1.6%) or A172 cells (45.3% ± 2.2%) in the ALA-PDT plus methadone group was even significantly higher than that in the ALA-PDT groups (20.6% ± 0.6% and 22.8% ± 1.9%, respectively) ($P < .05$), indicating methadone related enhancement of ALA-PDT induced apoptotic pathway. For the A172 cell line, it was observed that the percentage of apoptotic cells in the methadone only and the ALA plus methadone groups was significantly higher than in the untreated control group, implying that methadone only might also induce some tumor cell apoptosis.

3.5 | Cell cycle related effect of methadone and ALA-PDT

To investigate whether methadone influences the effect that ALA-PDT exerts on cell cycle phases, the cell cycle phase

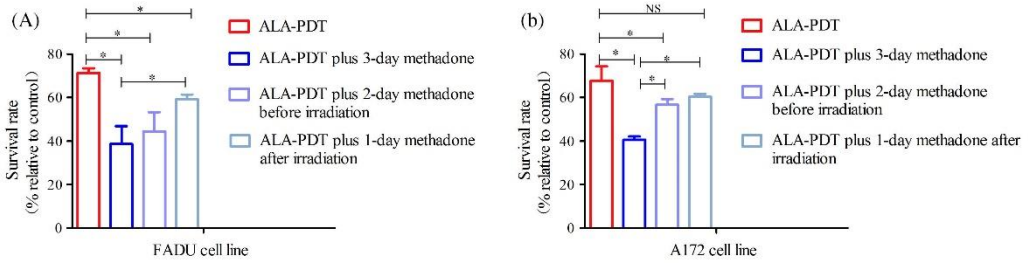


FIGURE 5 Methadone application protocol related effects on 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT). FADU cells (A) and A172 cells (B) were cultured in the presence of methadone for 1, 2, or 3 days as indicated in the legends (n = 9). *$P < .05$; NS: not significant

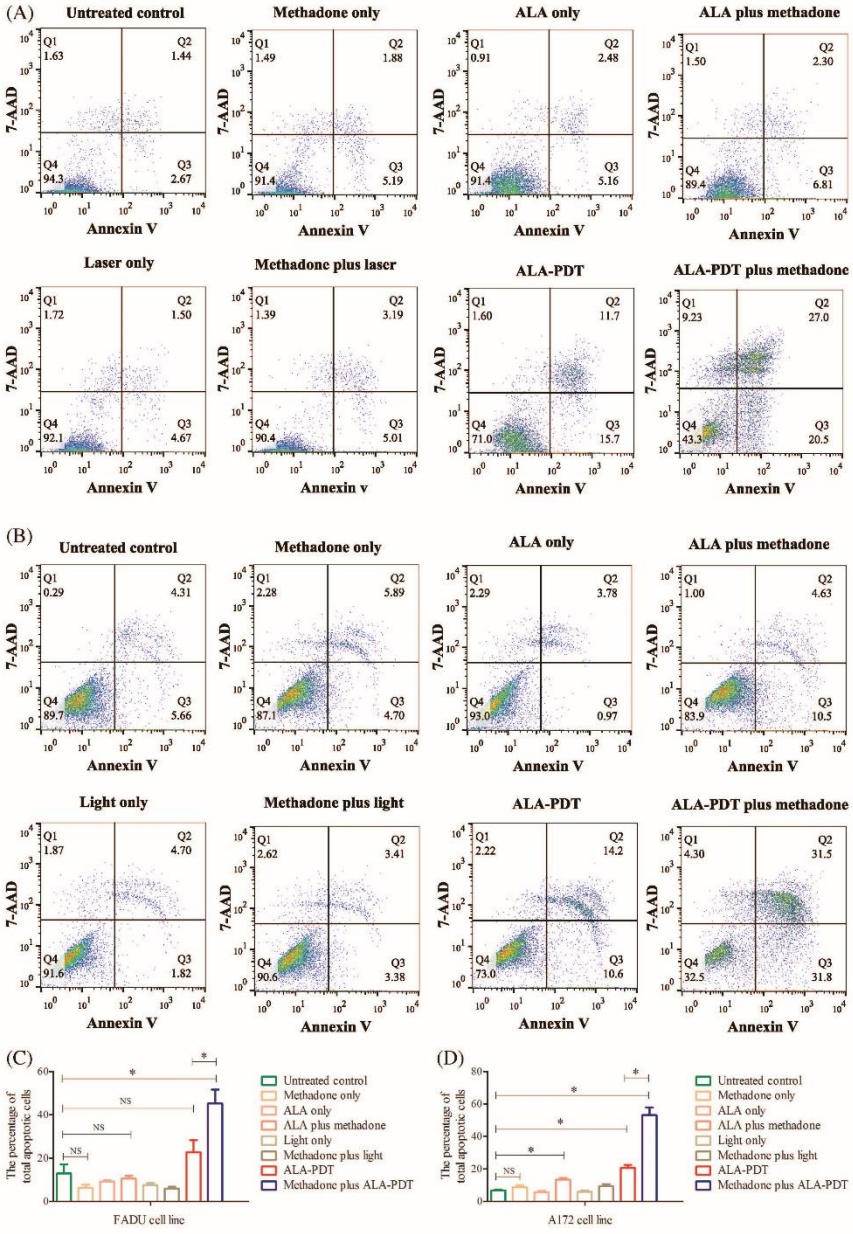


FIGURE 6 Methadone related enhancement of 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT)-induced apoptosis. FADU (A, C) and A172 cells (B, D) were cultured in the presence or absence of methadone for 3 days. Representative dot plots and percentage of apoptotic cells (mean ± SEM, n = 9) are shown. *$P < .05$

was determined by means of flow cytometry using 7-AAD DNA staining. As shown in Figure 7, for both FADU and A172 cells, ALA-PDT plus methadone significantly increased the number of cells found in the G0/G1 phase compared to ALA-PDT group ($P < .05$), methadone only ($P < .05$) and untreated controls ($P < .05$) (Figure 7A,C).

