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Toxicity Evaluation of 9-Thioansamitocin P3

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Abstract

In our efforts to extend the possible application of highly cytotoxic maytansine derivatives as a payload in drug delivery systems, we found that thiol group in 9-thioansamitocib P3 could be successfully used to create a disulfide linker to the carrier. Considering that the efficacy of any drug delivery system strongly depends on the properties of its payload we studied and recently published the data about anti-tumor activity of AP3SH. On the other hand, the toxicity of such systems is mostly determined by the toxicity of the small molecular payload. Here we present the results of toxicity evaluation of AP3SH in several in vitro and in vivo models. We found that the toxicity of AP3SH in these experiments is comparable to or even less than the toxicity of maytansine and DM1. Therefore, AP3SH could be considered as a promising small molecular payload in targeted antitumor drug delivery systems.

Keywords: 9-thioansamitocin; Maytansine; Maytansinoid; Acute toxicity; Repeated-dose toxicity.

Abbreviations: AP3SH: 9-Thioansamitocin-P3; PLT: Platelet Count; RET: Reticulocyte Count; LYM: Lymphocytes; NEUT: Neutrophils; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; WBC: White Blood Cells; RBC: Red Blood Cell Count; MONO: Monocytes; EOS: Eosinophils; A/G: Total Protein And Albumin/Globulin Ratio; GLO: Globulin; SD Rat: Sprague-Dawley Rat; FOB: Functional Observational Batteries.

Introduction

Maytansine and its derivatives comprise an important resource of payloads used in covalent anti-cancer conjugates of various types.

In most cases maytansine derivatives with C-3 ester group containing terminal thiol function such as DM1 and DM4 are applied (Figure 1). For example, the conjugation of maytansine derivative DM1 to antibodies led to Trastuzumab ematansine (Kadcyla), approved for breast and stomach cancers and to Lorvotuzumab, granted Orphan drug status for Merkel cell carcinoma. Several ADCs containing DM1 or DM4 are currently undergoing clinical evaluation for the treatment of different types of cancer [1,2]. Several examples of linking to small molecules aimed at critical pathways in tumor growth and survival were published [3,4]. Thus a conjugate containing DM1 and an immune checkpoint ligand based on zinc (II) bis-dipicolylamine (Zn-DPA) connected via cleavable disulfide linker was created and shown to be effective [4]. Another example includes a conjugate containing maytansinoid DM4 and tropomyosin kinase receptor-C (TrkC) which demonstrated a promising activity against metastatic breast tumors [3].

Some possibilities of using C-19 atom for attachment to a carrier via Pd-catalyzed Suzuki or Still coupling reactions were also demonstrated [5,6].

Recently we demonstrated that 9-thioansamitocyn (AP3SH) possesses excellent anti-tumor properties in both in vitro assays and in vivo U937 xenograft model. The pharmacological mechanisms include the blocking of tumor cells in G2 cycle phase and

inducing apoptosis [7]. The possibility of using the C-9 atom as a point of attachment to a macromolecular carrier via a disulfide linker was studied by us and this strategy was shown to be promising [8,9].

In the current work we describe the toxicity data of 9-thioansamitocyn (AP3SH) in *in vitro* and animal rat experiments.

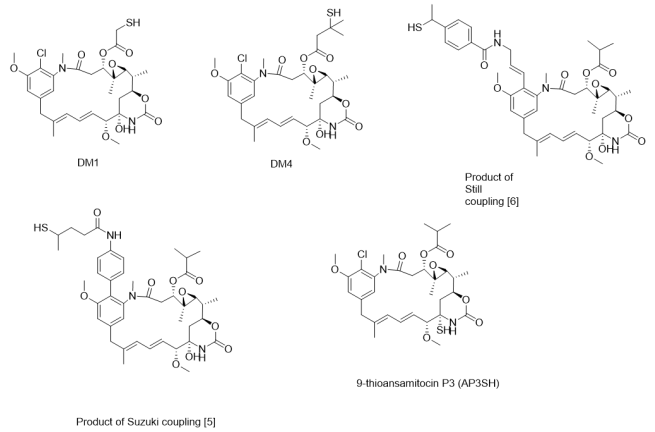

Figure 1: Maytansine derivatives which could be used for coupling.

