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Reversal of human multi-drug resistance leukaemic cells by stemofoline derivatives via inhibition of p-glycoprotein function

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Abstract
Our previous study reported multi-drug resistance (MDR) reversing properties of synthetic stemofoline derivatives (STFD), OH-A1, NH-B6 and NH-D6 on P-glycoprotein (P-gp) overexpressing leukaemic cells (K562/Adr); however, the mechanism was unclear. In this study, we further investigated whether the STFD reverse MDR through either the inhibition of P-gp function or expression in K562/Adr cells, or both. The P-gp functional studies showed that the STFD increased the accumulation of calcein-AM, rhodamine 123 and [14C]-doxorubicin in K562/Adr cells, while the effects have not been seen in their parental sensitive cancer cell line (K562). Further, the STFD did not alter the P-gp expression as determined by Western blotting. This study concludes that the STFD reverse MDR via the inhibition of P-gp function. The efficacy of the STFD to inhibit P-gp function followed the order: NH-B6 > OH-A1 > NH-D6. These compounds could be introduced as candidate molecules for treating cancers exhibiting P-gp-mediated MDR.

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Reversal of Human MDR Leukemic Cells by Stemofoline Derivatives via Inhibition of P-Glycoprotein Function

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Running title: Inhibition of P-Glycoprotein Function in Human MDR Cancer by Stemofoline Derivatives
Abstract: Our previous study reported multidrug resistance (MDR) reversing properties of synthetic stemofoline derivatives (STFD), OH-A1, NH-B6 and NH-D6 on P-glycoprotein (P-gp) over-expressing leukemic cells (K562/Adr), however, the mechanism was unclear. In this study, we further investigated whether the STFD reverse MDR through either the inhibition of P-gp function or expression in K562/Adr cells, or both. The P-gp functional studies showed that the STFD increased the accumulation of calcein-AM, rhodamine123 and [\(^{14}\)C]-doxorubicin in K562/Adr cells while the effects have not been seen in their parental sensitive cancer cell line (K562). The STFD did not alter the P-gp expression as determined by Western blotting. This study concludes that the STFD reverse MDR via inhibition of P-gp function. The efficacy of the STFD to inhibit P-gp function followed the order: NH-B6 > OH-A1 > NH-D6. These compounds could be introduced as candidate molecules for treating cancers exhibiting P-gp-mediated MDR.
Chemotherapy is one of the standard methods in the treatment of leukaemia, which is capable of inducing cancer cell death. However, many leukaemia patients experience recurrence and ultimately death because of treatment failure. Drug resistance is one cause of the failure that can be due to cell-intrinsic mechanisms or acquired de novo during the course of the treatment [1]. The development of resistance to chemotherapeutic agents is related to multiple mechanisms such as alterations in drug transport, changes in cellular proteins involved in detoxification, altered drug target, changes in DNA repair mechanisms and activation of key signalling pathways [2].

P-glycoprotein (P-gp), which is encoded by the MDR1 gene, is proven to be responsible for resistance to a variety of structurally and functionally unrelated anti-tumour drugs, including vinblastine, vincristine, doxorubicin and paclitaxel. This transporter uses the energy that is released upon hydrolysis of ATP to drive the transport of various molecules across the cell membrane. The efflux of anti-cancer drugs by this pump causes a decrease in intracellular drug accumulation leading to cancer cell survival and treatment failure [2].

Adjuvant chemotherapy against MDR cancer using agents from natural sources has therefore been of interest. For example, curcumin from Curcuma longa [3, 4], kuguacin J from Momordica charantia [5], and chelidonine from Chelidonium majus [6] have been reported as effective P-gp inhibitors in various types of human MDR cancer cells. The advantage of these dietary herbs is that they often exhibit little or virtually no side effects and do not add to a patient’s medication burden.