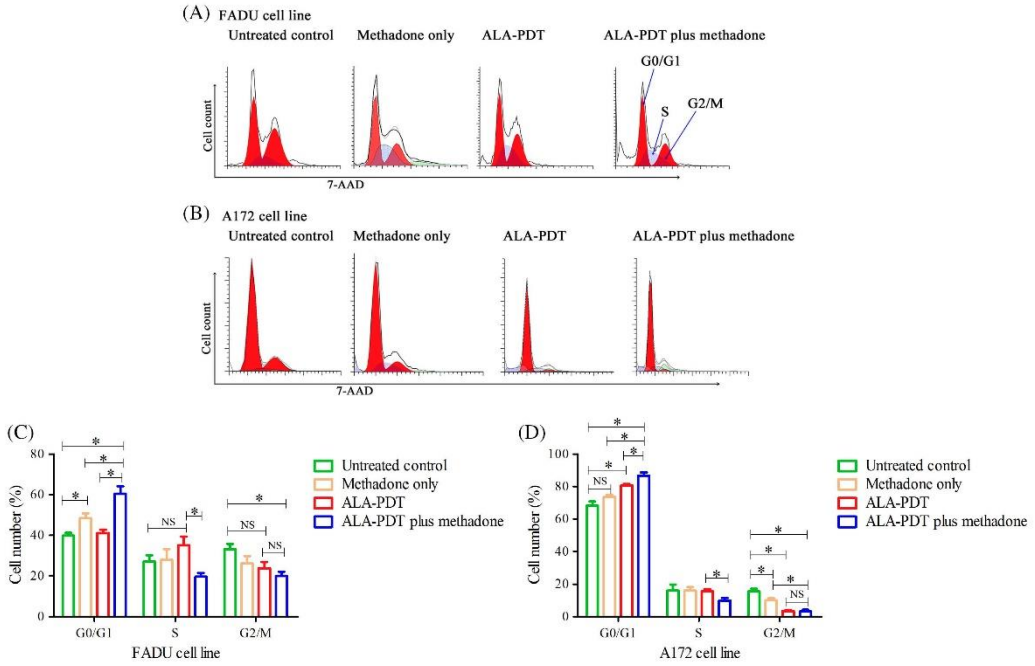


FIGURE 7 Influence of 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT) plus methadone on the cell cycle. Representative histograms for FADU (A) and A172 cells (B) are shown. In C and D the percentage of cells in the different cell cycle phases after treatment with methadone, ALA-PDT or ALA-PDT plus methadone for both cell lines are given (n = 9). *$P < .05$. NS: $P > .05$

Methadone only or ALA-PDT only show a higher percentage of the number of tumor cells in the G0/G1 phase than untreated controls, though not all differences are significant. These results suggest that methadone and ALA-PDT inhibit the proliferation of tumor cells mainly through G0/G1 phase arrest. This G0/G1 phase arrest was significantly enhanced by the combination of ALA-PDT plus methadone compared to methadone only or ALA-PDT only.

4 | DISCUSSION

So far, none of the known potentiating agents of ALA-PDT contributes to pain alleviation [11, 27, 28]. Methadone, as a long-acting opioid agonist, was found to enhance the toxicity of doxorubicin to tumor cells in vitro by re-enabling apoptosis [21, 29]. In ALA- PDT apoptosis is also an important cell death mechanism [11]. If methadone could enhance the effectiveness of ALA-PDT, an improved protocol based on a combination of ALA-PDT with methadone could be beneficial in the clinic, being less painful and more effective at the same time.

The results of the initial PDT-dose finding study showed that the FADU cell line was slightly more sensitive to ALA-PDT than the A172 cell line (Figure 2). It is well known that different cancer cell lines respond differently to ALA-PDT. In a cell survival study by Gederaas et al. [30], the A172 cells among a total of four different cell lines tested, showed the lowest cell death rate after ALA-PDT.

Despite the inconsistent effect on PpIX accumulation (Figure 3), methadone enhanced the ALA-PDT induced cytotoxicity in both cell lines quite considerably (Figure 4). This indicates that the increased susceptibility to low dose ALA-PDT induced by co-incubation with methadone is not due to an increased accumulation of PpIX, but might well have been enabled by a methadone-induced activation of apoptosis pathways as hypothesized in the context of a combination of chemotherapeutic drugs with methadone [29]. The results showed that both methadone incubated before and after irradiation contributed to the enhancement of ALA-PDT (Figure 5). This indicates that irrespective of the time-point of methadone incubation with respect to ALA-PDT, the supporting action of methadone was more pronounced at longer incubation times. As the strongest effect was observed at the longest incubation time, future settings should investigate even longer incubation times. However, this might require a reduction in

methadone concentration to avoid interference with effects induced by methadone alone.

The PpIX distribution in the flow cytometry histograms (Figure 3), although showing a single peak, still shows that cells are containing variable amounts of PpIX. This is similar to a clinical PDT scenario, be it invading glioblastoma cells or deeper lying skin SCC cells, which contain less PpIX or get less light than the average. If methadone could sensitize these cells to undergo apoptosis, which otherwise would not, this promises a clinical benefit.

For A172 cells, it was noted that the survival rate in ALA only, ALA plus methadone, light only or methadone plus light group was higher than in untreated control and the methadone group. The increase of cell proliferation after application of ALA was also reported for the colon adenocarcinoma cell line SW620 [31]. ALA, as an amino acid is a critical component in the body's synthesis of heme, which in turn is a major component of hemoglobin and also an essential component of cytochrome C [32, 33]. A low dosage of exogenous ALA may promote the production of cytochromes C which increases the mitochondrial activity conducive to cell proliferation [34]. With respect to light, the increase of cell proliferation after light application was also observed in epithelial cells [35], fibroblasts [36], and other cell types [37]. A low-dose of light can activate mitochondrial retrograde signaling leading to an upregulated expression of cell proliferation related genes to participate in this stimulation of cell proliferation [38]. However, such stimulated proliferation in the "ALA only" and "light only" groups was only observed with the A172 cell line, not with the FADU cell line. This indicates that these stimulations are cell line specific. It is well known that cells respond differently to low doses of light [39], with the effect ranging from inhibition to well above 20% increase in colony forming units [40], where the exact mechanisms are still under debate [41]. Also for ALA alone, it is plausible that cells may get a growth stimulus by promoted heme synthesis, but it remains unclear, why some do and others not.

The precise mechanism how methadone increases the cytotoxicity of ALA-PDT or chemotherapeutic drugs for tumor cells is still unclear. In this study, methadone significantly increased the apoptosis rates of tumor cells induced by sublethal ALA-PDT (Figure 6). This implies that the sensitizing effect of methadone for ALA-PDT was mainly conveyed by interaction with apoptosis pathways. This is supported by the observation that methadone can activate the intrinsic apoptosis pathway through the activation of caspase-9 and caspase-3 and down-regulation of BCL2L1, (BCL-X), BCL2 and XIAP to induce tumor cell death [17, 23, 29].

In this study, ALA-PDT and methadone alone slightly arrested the tumor cells in the G0/G1 phase to prevent the cells from entering the replicative phase. This effect was improved by the combination of ALA-PDT and methadone (Figure 7). It was reported that ALA-PDT induced the cell cycle phase arrest of tumor cells predominantly by the upregulation of P53 and DNA damage [42, 43]. Both the upregulation of P53 and DNA damage might activate the checkpoints of the cell cycle, leading to the inhibition of tumor cells proliferation [44]. However, the exact mechanism by which methadone influences the cell cycle remains to be investigated.

5 | CONCLUSION

This investigation showed the enhancement of methadone to ALA-PDT by activation of apoptosis pathways and increased cell cycle phase arrest. A clear and consistent evidence that methadone leads to an increased accumulation of PpIX could not be found. The additional interaction of methadone and ALA-PDT should be tested in further cell lines. The exact mechanisms how methadone may influence the effectiveness of ALA-PDT for tumor cells should be investigated.

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Please see Supporting Information online.