Materials and methods

All experimental animals were managed in strict accordance with the Shanghai Medici Experimental Animal Use and Management guidelines.

Acute toxicity study of AP3SH in SD rats

A total of 46 SD rats (23M, 23F) were transferred to this study for administration and randomly assigned to four groups based on their body weight and sex, with five animals per sex in each group. Group 1 was the vehicle control group (0 mg/kg); Groups 2-4 were AP3SH treatment groups with doses of 0.1, 0.2, and 0.4 mg/kg, respectively. All animals were given one dose of vehicle (Group 1) or AP3SH (Groups 2-4) via intravenous bolus injection and then were observed for 14 days. The cage-side observation was conducted twice a day and detailed clinical observation - once a day during the experimental period. Food consumption was measured on Day 2, Day 3, Day 4, Day 8 and Day 12. On Day 15, all surviving animals were euthanized by inhalation of carbon dioxide, and a gross necropsy examination was performed.

2-week dose range finding toxicity study in SD rats (14 consequent day administrations)

40 healthy SD rats (males and females) were randomly assigned into 4 groups based on their body weights and genders, with 5 animals per sex. Group 1 received only a vehicle control; groups 2-4 received AP3SH at doses of 0.025, 0.05, and 0.2 mg/kg, respectively for 14 consecutive days.

4-week repeated dose toxicity study in SD rats with a 4-week recovery period (once a week administration)

60 males and 60 females SD Rats were randomly assigned to 4 groups based on their body weights and genders (15 animals/sex/group). Group 1 received a vehicle (0 mg/kg), Groups 2-4 - 0.12 mg/kg, 0.30 mg/kg, and 0.60 mg/kg of AP3-SH via intravenous bolus on Day 1, Day 8, Day 15, Day 22, and Day 29. Animals were followed during a four-week recovery period.

***In vitro* mammalian chromosomal aberration test in Chinese Hamster Lung (CHL) cells**

The dose levels of AP3SH were selected as follows (two replicates were set in all groups):

1. 4 hours, with rat liver S9 metabolic activation system: 0.020 μg/mL, 0.015 μg/mL, 0.010 μg/mL, 0.008 μg/mL, 0.004 μg/mL, and 0.002 μg/mL;
2. 4 hours, without rat liver S9 metabolic activation system: 0.015 μg/mL, 0.010 μg/mL, 0.008 μg/mL, 0.004 μg/mL, 0.002 μg/mL, and 0.001 μg/mL;
3. 24 hours, without rat liver S9 metabolic activation system: 0.008 μg/mL, 0.006 μg/mL, 0.004 μg/mL, 0.003 μg/mL, 0.002 μg/mL, and 0.001 μg/mL;

Negative control group (DMSO) and positive control groups (Cyclophosphamide or Mitomycin C) were also set. For treatment groups, cells were exposed to AP3SH for 3-4 hours or 23-24 hours, then the culture medium was discarded, the cells were harvested and the smears were prepared.

The chromosomes of 300 metaphase cells in the negative control group and the AP3SH treatment groups, and the chromosomes of 100 metaphase cells - in the positive control group were observed to calculate the chromosomal aberration rate.

***In vivo* SD rat bone marrow micronucleus test**

60 male SD rats were randomly assigned to 5 groups according to their body weight. The animals in Group 1 were given 5% DMSO + 5% Tween 80+ 90% Sodium Chloride Injection as vehicle control (0 mg/kg), in Groups 2-4 were given AP3SH at dose levels of 0.05, 0.10, and 0.20 mg/kg respectively, and Group 5 were given Cyclophosphamide (CP) at 40 mg/kg as the positive control. The animals in Groups 1-4 were administered via intravenous injection once a day for two consecutive days, with an interval of 24±1 hours. The animals in Group 5 were dosed with CP once on Day 2 via intraperitoneal injection. All surviving animals were euthanized and the bone marrow was collected from femurs to prepare the bone marrow smears. After being fixed and stained, the slides were scored.