In our previous study, twelve STFD derivatives were synthesized, and their cytotoxicity and effect on doxorubicin (DOX) and paclitaxel (PTX) sensitivity in drug-resistant (K562/Adr) and drug-sensitive (K562) cells was determined. [7]. We found that three active STFD including OH-A1, NH-B6 and NH-D6 could re-sensitize the human MDR leukaemic cell to the chemotherapeutic drugs [7]. However, the mode of action of the STFD on P-gp inhibition
was unclear. In this study, we therefore aimed to investigate whether the STFD can reverse MDR through inhibition of P-gp function or expression in human MDR leukaemic cells (K562/Adr), or both.
Materials and Methods

Drugs and reagents

Doxorubicin (DOX), paclitaxel (PTX), cyclosporin A (CSA), verapamil (VER), calcein-AM (C-AM), rhodamine 123 (Rho123), anti-P-gp antibody, anti-β-actin antibody, HRP-conjugated mouse anti-mouse IgG antibody and foetal bovine serum were obtained from Sigma Chemical Company (St. Louis, MO, USA). [14C]-DOX (0.05 µCi/mmol) was purchased from Perkin-Elmer Life Sciences. Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI1640) were purchased from Gibco BRL (Grand Island, NY, USA). Modified radioimmunoprecipitation assay (RIPA) lysis buffer, protease inhibitor cocktail and chemiluminescent Immunoblotting reagents were obtained from Thermo Science Company (Thermo Scientific, Rockford, IL, USA) Guava® cell cycle reagent was purchased from Merck KGaA (Guava Technologies, Hayward, CA, USA).

Cell lines and cell culture

A MDR leukaemic cell line (K562/Adr) and a drug-sensitive leukaemic cell line (K562) were purchased from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) and The American type Culture Collection (ATCC, Manassas, VA, USA), respectively. The cells were cultured in RPMI1640 with 10% foetal bovine serum (FBS), L-glutamine (5 mM), penicillin (50 IU/mL) and streptomycin (50 mg/mL). DOX (700 nM) was added only to the K562/Adr culture medium. Human fibroblasts were isolated from scar tissue from females who had caesarean birth for the second time. The fibroblast cells were cultured in DMEM with 4.5 g of glucose/L plus 10% foetal bovine serum (FBS), L-glutamine (5 mM), penicillin (50 IU/mL) and streptomycin (50 mg/mL). When the cells reached 70–80% confluence, they were harvested and plated either for subsequent passages or STFD treatments.
Preparation of human peripheral blood mononuclear cells (PBMCs)

Blood samples were obtained from five healthy volunteers by intravenous intervention and kept in a heparinized tube. Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-hypaque, according to the manufacturer’s instructions. The mononuclear cells were carefully collected and rinsed twice with ice-cold PBS. The PBMCs were maintained in the same condition as K562 cells.

Stemofoline (STF) and stemofoline derivatives (STFD) (fig. 1)

STF, NH-B6 and NH-D6 were prepared from didehydrostemofoline (OH-A1) according to the literature [8].

Cell cycle analysis

K562/Adr cells were plated and incubated for 24 hr. The cells were treated with various doses of DOX or PTX with or without 5 µM of STFD for 48 hr. The cells were collected and fixed in 95% of ethanol overnight and stained with Guava® Cell Cycle reagent. The cell cycle phase distributions were determined on a Guava® PCA Instrument using Guava® easyCyte HT Software. In each experiment, determinations were carried out in triplicate.

P-gp fluorescent substrate accumulation assay

P-gp fluorescent substrate accumulation, K562 and K562/Adr cells were stained with Rho123 (2 µg/mL) for 30 min. or C-AM (0.1 µM) for 15 min. at 37°C in 0.1% BSA-PBS either with or without CSA (5 µM), VER (20 µM), STF (40 µM) and the STFD (0-40 µM). The cells were then washed twice with ice-cold PBS and re-suspended in 0.1% BSA-PBS. The percentages of Rho123 or C-AM accumulation were detected by flow cytometry [9-11]. In each experiment, determinations were carried out in triplicate.
Radioactive chemotherapeutic drug \([^{14}\text{C}]\)-DOX accumulation assay

K562 and K562/Adr cells were plated and treated with 0-40 µM of STFD in the presence of \([^{14}\text{C}]\)-DOX (0.05 µCi) for 60 min. CSA and VER were used as positive controls. After that, the radiation of \([^{14}\text{C}]\)-DOX in the treated cells was detected using a liquid scintillation beta-counter as previously described [9]. In each experiment, determinations were carried out in triplicate.

Western blot analysis

K562/Adr cells were plated for 24 hr and treated with 5 µM of the STFD for 48 hr. The treated cells were washed with ice-cold PBS, and the cells pellet was centrifuged at 4°C for 10 min. The cell lysates were prepared (10 µg/lane) and separated on 10% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. The membranes were incubated sequentially with mouse monoclonal anti-P-gp or anti-β-actin antibody (1:10,000) and HRP-conjugated mouse anti-mouse IgG (1:10,000) [12, 13].