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PUBLICATION II

MOP-dependent enhancement of methadone on the effectiveness of ALA-PDT for A172 cells by upregulating phosphorylated JNK and BCL2

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Short version:

Methadone, as a long-acting opioid analgesic, shows an ability to sensitize the treatment of 5-ALA-PDT for glioblastoma cells (A172) *in vitro* by promoting apoptosis. However, the mechanisms how methadone enhances the effectiveness of 5-ALA-PDT for tumor cells remains to be clarified. The expression of mu opioid receptor (MOP), apoptosis, phosphorylated c-Jun N-terminal kinase (JNK) and phosphorylated apoptosis regulator B cell lymphoma 2 (BCL2) were measured by flow cytometry. Cytotoxicity was determined using Cell Counting Kit-8 (CCK-8). A MOP antagonist, naloxone, was used to evaluate the role of MOP in the abovementioned process. It was found that A172 cells show the expression of MOP and that naloxone inhibits the enhancement of the methadone effect on apoptosis following 5-ALA-PDT ($p<0.05$). The expression of phosphorylated JNK and BCL2 induced by 5-ALA-PDT were enhanced in the presence of methadone ($p<0.05$). These methadone effects were also inhibited by naloxone ($p<0.05$). The results suggest that apoptosis induced by 5-ALA-PDT is enhanced by methadone, mostly MOP-mediated, through the upregulation of phosphorylated JNK and BCL2, leading to improved cytotoxicity of 5-ALA-PDT in A172 cells.

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MOP-dependent enhancement of methadone on the effectiveness of ALA-PDT for A172 cells by upregulating phosphorylated JNK and BCL2

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ABSTRACT

Background: Methadone, as a long-acting opioid analgesic, shows an ability to sensitize the treatment of ALA-PDT for glioblastoma cells (A172) *in vitro* by promoting apoptosis. However, the mechanisms how methadone enhances the effectiveness of ALA-PDT for tumor cells remains to be clarified.

Methods: The expression of mu opioid receptor (MOP), apoptosis, phosphorylated c-Jun N-terminal kinase (JNK) and phosphorylated apoptosis regulator B cell lymphoma 2 (BCL2) were measured by flow cytometry. Cytotoxicity was determined using Cell Counting Kit-8 (CCK-8). A MOP antagonist, naloxone, was used to evaluate the role of MOP in the above process.

Results: It was found that A172 cells show the expression of MOP and that naloxone inhibits the enhancement of the methadone effect on apoptosis following ALA-PDT ($p < 0.05$). Phosphorylated JNK and BCL2 induced by ALA-PDT were promoted in the presence of methadone ($p < 0.05$). These methadone effects were also inhibited by naloxone ($p < 0.05$).

Conclusions: The results suggest that apoptosis induced by ALA-PDT is enhanced by methadone, mostly MOP-mediated, through the upregulation of accumulation of phosphorylated JNK and BCL2, leading to a promotion of cytotoxicity of ALA-PDT for A172 cells.

1. Introduction

5-Aminolevulinic acid-based photodynamic therapy (ALA-PDT) is a well-established non-invasive antitumor treatment in dermatology [1,2]. It is also applied for the treatment of glioblastoma [3,4]. The main drawback of ALA-PDT is its limited effectiveness on invasively growing or large tumors [5,6]. To improve the effectiveness of ALA-PDT, several potentiating agents, such as the iron chelator deferoxamine, calcitriol, methotrexate, gefitinib, metformin and imiquimod have been investigated in combination with ALA-PDT [7–13]. However, the above potentiating effects have not reached full satisfaction.

Pain is one of the main symptoms of cancers [14]. ALA-PDT may also induce severe pain during or after irradiation, which limits the application of ALA-PDT especially in dermatology [15,16]. Methadone, as a long-acting opioid analgesic [17] is used as a pain medication for cancer patients. Interestingly, methadone has also been shown to sensitize glioblastoma, leukemia, and other tumors to the treatment of doxorubicin or cisplatin *in vitro* and *in vivo* [18–23]. In our previous research, the sensitization effect of methadone was also observed on ALA-PDT with a sublethal treatment parameter set for glioblastoma cells (A172) *in vitro* by promoting apoptosis [24].

Methadone is an opioid receptor agonist with a high affinity to the mu opioid receptor (MOP) [25]. The mechanisms of methadone reported to enhance the cytotoxicity of chemotherapy for tumor cells include: 1) Activation of MOP leads to the reduction of cyclic adenosine monophosphate (cAMP) production *via* inhibition of adenylyl cyclase [26]. cAMP as a second messenger controls cell proliferation, differentiation, and apoptosis. The downregulation of cAMP induced by MOP

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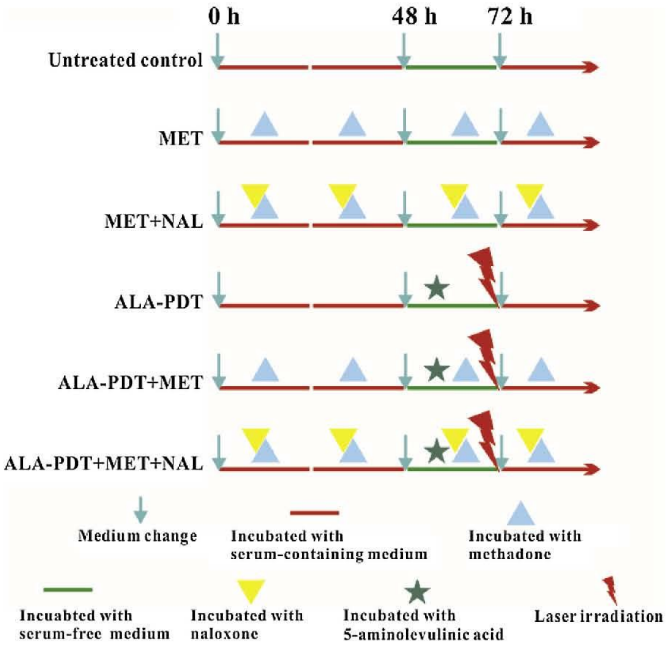

Fig. 1. Experiment groups and flow chart for treatments and drug administration. The serum-free medium was used during treatment with 5-aminolevulinic acid. Tumor cells were irradiated after a 24 h incubation with 5-aminolevulinic acid.

activation using methadone sensitizes tumor cells to doxorubicin-induced apoptosis [19,22]. 2) Methadone induces death of tumor cells through the activation of caspase-9 and caspase-3, down-regulation of B-cell lymphoma-extra large (BCL-XL) and X chromosome–linked inhibitor of apoptosis (XIAP) [27]. However, the mechanism how methadone enhances the effectiveness of ALA-PDT for tumor cells remains to be clarified.