Effects of a single intravenous injection of AP3SH on the central nervous system of SD rats

20 SD rats were randomly divided into 2 groups (vehicle and treatment groups), 10 animals in each group (5 males and 5 females) according to body weight and gender. Vehicle and AP3SH (0.4 mg/kg) were given by intravenous bolus. Detailed clinical observation was conducted on all animals once on the dosing day (before dosing) and 4 times after administration. FOB test [10] was performed on all animals before dosing (0 hour) and at 15 minutes, 30±3 minutes, 1 hour ±6 minutes, 24±1 hours, 48±1 hours, and 96±1 hours post-dosing.

Effects of a single intravenous injection of AP3SH on the respiration system of SD rats

20 SD rats were randomly divided into vehicle and AP3SH treatment groups, 10 animals in each group (5 males and 5 females) according to body weight and gender. Vehicle or AP3SH (0.4 mg/kg) was given by intravenous bolus. Detailed clinical observation was conducted on all animals once on the dosing day (before dosing) and 4 times post-dosing. The animals were put in the Whole-Body Plethysmography (WBP) chamber before administration and 15±2 minutes, 30±3 minutes, 1 hour ±6

minutes, 2 ±1 hours, 48±1 hours, and 96±1 hours post-dosing and respiratory parameters were recorded and analyzed.

Ames assay

The mutagenic activity of AP3SH was evaluated in five histidine-requiring S. typhimurium mutant strains TA1535, TA1537, TA98, TA100 and TA102. Tested bacteria were exposed to five concentrations ranging from 39.1 to 5000 µg/plate, with and without S9 mixture, respectively. Three parallel plates were tested in each concentration. Negative and positive controls were run simultaneously. All the tests were performed at least twice.

Results

Acute toxicity study of AP3SH in SD rats

All animals survived to scheduled necropsy on Day 15. No abnormalities in detailed clinical observation, body weight, food consumption, or gross necropsy were noted neither in the vehicle or experimental groups. Therefore, the maximal tolerance dose (MTD) of AP3SH in SD rats was >0.4 mg/kg.

2-week dose range finding toxicity study of AP3SH in SD rats (14 consequent day administrations)

During the experiment, in the toxicity group of 0.2 mg/kg dose, three male (3/5) and four female animals (4/5) died. Detailed observation showed material around the nose and activity decreased, as well as piloerection in male animals, perianal contamination, and soft and discolored feces. The body weight and food consumption decreased significantly. In blood WBC, NEUT, and LYM decreased significantly and RBC, HGB, HCT, RET, and PLT decreased, CRE, Urea, P, K, TG, and CK increased significantly, and urine analysis showed an increase in Bilirubin, Occult Blood and Protein. Gross observations showed small spleen and thymus volumes, discoloration of stomach and intestines in two male animals and discoloration of lungs, bronchi and one kidney in two female animals.

In the 0.05 mg/kg dose group decrease in RBC, HGB, HCT, RET, and A/G , an increase in PLT, GLO, and a significant increase in ALT, AST, ALP, TBIL, and TCHO were observed. The body weight and food consumption decreased significantly.

In the 0.025 mg/kg dose group a decrease in A/G and an increase in GLO were noted, there were no other abnormal indicators.

In this study, the Severely toxic dose to 10% of the animals (STD_{10}) was determined to be 0.05 mg/kg.

4-week repeated dose toxicity study in SD rats with a 4-week recovery period (once a week administration)

One female animal in group 0.6 mg/kg (1/30, 3.3%) died during dosing on Day 15. Based on clinical symptoms (unkempt appearance, tail edema, red ocular discharge), changes in food intake, and anatomical observations, the cause of death was supposed to be related to the marked suppression of hematopoietic and immune systems.