Cytotoxicity of STFD on human fibroblasts and peripheral blood mononuclear cells (PBMCs) by MTT assay

Fibroblasts were plated at 3×10³ cells/well while PBMCs were plated at 8x10⁴ cells/well in 96-well plates for 24 hr. Next, the various concentrations of the STFD (0-50 µM) were added, and the cells were then incubated for 48 hr. The cell survival was assessed by means of the MTT colourimetric assay. In each experiment, determinations were carried out in triplicate.

Statistical analysis
The results were presented as means ± S.D. from duplicate or triplicate samples of three independent experiments (n = 3). The cytotoxicity of STFD on human PBMCs were presented as means ± S.D. from five independent experiments (n = 5). Statistical analysis was calculated by mean ± S.D. using one-way ANOVA, Tukey’s correction and Dunnett’s multiple comparison tests. Statistical significance was considered when $p < 0.05$. All statistical analyses were performed using the Prism 4.0 software.
Results

**STFD enhanced chemotherapeutic drugs sensitivity in MDR leukaemic cells**

DOX is a cell cycle non-specific agent and a drug of choice for treatment of leukaemic patients, which produces ROS and inhibits topoisomerase II leading to chromatin aggregation [14], while PTX is a highly specific P-gp substrate anti-cancer drug that inhibits microtubule depolarization within the cancer cells leading to G2/M phase arrest [15, 16]. Our previous studies found that co-treatment of STFD could increase cytotoxicity of DOX and PTX in the MDR leukaemic cells using the MTT assay [7]. In this study, cell cycle determination in K562/Adr cells was performed to confirm that the cytotoxicity or growth inhibition was due to the action of the anti-cancer drugs. DOX single treatment caused cell accumulation at the G2/M phase while the accumulation was shifted to sub-G1 (cell death) and S populations when the cells were co-treated with STFD, especially NH-B6 (fig. 2A-2D). Combination treatment of PTX and STFD in K562/Adr cells significantly enhanced G2/M arrest whereas the cell cycle profile was not altered in the PTX single treatment when compared to the control (fig. 2D-2G). These results have suggested that the cytotoxicity and growth inhibition were due to the effect of the anti-cancer drugs which were accumulated in the MDR cells by the STFD. STFD alone did not change the cell cycle profile when compared to the vehicle control.

**STDF increased the accumulation of P-gp fluorescent substrates**

C-AM and Rho123 have been known to be substrates for P-gp that could be used to determine whether the STFD modulate intracellular drug levels by inhibiting P-gp function [9, 10]. The accumulation of C-AM and Rho123 was determined using flow cytometry. It was found that, OH-A1, NH-B6 and NH-D6 dramatically increased C-AM accumulation in K562/Adr cells in a dose-dependent manner. CSA and VER were used as positive controls.
Compared to the controls, the accumulation was enhanced when the cells were treated with 5, 10, 20, 40 µM of the STFD by 18.43- ($p<0.001$), 24.90- ($p<0.001$), 39.91- ($p<0.001$) and 44.37- ($p<0.001$) fold, respectively, in OH-A1 treatment, 4.51-, 7.31-, 47.35- ($P<0.001$) and 47.46- ($P<0.001$) fold, respectively, in NH-B6 treatment, and 11.91- ($P<0.001$), 18.91- ($P<0.001$), 37.30- ($P<0.001$) and 44.80- ($P<0.001$) fold, in NH-D6 treatment (fig. 4a). At lower concentration (5 and 10 µM), OH-A1 and NH-D6 had higher efficacy to increase C-AM accumulation than NH-B6 ($P<0.001$), while at higher concentrations (20 and 40 µM), NH-B6 showed the highest efficacy compared to OH-A1 and NH-D6 ($P<0.01$). These effects were not observed in drug-sensitive K562 cells (fig. 4d).

In K562/Adr cells, 5, 10, 20 and 40 µM of NH-B6 dramatically increased the accumulation of Rho123 by 2.37-, 3.68- ($P<0.001$), 4.56- ($P<0.001$) and 4.67- ($P<0.001$) fold. Whereas, OH-A1 and NH-D6 (5, 10 20 and 40 µM) slightly increased Rho123 accumulation by 1.31-, 1.62-, 2.00- and 2.25-fold and 1.13-, 1.24-, 1.60- and 1.78-fold, respectively (fig. 4b). These effects were not observed in drug sensitive K562 cells (fig. 4e). The Rho123 accumulation assay revealed that NH-B6 exhibited the highest efficacy compared to OH-A1 ($P<0.05$) and NH-D6 ($P<0.05$). These results imply that the STFD could inhibit P-gp function that resulted in an increase of the accumulation of P-gp substrates. The efficacy of the STFD to inhibit P-gp function followed the order of NH-B6 > OH-A1 > NH-D6.