The c-Jun N-terminal kinase (JNK) activity pathway, also named stress-activated protein kinase pathway (SAPK) is essential for providing a cellular response to many types of stress, including reactive oxygen species, ultraviolet, γ-irradiation, and anticancer drugs (cisplatin, doxorubicin, or etoposide) [28,29]. ALA-PDT initiates apoptotic cell death *via* activation of the JNK pathway in HaCaT cells, human non-small cell lung carcinoma cells and oral cancer Ca9-22 cells [30–33]. Activated MOP also shows the ability to initiate the JNK pathway [34,35]. Therefore, the question arises, whether methadone as a MOP agonist can activate JNK and enhance the activation of JNK induced by ALA-PDT in A172 cells.

The JNK pathway shows the ability to induce phosphorylation of B-cell lymphoma-2 (BCL2). Non-phosphorylated BCL2 is a 26-kDa integral membrane oncoprotein which shows the ability to suppress apoptosis [36]. However, the exact mechanism of apoptosis regulation by phosphorylated BCL2 is unclear yet and conflicting results have been reported [37,38]. On the one hand, growth factor or nicotine-induced BCL2 phosphorylation was found to suppress apoptosis that plays a role in resistance to chemotherapy [39–42]. On the other hand, paclitaxel-induced BCL2 phosphorylation showed the ability to promote apoptosis in several tumor cell lines indicating that phosphorylation inhibits BCL2 anti-apoptotic function [43–47]. Hypericin mediated PDT was also found to induce BCL2 phosphorylation and promotes apoptosis of tumor cells [33]. However, methadone was reported to downregulate BCL2 phosphorylation and to promote cytotoxicity in leukemia cells [48]. Therefore, the effects of ALA-PDT plus methadone on BCL2 phosphorylation and apoptosis are very intriguing and worth exploring.

Generally, it would be interesting to explore the mechanism how methadone enhances the effectiveness of ALA-PDT for A172 cells. We therefore investigated, whether the activation of apoptosis is caused by a MOP-dependent activation of the JNK pathway. We also addressed the relationship between apoptosis and phosphorylated BCL2.

2. Methods

2.1. Cell culture

The glioblastoma cell line A172 and the chronic myelogenous leukemia cell line K562 were purchased from the American Type Culture Collection and cultured in DMEM medium (Gibco, Thermo Fisher Scientific, Germany) supplemented with 10 % fetal bovine serum (FBS, Biochrom AG, Germany), penicillin (100 IU/mL), streptomycin (100 µg/mL) and MEM non-essential amino acids (Gibco, Thermo Fisher Scientific, Germany). The cells were kept at 37 °C in a humidified atmosphere with 5 % CO_2 and passaged using 0.05 % trypsin/0.02 % EDTA every three days.

2.2. Measurement of the expression of mu opioid receptor

MOP expressions on A172 and K562 cells were determined by flow cytometry staining with opioid receptor mu 1 (OPRM1) polyclonal antibody (44-308G; Invitrogen, Thermo Fisher Scientific, Germany). Tumor cells were harvested from a 6-well plate into flow cytometry tubes and washed with phosphate-buffered saline (PBS) twice. 1×10^6 of cells were re-suspended in 100 µL PBS and treated with 5 µL OPRM1 polyclonal antibody for 1 h. After washing with PBS, cells were re-suspended in 250 µL PBS and treated with 1 µL goat anti-rabbit IgG

cross-adsorbed secondary antibody (FITC conjugate) (Invitrogen, Thermo Fisher Scientific, Germany) for 1 h. Finally, MOP was measured using a flow cytometer (FACSCalibur, Becton Dickinson Heidelberg, Germany). The fluorescence was excited by a 488 nm diode laser and measured in the FL1 (530/30 bandpass filter) photomultiplier tube of the flow cytometer. The mean fluorescence intensity (MFI) was calculated using a FlowJo v10 Workspace (FlowJo LLC, OR, USA). The value of MOP MFI of tumor cells was calculated by subtracting the mean MFI of tumor cells stained only with secondary antibody.

2.3. Experimental groups

The experiments in this study consisted of untreated control, methadone (MET) only, methadone plus naloxone (MET + NAL), ALA-PDT, ALA-PDT plus methadone (ALA-PDT + MET) and ALA-PDT plus methadone and naloxone (ALA-PDT + MET + NAL) groups. A172 cells were used to investigate the methadone effect on ALA-PDT for glioblastoma cells. The flow chart for administrations of drugs and treatments in each group are shown in Fig. 1. Methadone used in this study was D, L-methadone (Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany) at a final concentration of 0.065 μM.

2.4. Photodynamic therapy

A sublethal treatment parameter set of ALA-PDT was used in this study. The protocol of ALA-PDT was similar as we described in our previous work [24]. ALA (Fagron GmbH & Co. KG, Barsbüttel, Germany) solutions were freshly prepared in serum free medium each time. All drug and laser treatments were performed as Fig. 1 shows. A172 cells were incubated in serum-free medium containing 1 mM ALA for 24 h. Cells were irradiated with laser light delivered by a fiber (core diameter: 600 μm) with custom-made projection optics connected to a 635 nm Ceralas diode laser (CeramOptec GmbH, Bonn, Germany) at an irradiation of 100 mW/cm^2 for 2 J/cm^2 in a temperature stabilized (T = 37 °C) light tight box.

2.5. Quantification of apoptotic cells

A172 cells (1×10^6 per well) were plated in 6-well plates and treated according to the protocols in Fig. 1. Cells were collected into flow cytometry tubes at 24 h after irradiation. Then, cells were washed with 1 mL PBS twice and re-suspended in 200 μL of annexin V buffer with 5 μL annexin V-FITC and 5 μL 7-aminoactinomycin D (7-AAD, BD Biosciences, Heidelberg, Germany) for 20 min. The apoptosis rate was determined by flow cytometry. Annexin V-positive cells were considered as apoptotic.

2.6. Measurement of the expressions of JNK and phosphorylated BCL2

A172 cells were harvested from 6-well plates and washed with PBS twice. Cells were treated with 100 μL of fixing medium from the FIX & PERM Kit GAS-002 (Nordic-MUbio, Susteren, Netherlands) according to the manufacturer's protocol. After washing with 4 mL of PBS, cells were treated with 100 μL of permeabilization medium and 2 μL of monoclonal mouse anti-phospho-SAPK/JNK (Thr183/Tyr185) (G9) antibody (Alexa Fluor® 647 conjugate) or 2 μL of rabbit anti-phospho-BCL2 (Ser70) (5H2) monoclonal antibody (Alexa Fluor® 488 conjugate) (Cell Signaling Technology, Frankfurt, Germany) to measure the expression of JNK and phosphorylated BCL2, respectively [49]. After 1 h of incubation under light protection, expressions of phopho-JNK and phopho-BCL2 were quantified using a flow cytometer (FACSCalibur, Becton Dickinson Heidelberg, Germany). The fluorescence of anti-JNK antibodies was excited by a 635 nm red diode laser and measured in the FL4 (661/16 bandpass filter) photomultiplier tube of the flow cytometer. The fluorescence of anti-BCL2 antibodies was excited by a 488 nm red diode laser and measured in the FL1 (530/30 bandpass filter) photomultiplier tube of the flow cytometer. The MFI was calculated using the FlowJo v10 Workspace. The final MFI of JNK or BCL2 was calculated by subtracting the mean MFI without staining.