The decrease of WBC, RBC, MONO, #EOS, and EOS% (only in females) and increase of ALT, AST, and ALP in the medium and high dose groups were observed. Changes in organ weight, including the increase of liver, and the decrease of testes, thymus gland, ovaries, and epididymites were dose-dependent and correlated with histopathological findings. The lesions in hematolymphoid system (bone marrow, thymus, spleen, mandibular lymph node, mesenteric lymph node), gastrointestinal tract (stomach, small intestine, large intestine and esophagus), parotid, skin, liver, kidney, eyes, male reproductive system (testes, epididymides, prostate, seminal vesicles), female reproductive system (vagina, uterus with cervix, ovaries), thyroid gland, mammary gland and pancreas were considered to be AP3SH-related in medium and high doses groups. All the lesions except the spleen, testes, and epididymides were fully recovered at the end of the recovery period. No significant changes in body weight, food consumption, ophthalmic examination, coagulation, or urinalysis were noted during the study.

Based on this study, STD_{10} of AP3SH in SD rats was estimated to be 0.6 mg/kg when administrated via intravenous bolus injection once a week for 4 consecutive weeks (total 5 doses).

***In Vitro* mammalian chromosomal aberration test in Chinese Hamster Lung (CHL) cells**

There was no statistical difference in chromosomal aberration rate compared to the negative control group ($p>0.05$) in any of the treatment groups with or without metabolic activation. Therefore, 0.001-0.015 µg/mL AP3SH showed no chromosome aberration effect on mammalian cells and low risk of potential mutagenic properties.

***In vivo* SD rat bone marrow micronucleus test**

All animals survived to the completion of the experiment; the body weights were within the normal range. Soft feces were observed in 1 male animal in the 0.05 mg/kg group and 1 male animal in the 0.10 mg/kg group, dark red material around the eyes was noted in 1 male rat in the 0.20 mg/kg group, and no other abnormality was observed. The portions of polychromatic erythrocytes (PCE) decreased in female rats in the 0.10 mg/kg (34.35%) and the 0.20 mg/kg (31.56%) dose groups and male rats in 0.10 mg/kg (41.50%) and 0.20 mg/kg (27.44%) dose groups compared to the vehicle group (female: 50.24%, male: 49.66%). The portions of PCE in rats in the 0.05 mg/kg dose group were the same as in the vehicle group. The ratios of micronucleus were 0.90, 3.43 and 8.28 in male rats and 0.65, 3.14, and 7.06‰ in female rats in the 0.05, 0.10, and 0.20 mg/kg AP3SH dose groups and increased in a dose-dependent manner.

Therefore, AP3SH could damage the erythroblast chromosome or mitosis in SD rats, and induce the formation of micronucleus of mice erythrocytes at 0.10-0.20 mg/kg dose level.

Effects of a single intravenous injection of AP3SH on the central nervous system of SD rats

No abnormality in detailed clinical observation was observed during the experiment. Before and after administration, there were no abnormal changes in the body temperature among all animals. Before and after administration, there were no test article-related changes in the FOB test parameters. Therefore, under the experiment condition, there was no significant effect on the central nervous system of SD rats treated with AP3SH (0.4 mg/kg) via a single intravenous bolus injection.

Effects of a single intravenous injection of AP3SH on the respiration system of SD rats

There were no abnormal changes in detailed clinical observation at all time points in all animals during the experiment. Compared with the vehicle group, there were no statistically significant differences in the AP3SH group in respiratory rate,

minute volume, tidal volume, enhanced pause, inspiratory time, and expiratory time at all time points during the experiment. Therefore, there was no significant effect on the respiratory system of SD rats treated with AP3SH (0.4 mg/kg) via a single intravenous bolus injection.

Ames mutation

An Ames assay was performed in order to detect whether AP3SH induces bacterial reverse mutation on five strains of *Salmonella typhimurium* with different concentrations, to evaluate the potential genotoxicity, and to predict the mutagenicity and carcinogenicity of AP3SH.