**STFD increase radioactive-labelled chemotherapeutic drug, [14C]-DOX accumulation**

Previous experiments showed that the ability of the STFD to increase the accumulation of P-gp fluorescent substrates in K562/Adr cells which further suggested that the STFD could inhibit P-gp function. In this current study, DOX, labelled with [14C], was used as a P-gp substrate to confirm the effect of the STFD on P-gp function. CSA and VER were used as positive controls (data not shown). It was found that OH-A1, NH-B6 and NH-D6
dramatically increased $[^{14}\text{C}]-\text{DOX}$ accumulation in K562/Adr cells in a dose-dependent manner. Compared to the controls, the accumulation was enhanced when the cells were treated with 5, 10, 20, 40 M of the STFD by 2.04- ($P<0.01$), 2.26- ($P<0.001$), 2.75- ($P<0.001$) and 2.26- ($P<0.001$) fold, respectively, in OH-A1 treatment, 1.49-, 1.58-, 2.20- ($P<0.001$) and 2.48- ($P<0.001$) fold, respectively, in NH-B6 treatment, and 1.78- ($P<0.05$), 2.06- ($P<0.001$), 2.29- ($P<0.001$) and 2.16- ($P<0.01$) fold, respectively, in NH-D6 treatment (fig. 4c). These effects were not found in drug-sensitive K562 cells (fig. 4f). The increased $[^{14}\text{C}]-\text{DOX}$ accumulation was not statistically different among the STFD treatments. The results confirmed that the STFD could indeed inhibit the P-gp function, resulting in an increase in the accumulation P-gp substrates, not only the fluorescent substrates but also the chemotherapeutic drug.
**STFD did not alter P-gp expression**

Over-expression of the P-gp has been well established as the cause of the MDR phenotype *in vitro* on selected drug-resistant cells [2]. K562/Adr cells have been shown to express P-gp at a high level on their plasma membrane. The MDR reversal by the chemosensitizers might be due to their inhibition of the P-gp expression. STFD were assessed of their ability to modulate the P-gp expression via Western blot analysis. It was found that P-gp expression did not differ between the control and the treatment groups (fig. 5). These results suggested that the MDR reversing property of the STFD was not involved with the down-regulation of the P-gp expression.

**Effects of STFD on the cytotoxicity of human fibroblasts and PBMCs**

Previous studies have shown that 0-40 µM of the STFD were not toxic to K562 and K562/Adr cells using the MTT assay [7]. In this current study, the cytotoxicity of the STFD on human normal cells was investigated. We found that the survival of human fibroblasts (normal human skin tissue primary cells) and PBMCs (normal human white blood cells) was more than 90% after the treatment (fig. 6a-b).
**Discussion**

Several studies have described an increase in the P-gp expression after initial chemotherapy treatment that has repeatedly been linked to poor outcomes in some forms of leukaemia [17, 18]. Chemosensitizers or MDR modulators are agents that can affect the drug accumulation detected in the MDR cells, but there is no potentiation of drug cytotoxicity in sensitive cells. The magnitude of chemosensitizers’ effects can be determined by comparing the IC$_{50}$ values of a cytotoxic drug in the absence and presence of a relatively non-toxic and fixed concentration of a chemosensitizer [18]. VER and CSA have been reported as MDR modulators [4, 7, 9, 13, 19] but they have limited clinical use due to their side effects, such as hypertension and immunosuppression [20]. Tyrosine Kinase Inhibitors (TKI) are a class of commercial, adjuvant agents used against MDR cancers, for example imatinib, [21] which has shown adverse effects in the thyroid gland and the heart and also can lead to TKI resistance cancers [22, 23]. Our recent studies aimed to discover and develop high potential MDR modulators that have no toxicity or adverse drug effects.