2.7. Measurement of cytotoxicity

The cytotoxicity was assessed based on survival rates as determined by the Cell Counting Kit-8 (CCK-8) method [6]. To each well of a 96-well plate, 10 μL of CCK-8 (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) solution were added. After 2 h of incubation, the optical density of each well was measured at λ = 450 nm using an ELISA Microplate Reader (EMax® Endpoint ELISA Microplate Reader, Molecular Devices, San Jose, CA, USA). The cell survival rate (CSR) in % was calculated as:

$$\left(CSR[\%] = \frac{optical\ density\ value\ in\ treated\ well - blank}{optical\ density\ value\ in\ control\ well - blank} \times 100 \right).$$

2.8. Statistical methods

All experiments in this study were performed in triplicates and biologically repeated three times. Statistical tests were performed using SPSS 13.0 software (IBM Corporation, Armonk, NY, USA). Mann-Whitney-U-test was used to compare the expressions of MOP, phosphorylated JNK, phosphorylated BCL2, apoptosis and cytotoxicity between different groups. P-values < 0.05 were considered statistically significant.

3. Results

3.1. A172 cells express MOP

The expression of MOP on A172 cells was measured using flow cytometry. K562 cells were used as a positive control known to express MOP [50]. Fluorescence of A172 cells stained by both anti-MOP antibody and secondary antibody was stronger than that obtained only with secondary antibody, indicating MOP expression on A172 cells (Fig. 2A). K562 cells show a more apparent fluorescence difference between cells stained with or without anti-MOP antibody. The MOP MFI (mean ± SEM) (Fig. 2B) of A172 cells was 124.0 ± 19.2 a.u. which was lower than that of K562 cells (482.6 ± 20.3), indicating A172 cells did express MOP but less than K562 cells.

3.2. MOP antagonist inhibits the sensitizing effect of methadone on ALA-PDT-induced apoptosis

To explore whether methadone enhances the ALA-PDT-induced apoptosis of A172 cells by binding with MOP, a MOP antagonist, naloxone [51] was used to competitively bind the MOP. Apoptosis was measured using flow cytometry. The administrations of drugs and treatments of the experimental groups are sketched in Fig. 1. A172 cells were treated with 0.065 μM of D, L-methadone in the presence of varying concentrations of naloxone (0.065, 0.325, 0.65 or 1.3 μM) as shown in Fig. 3. The percentage of apoptotic A172 cells (mean ± SEM) in the untreated control group was 5.1 % ± 0.3 %. The fraction of apoptotic cells in MET and MET + 0.65 μM NAL groups was 11.1 % ± 0.9 % and 13.0 % ± 0.9 %, respectively. Both were higher than in the untreated control group ($p < 0.05$). The fraction of apoptotic cells in the ALA-PDT group was 23.2 % ± 1.4 %, which was significantly higher than in the untreated control, MET or MET + 0.65 μM NAL group ($p < 0.05$). The fraction of apoptotic cells in the ALA-PDT + MET group was 48.0 % ± 2.1 % which was higher than in the ALA-PDT group ($p < 0.05$). Apoptosis in ALA-PDT + MET + NAL groups decreased with increasing concentrations of naloxone. In the ALA-PDT + MET + 0.065 μM and 0.325 μM NAL groups, the apoptosis

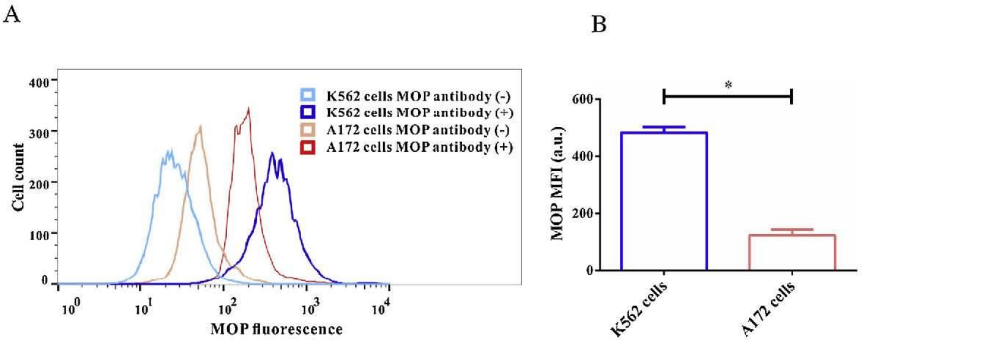

Fig. 2. A172 cells express mu opioid receptor (MOP). The expressions of MOP on A172 cells and K562 cells were measured by flow cytometry staining with OPRM1 polyclonal antibody. Representative histograms (A) and mean MOP MFI (± SEM) (B) are shown (n = 9). MOP antibody (+) cells were stained with both OPRM1 polyclonal antibody and FITC-conjugated secondary antibody; MOP antibody (-): cells were stained with secondary antibody only. *: $p < 0.05$.

rates decreased to 38.0 % ± 0.7 % and 35.5 % ± 1.1 %. Both were significantly lower than ALA-PDT + MET group ($p < 0.05$). In the ALA-PDT + MET + 0.65 μM NAL group, the apoptosis rates further decreased to 29.9 % ± 1.8 % which was significantly lower than that in ALA-PDT + MET + 0.325 μM NAL group ($p < 0.05$). There was no significant difference between the ALA-PDT + MET + 0.65 μM NAL group and the ALA-PDT + MET + 1.3 μM NAL group (28.8 % ± 1.1 %) ($p > 0.05$). The above results show that methadone enhances the ALA-PDT induced apoptosis of A172 cells and that this effect can largely be inhibited by the MOP antagonist naloxone.

3.3. Accumulation of phosphorylated JNK induced by ALA-PDT is promoted by methadone

Phosphorylated JNK was quantified in permeabilized cells using flow cytometry. Phosphorylated JNKs in the ALA-PDT and ALA-PDT + MET groups were determined at 0.5, 3, 6 and 12 h after irradiation (Fig. 4). At all time-points, the amount of phosphorylated JNK in the ALA-PDT + MET group was significantly higher than that in the ALA-PDT group ($p < 0.05$). Of note, the active JNK in ALA-PDT + MET group increased with time. The MFI of phospho-JNK at 12 h after irradiation was significantly higher than that of 0.5 h ($p < 0.05$). In the ALA-PDT group, compared to that at 0.5 h, there was no significant change of the activity of JNK at different time-points ($p > 0.05$).