In our previous study, three stemona alkaloids, stemocurtisine, oxystemokerrine and stemofoline (STF), were isolated from *Stemona burkillii* and investigated for their biological activities. We found that STF exhibited the most potent MDR reversal effect in human MDR cervical carcinoma cells [19]. While these alkaloids all have relatively complex multi-cyclic ring structures, ring A in the former two alkaloids is six-membered (piperidine ring) while the one in STF is a five-membered ring (pyrrolidine ring) [8, 24]. Furthermore, STF has a more complex caged structure (fig. 1) than that of stemocurtisine and oxystemokerrine. These differences in the ring structure may contribute to the deference in their abilities to reverse MDR. Structural modification of STF might enhance its biological activity leading to more effective MDR reversing agents for cancer therapy. Subsequently, we then studied the effect of synthetic STFD on the P-gp mediated multidrug resistance in human MDR cancer cells.
Eleven STFDs, including five alcohol derivatives and six amine derivatives were semi-synthesized from didehydrostemofoline (OH-A1) with designed C-3 side chain chemical modifications [8, 24]. It was found that co-treatment of didehydrostemofoline (OH-A1) and two amine derivatives (NH-B6 and NH-D6) dramatically increased sensitivity of the P-gp over-expressing human MDR leukaemic (K562/Adr) and cervical carcinoma (KB-V1) cells to the antineoplastic drugs but the mechanism was not defined [7].

P-gp-mediated MDR could be altered either by the inhibition of P-gp function and expression, or both. A short-time period exposure (15-30 min.) of the MDR cancer cells to P-gp substrates, with or without chemosensitizers, could be used to examine the inhibition of the P-gp function. The P-gp substrate accumulation (C-AM, Rho123 and [14C]-DOX) assays were then performed to study whether the STFD modulated the P-gp function in K562/Adr cells. It was found that OH-A1, NH-B6 and NH-D6 significantly increased accumulation of C-AM, Rho123 and [14C]-DOX in a dose-dependent manner when compared to the controls. Based on C-AM and Rho123 accumulation, at high concentration of STFD, NH-B6 showed much higher efficacy than OH-A1 and NH-D6, respectively. In the [14C]-DOX experiments, OH-A1, NH-B6 and NH-D6 increased accumulation of [14C]-DOX in a dose-dependent manner. STF and the STFD have the same core nucleus structure but different (C-3) side chain structures. In previous studies, it was found that the hydroxy group (-OH) of the alcohol derivatives may reduce the MDR reversing property [7]. The benzylamino side chain of NH-B6, which might be protonated at physiological pH, may be responsible for this compound’s enhanced efficacy over OH-A1 and NH-D6, which have neutral alkene and carbamate side chains, respectively. Our results showed that the STFD altered the P-gp function but not the expression in K562/Adr cells. These results suggested that the STFD reversed MDR through the inhibition of the P-gp function. Similar to other reports, kuguacin J, tetrandrine, schisandrin B and HZ08 inhibited only the P-gp function and not the
expression [5, 25, 26]. However, other natural products, such as curcumin, α-asarone and β-asarone have been reported as effective chemosensitizers able to down-regulate both MDR1 gene expression and the P-gp function in various types of human MDR cancer cells [3, 4, 27]. The P-gp contains six putative transmembrane helices and one nucleotide binding/utilization domain (NBD), also called the ATP site [28]. In order to inhibit the drug export function of the P-gp, a class of compounds known as modulators, can block its function via direct interaction with the P-gp and appear to compete with drugs for the substrate-binding site(s) on the protein. Our previous study reported that STF isolated from *Stemona burkillii* inhibited the P-gp function by acting on the substrate-binding site determined by photoaffinity labelling assay [9]. For additional studies on drug P-gp binding, P-gp ATPase activity was stimulated by STF in a concentration-dependent manner. Therefore, the action of the STFD may interact directly with the P-gp and inhibit its activity in a similar manner to that of STF. The multi-cyclic ring, the pyrrolidine ring contained in STF and STFD structure, may incorporate into ligand-binding site that could inhibit the P-gp function. In another study, a cell-based screening technique was used to screen the NCI Structural Diversity Set for small-molecule compounds capable of reversing MDR [29]. One of the most potent small molecules in this screening was tetrandrine, a bis-benzylisoquinoline alkaloid compound isolated from the root of *Stephania tetrandra*. Tetrandrine has been reported to reverse drug resistance via simulating ATPase activity. However, it did not alter the protein expression level of the P-gp. The MDR modulating effect of tetrandrine was similar to our finding using natural stemofoline isolated from *Stemona burkillii* in a previous report [9]. We have demonstrated that the STFD showed MDR reversal properties by inhibiting the P-gp function leading to an increased intracellular chemotherapeutic drug accumulation. These findings provide additional evidence for the development of STFD as new potential chemo-sensitizers to improve the efficiency of anticancer drugs in the treatment of cancer.
Up to 50 µM, STFDs were not cytotoxic to both sensitive (K562) and resistant cell lines (K562/Adr) (data not shown). The growth inhibition curves were similar in matched cell lines that did not express the P-gp, suggesting that STFDs were not P-gp substrates. In addition, STFDs were not cytotoxic to human normal fibroblasts and PBMCs in an *ex vivo* model. Interestingly, these compounds could be readily dissolved in water or saline in all concentrations used in these experiments. Stemofoline and its derivatives have the potential as a treatment strategy for cancer patients due their ability to restore sensitivity to chemotherapy or prevent resistance in cancer cells. However, for the MDR reversal property, the pharmacokinetic and pharmaceutical safety of the STFD in animal models should be further investigated before moving to clinical studies for development of these agents as effective adjuvants for use in combination with conventional chemotherapy for cancer patients.
References