3.4. The stimulatory effect of methadone on ALA-PDT-induced phosphorylation of JNK is inhibited by MOP antagonist naloxone

The amounts of phosphorylated JNK at 3 h after irradiation were determined in the following A172 cells treatment groups: untreated control, MET only, NAL only, MET + NAL, ALA-PDT, ALA-PDT + MET and ALA-PDT + MET + NAL (Fig. 5). The concentration of naloxone used was 0.65 μM. The phosphorylated JNK MFI in the untreated control group was 8.3 ± 0.5. There was no significant differences of phosphorylated JNK MFI between untreated control group and the MET only (7.8 ± 0.3), NAL only (7.4 ± 0.2), or MET + NAL groups (7.6 ± 0.3) ($p > 0.05$). The phosphorylated JNK MFI in the ALA-PDT group (29.1 ± 1.7) was significantly higher than that in the untreated control group ($p < 0.05$). The phosphorylated JNK MFI in ALA-PDT + MET group (60.7 ± 2.5) was higher than that in the ALA-PDT group ($p < 0.05$). The phosphorylated JNK MFI in the ALA-PDT + MET + NAL group (47.4 % ± 1.0 %) was lower than that in the ALA-PDT + MET group ($p < 0.05$) but higher than that in the ALA-PDT group ($p < 0.05$). The above results indicate that ALA-PDT increases the amount of phosphorylated JNK, which could be promoted by the addition of methadone. This effect of methadone on the accumulation of phosphorylated JNK induced by ALA-PDT could be partly inhibited by the MOP antagonist naloxone.

3.5. The amount of phosphorylated BCL2 after ALA-PDT is increased by methadone

To explore the correlational relationship between phosphorylated BCL2 and apoptosis of ALA-PDT treated A172 cells, phosphorylated BCL2 was quantified by flow cytometry. First, phosphorylated BCL2 was determined in the ALA-PDT and ALA-PDT + MET groups at 0.5, 3, 6 and 12 h after irradiation (Fig. 6). At all time-points, the phosphorylated BCL2 in the ALA-PDT + MET group were significantly higher than that in the ALA-PDT group ($p < 0.05$). The value of phosphorylated BCL2 MFI at 3 h after irradiation was higher than that at 0.5 h in the ALA-PDT + MET group ($p < 0.05$). In the ALA-PDT group, compared to that at 0.5 h, there was no significant change of phosphorylated BCL2 at different time-points ($p > 0.05$). Methadone significantly promotes the expression of phosphorylated BCL2 in the presence of ALA-PDT.

3.6. The stimulatory effect of methadone on ALA-PDT-induced phosphorylation of BCL2 is inhibited by the MOP antagonist naloxone

Phosphorylated BCL2 was quantified in A172 cells at 3 h after irradiation in the untreated, MET only, NAL only, MET + NAL, ALA-PDT, ALA-PDT + MET and ALA-PDT + MET + NAL groups by flow cytometer. The concentrations of naloxone used with the NAL only and ALA-PDT + MET + NAL groups were 0.65 μM. The results are shown in Fig. 7. The highest amount of phosphorylated BCL2 was recorded with the ALA-PDT + MET group (50.1 ± 1.5), where neither MET only nor ALA-PDT only was significantly different from the untreated control (18.8 ± 1.4). Co-incubation with naloxone partly inhibited the effect of methadone, leading to a phosphorylated BCL2 MFI of 43.4 ± 1.0.

3.7. MOP antagonist naloxone inhibits the methadone-mediated enhancement of ALA-PDT-induced cytotoxicity

To clarify whether the MOP antagonist naloxone reverses the effect of methadone to enhance the cytotoxicity of ALA-PDT for A172 cells, the survival was measured using the CCK8 assay in untreated control, MET only, NAL only, MET only, NAL only, MET + NAL, ALA-PDT, ALA-PDT + MET and ALA-PDT + MET + NAL groups (Fig. 8). There was no significant change of survival rates in the MET only (100.3 % ± 2.6 %), NAL only (107.8 % ± 1.8 %), or MET + NAL groups (109.2

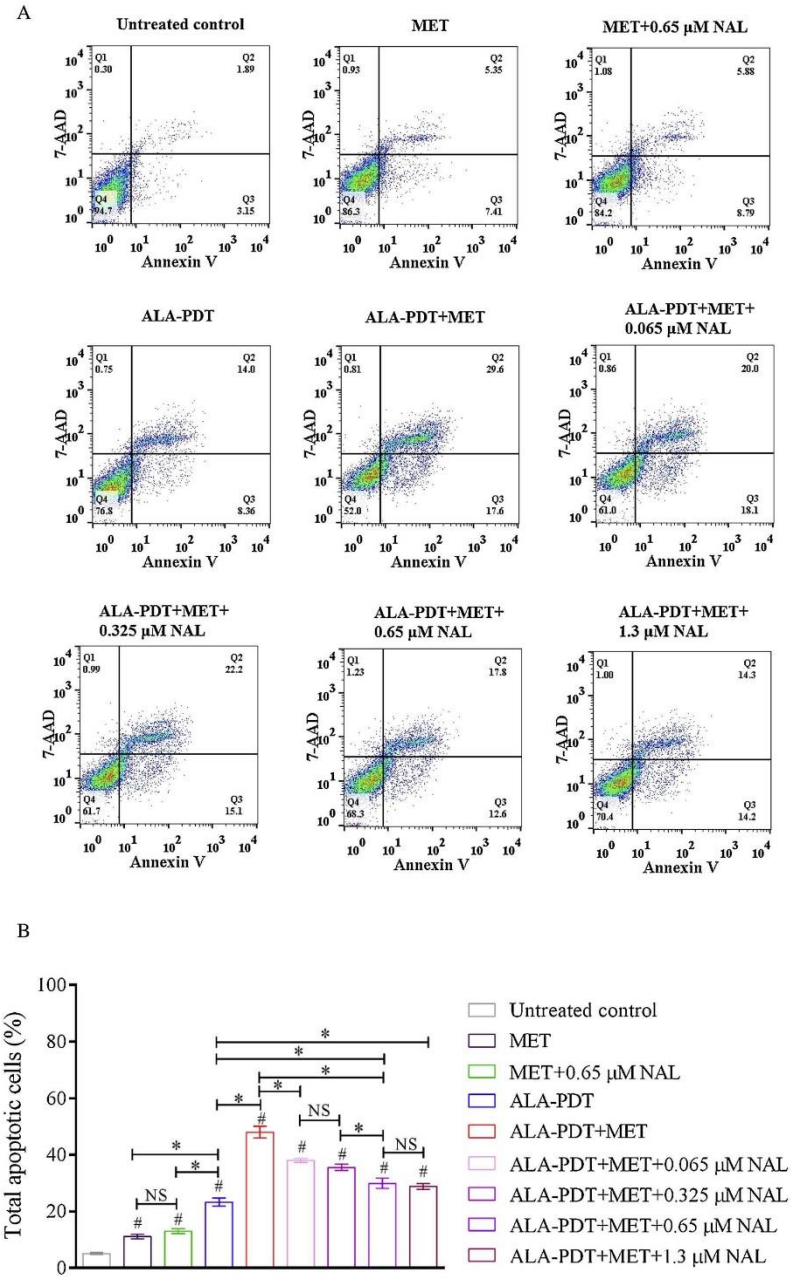

Fig. 3. Naloxone inhibits the sensitizing effect of methadone on ALA-PDT-induced apoptosis. A172 cells were treated as outlined in Fig. 1. Apoptosis was determined by with annexin V and 7-amino-actinomycin D followed by flow cytometry staining. Annexin V-positive cells were considered apoptotic. Representative dot plots (A) and percentage of apoptotic cells (mean ± SEM) (B) are shown (n = 9). *: $p < 0.05$. #: $p < 0.05$, compared to untreated control.