Legend of figures

Fig. 1. Structure of stemofoline and its derivatives. Stemofoline (STF) and two amine derivatives NH-B6 and NH-D6 were prepared from didehydrostemofoline (OH-A1).

Fig. 2. Stemofoline derivatives (STFD) enhanced sensitivity of K562/Adr cells to doxorubicin (DOX). The graphs represent Sub-G1 (A), G1 (B), S (C) and G2/M (D) cell distribution. K562/Adr cells were treated with various doses of DOX with or without 5 µM of STFD for 48 hr. The cells were collected and fixed in 95% of ethanol overnight and stained with Guava® Cell Cycle reagent. The cell cycle phase distributions were determined on a Guava® PCA Instrument using Guava® easyCyte HT Software. The mean value from three independent experiments is shown and error bars indicate SD (n = 3). *p< 0.05, **p< 0.01 and ***p< 0.001, versus control treated without the STFD.

Fig. 3. Stemofoline derivatives (STFD) enhanced sensitivity of K562/Adr cells to paclitaxel (PTX). The graphs represent Sub-G1 (A), G1 (B), S (C) and G2/M (D) cell distribution. K562/Adr cells were treated with various doses of PTX with or without 5 µM of STFD for 48 hr. The cells were collected and fixed in 95% of ethanol overnight and stained with Guava® Cell Cycle reagent. The cell cycle phase distributions were determined on a Guava® PCA Instrument using Guava® easyCyte HT Software. The mean value from three independent experiments is shown and error bars indicate SD (n = 3). *p< 0.05, **p< 0.01 and ***p< 0.001, versus control treated without the STFD.
Fig. 4. The effect of the stemofoline derivatives (STFD) on accumulation of P-gp substrate. Calcein-AM (C-AM) (A, D) and rhodamine 123 (Rho123) (B, E). Leukaemic cells were stained with 2 µg/mL of Rho123 for 30 min. or 0.1 µM of C-AM for 15 min. in 0.1% BSA-PBS with or without 0-40 µM of the STFD and analysed by flow cytometry. Radioactive-labelled chemotherapeutic drug, [14-C]-doxorubicin ([14C]-DOX) (C, F). The cells were treated with 0-40 µM of STFD in the presence 0.05 µCi of [14C]-DOX for 60 min. After that, the radiation of [14C]-DOX in the treated cells was detected using a liquid scintillation beta-counter as previously described. The mean value from three independent experiments is shown and error bars indicate SD (n = 3). *p<0.05 and ***p< 0.001, versus control treated without the STFD.

Fig. 5. The effect of the stemofoline derivatives (STFD) on P-gp expression in K562/Adr cells. The cells were treated with 5 µM of the STFD for 48 hr. The P-gp expression was assessed by Western blot analysis. The data are representative of three independent experiments.

Fig. 6. Cytotoxicity of stemofoline derivatives (STFD) on human fibroblasts (A) and peripheral blood mononuclear cells (PBMCs) (B). Fibroblasts or PBMCs were treated with STFD (0-50 µM) for 48 hr. The cell survival was assessed by means of the MTT colourimetric assay. The mean value from three independent experiments is shown, and error bars indicate SD (n = 5).
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