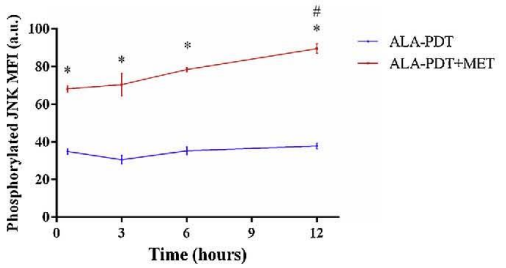

Fig. 4. Methadone enhances the effect of ALA-PDT on the accumulation of phosphorylated JNK with time. A172 cells were treated as outlined in Fig. 1. Phosphorylated JNK MFI measured by flow cytometry are shown (n = 9). *: $p < 0.05$, compared to the phosphorylated JNK MFI in ALA-PDT group at the same time point. #: $p < 0.05$, compared to the JNK MFI at 0.5 h.

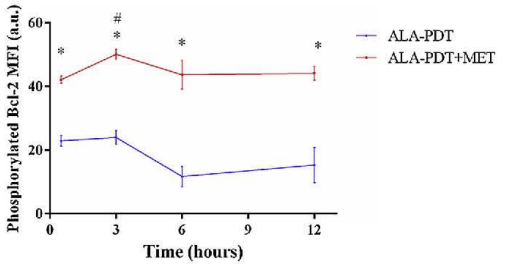

Fig. 6. The expression of phosphorylated BCL2 induced by ALA-PDT was up-regulated by methadone. A172 cells were treated as outlined in Fig. 1. Mean phosphorylated BCL2 MFI measured by flow cytometry are shown (n = 9). *: $p < 0.05$, compared to the phosphorylated BCL2 MFI in the ALA-PDT group at the same time point. #: $p < 0.05$, compared to the phosphorylated BCL2 MFI at 0.5 h.

% ± 3.4 %), compared to that in the untreated control group (100.0 % ± 2.8 %) ($p > 0.05$). Cell survival in the ALA-PDT group decreased to 77.0 % ± 6.2 %, which was significantly lower than that in the untreated control group ($p < 0.05$). A further significant drop in survival was observed in the ALA-PDT + MET group (38.1 % ± 3.8 %) compared to the ALA-PDT group ($p < 0.05$). However, in the ALA-PDT + MET + NAL group, survival was partially restored (67.6 % ± 7.7 %), which was significantly higher than that in ALA-PDT + MET group ($p < 0.05$). The above results indicate that the MOP antagonist, naloxone can partially abrogate the methadone-mediated enhancement of ALA-PDT-induced cytotoxicity in A172 cells.

4. Discussion

Methadone had been found to enhance ALA-PDT-induced apoptosis of A172 cells *in vitro* [24]. In the previous study, it was found this enhancement did not rely on the increase of 5-ALA-induced PpIX. The methadone effects on the apoptosis induction between different time-points of incubation were compared. Both before and after irradiation, methadone showed the ability to enhance the effect of ALA-PDT on tumor cells. This supporting action of methadone is positively correlated with the length of incubation times [24].

In this study, methadone was applied in the whole ALA-PDT period including pre-irradiation and post-irradiation (Fig. 1). It was investigated whether this methadone effect on ALA-PDT-induced apoptosis in A172 cells is MOP-dependent. The role of MOP in mediating the methadone effect on the ALA-PDT-induced activation of the JNK pathway, BCL2 phosphorylation and cytotoxicity was also analyzed.

Classical opioid receptors consist of MOP, kappa opioid receptors (KOP) and delta opioid receptors (DOP) [24,52]. MOP expression was found in U87 and U251 glioblastoma cells [53]. A172 as another glioblastoma cell line was also proved to express the opioid receptor [19]. In this study the expression of MOP in A172 cells was measured and compared with the expression of MOP in K562 cells, which served as positive control. The result showed that A172 cells do express MOP (Fig. 2), and that delivery of the MOP agonist naloxone at least partly reverses the methadone effect on ALA-PDT through binding to MOP [25].

To reduce the interference of PpIX fluorescence, 7-AAD was used instead of propidium iodide to stain the cells with annexin V for the measurement of apoptosis by flow cytometry. It was found that methadone enhances ALA-PDT-induced apoptosis in A172 cells and this methadone effect was significantly inhibited by MOP antagonist naloxone in a dose dependent manner. Maximum inhibition was observed at 1.3 μM naloxone (Fig. 3). The above results imply that the methadone effect on apoptosis is mostly MOP-dependent.

In this study, it was also found that JNK was activated by ALA-PDT through phosphorylation of threonine (Thr) 183 and tyrosine (Tyr) 185. This effect was further enhanced by addition of methadone. Involvement of the methadone target MOP is supported by the observation that the antagonist naloxone is able to inhibit this methadone effect (Figs. 4 and 5). Phosphorylated JNK showed a positive correlation with apoptosis in this study. Phosphorylated JNK can promote

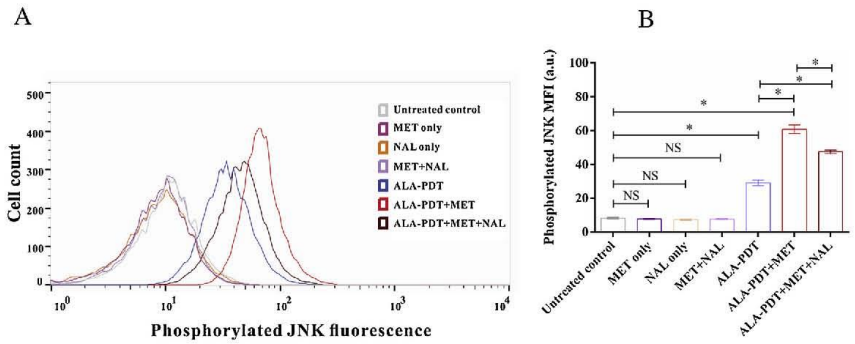

Fig. 5. Naloxone partially inhibits the stimulatory effect of methadone on ALA-PDT-induced phosphorylation of JNK. A172 cells were treated as outlined in Fig. 1. Representative histograms for phosphorylated JNK (A) and mean phosphorylated JNK MFI (B) measured by flow cytometry are shown (n = 9). *: $p < 0.05$; NS: $p > 0.05$.

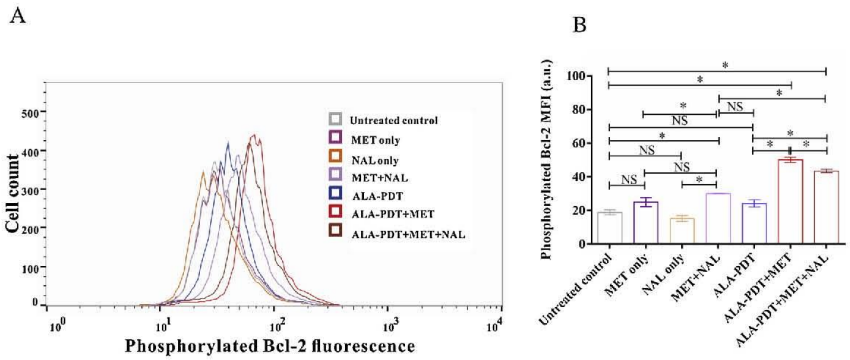

Fig. 7. Naloxone partially inhibits the stimulatory effect of methadone on ALA-PDT-induced phosphorylation of BCL2. A172 cells were treated as outlined in Fig. 1. Representative histograms for phosphorylated BCL2 (A) and mean phosphorylated BCL2 MFI (B) measured by flow cytometry are shown (n = 9). *: $p < 0.05$. NS: $p > 0.05$.

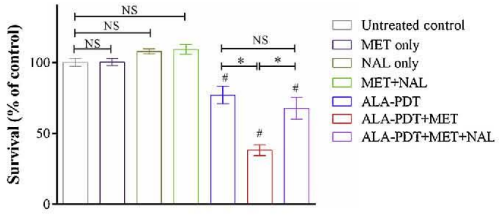

Fig. 8. Naloxone inhibits the methadone-mediated enhancement of ALA-PDT-induced cytotoxicity in A172 cells. A172 cells were treated as outlined in Fig. 1. The survival was measured by the CCK8 method (n = 9). Untreated cells were used as control group. *: $p < 0.05$. NS: $p > 0.05$. #: $p < 0.05$ compared to untreated control group.

apoptosis by multiple mechanisms [54]. In one mechanism activated JNK translocates to the nucleus, activates c-Jun and other target transcription factors by phosphorylation, and promotes apoptosis by increasing the expression of pro-apoptotic genes. In another mechanism directed at mitochondrial apoptotic proteins, activated JNK translocates to mitochondria, phosphorylates the BCL2 protein to antagonize the anti-apoptotic activity of BCL2 [55].

Phosphorylated JNK can further induce the phosphorylation of BCL2 at Thr56, serine (Ser) 70, Thr74, and Ser87 sites in the BCL2 loop region resulting in inactivation of its anti-apoptotic function [37,56]. Among these loop residues, Ser70 is the most frequently observed loop residue to be phosphorylated [37,57]. In this study, the degree of phosphorylation of BCL2 at Ser70 was measured. The results showed that the amount of phosphorylated Ser70 of BCL2 in the presence of ALA-PDT was significantly increased by addition of methadone. Compared to the ALA-PDT group, inclusion of methadone resulted in a higher apoptosis rate, more phosphorylated active JNK and more phosphorylated Ser70 in BCL2. This methadone-mediated enhancement of ALA-PDT could largely be inhibited by the MOP antagonist naloxone (Figs. 3–7). The above implies that methadone enhances the effect of ALA-PDT by the activation of the JNK pathway and initiation of apoptosis in a MOP-dependent manner. Phosphorylated Ser70 of BCL2 might be upregulated by the activity of JNK and promote the apoptosis induced by ALA-PDT in A172 cells (Fig. 9) [43–47,55].

Under sublethal treatment conditions of ALA-PDT, apoptosis is the main cause of cytotoxicity [58]. Methadone enhances the cytotoxicity of ALA-PDT through promoting apoptosis. In the present study it is shown that the MOP antagonist, naloxone at least partly inhibits the

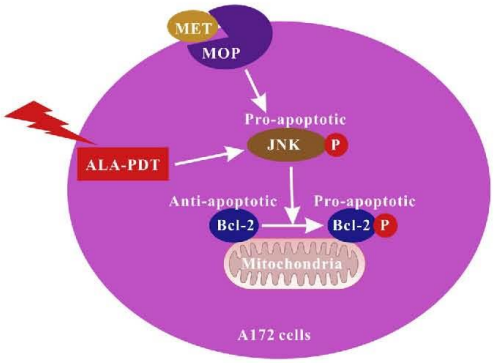

Fig. 9. Possible mechanism based on the results of this study how methadone enhances the effectiveness of ALA-PDT in A172 cells. MET: methadone; MOP: mu opioid receptor; ALA-PDT: 5-aminolevulinic acid-based photodynamic therapy; JNK: c-Jun N-terminal kinase; P: phosphorylation; BCL2: B-cell lymphoma-2.

methadone effect on ALA-PDT-induced apoptosis resulting in an increase of survival (Fig. 8). Naloxone as a MOP antagonist, could inhibit the methadone effect through the inhibition of the activation of JNK, decrease of Ser70 phoshorylation of BCL2 and apoptosis.

In summary, this study is the first report to show A172 cells express MOP, the methadone effect on the ALA-PDT-induced apoptosis for A172 cells is MOP-dependent, and the methadone effect on the ALA-PDT-induced activation of JNK pathway, BCL2 phosphorylation contributes to the cytotoxicity of ALA-PDT for A172 cells. The expression of phosphorylated Ser70 of BCL2 showed a positive correlation with the apoptosis rate induced by ALA-PDT in this study. The relationship between the efficacy of methadone and the amount of MOP expressed in tumor cells would be interesting to be explored in future studies. In addition, methadone may also enhance the effect of other photosensitizer-based PDT for tumors. A higher concentration of methadone may have a stronger effect on improving the efficacy of PDT on tumor cells. However, for the transfer to the clinic, more studies on patients have to be carried out in order to evaluate the effectiveness of methadone and to find a suitable methadone concentration with a strong therapeutic effect but least possible side effects.

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ABBREVIATIONS

Abbreviation	Full name
5-ALA-PDT	5-Aminolevulinic acid - photodynamic therapy
ABCG2	ATP Binding Cassette Subfamily G Member 2
AK	Actinic keratosis
BCC	Basal cell carcinoma
BCL2	B-cell lymphoma-2
BCLxL	B-cell lymphoma-extra large
cAMP	Cyclic adenosine monophosphate
CCK-8	Cell Counting Kit-8
CCL-8	C-C motif chemokine ligand 8
CXCL13	chemokine C-X-C motif ligand 13
FDA	Food and drug administration
HGGs	High-grade gliomas
IL	Interleukin
iPDT	Interstitial photodynamic therapy
JNK	c-Jun N-terminal kinase
MFI	Mean Fluorescence Intensity
MOP	Mu pioid receptor
OPRM1	Opioid receptor mu 1
ROS	Reactive oxygen species
PpIX	Protoporphyrin IX
RIP3	Receptor-interacting protein 3
SCC	squamous cell carcinoma
SAPK	Stress activated protein kinase pathway

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