AP3SH at terminal doses 2500.0, 1250.0, 625.0, 312.5, 156.3, 78.2 and 39.1 µg/plate were tested in five strains of *Salmonella typhimurium* (TA1537, TA98, TA100, TA102 and TA1535). Each strain was treated by two conditions, with (+S9) or without a metabolic activation system (-S9). After incubating with AP3SH for 66-67 hours, revertant colony numbers were counted to verify the mutagenicity of AP3SH. Under the used conditions, no mutagenicity to test strains TA1537, TA98, TA100, TA102, TA1535 was observed.

Discussion

Maytansine isolated by Kupchan and coworkers from the Ethiopian shrub, Maytenus serrata, in 1972 [11], demonstrated extremely high cytotoxic and antitumor activity both in vitro and in animal xenograft models. Several Clinical trials of Phase I and Phase II against 35 tumor types have been performed. However, high systemic toxicity and low therapeutic indexes led to the termination of all Clinical Trials and the closing of the Investigational New Drug application (IND) for maytansine.

Later maytansine derivatives bearing a handle that can be used for attachment to a targeting carrier gained the attention of researchers. This approach allows to reduction of systemic toxicity due to decreased exposure of the healthy organs to the active component and increased efficacy achieved by its delivery directly to the tumor site. In most cases, C-3 derivatives of maytansine such as DM1 and DM4 are used. In the course of our work, we investigated other possibilities for attachment of maytansine derivatives to a carrier and found that C-9 derivative 9-thioansamitocyn P3 can be successfully linked via disulfide bond and serve as an anti-tumor payload in drug delivery systems. After cleavage of a disulfide bond in a reductive environment of a tumor AP3SH is released. Excellent anti-tumor properties of this compound have been shown by us [7], but we also felt necessary to study the toxicity of AP3SH and compare it with the data published for other maytansine derivatives.

Maytansine and its C-3 derivatives DM1 and DM4 are known to possess high systemic toxicity in animal models. LD_{10} published for maytansine in rats accounts for 0.4-0.48 mg/kg [12,13] and this dose causes necrotizing lesions in the gastrointestinal tract, mucosa, thymus, spleen, bone marrow, and testes. A single intravenous dose of DM1 was only tolerated up to 0.2 mg/kg; doses ≥0.4 mg/kg were associated with mortality 2 or 3 days postdose and a decrease in body weight [14]. The authors mentioned dose-dependent effects on the liver, bone marrow/hematologic systems, and lymphoid organs. Hematologic changes included decreased PLT, RET, and LYM, as well as increased ALT, AST, and NEUT, which correlated with microscopic observations of mild bone marrow hypocellularity and minimal to moderate lymphoid depletion or necrosis in the lymph nodes, thymus, and spleen.

In our experiments, the acute single-dose toxicity of AP3SH after intravenous administration was evaluated in 46 male and female SD rats. No death or abnormalities in gross necropsy were observed so the Maximal Tolerance Dose (MTD) of AP3SH in rats was evaluated as >0.4 mg/kg. This data allowed us to suggest less toxicity of AP3SH in rats compared to maytansine and DM1.

However, once a week repeated administration of AP3SH at doses 0.6, 0.3, and 0.12 mg/kg dose for 4 weeks was accompanied by hematological and macroscopic abnormalities in animals of middle and high doses similar to those observed for maytansine and DM1. Levels of WBC, RBC, MONO, and EOS (only in females) were decreased and ALT, AST, and ALP were increased. The gross observation demonstrated the increase of the liver, and decrease of testes, thymus gland, ovaries, and epididymites and lesions in the gastrointestinal tract, hematolymphoid, reproductive, and some other systems. However, all the lesions except the spleen, testes, and epididymides were fully recovered by the end of the study. Only one incidence of death was observed in the high-dose group (1/30). Based on the results of the 4-week study, a 10% severely toxic dose (STD_{10}) of AP3SH was estimated as 0.6 mg/kg in rats.

Daily injections of AP3SH at doses 0.025, 0.05, and 0.2 mg/kg during 14 consecutive days showed clear signs of toxicity including deaths of most animals in the high-dose group. No deaths were recorded in the middle dose group however body weight and food consumption decreased significantly and hematological abnormalities were observed. In the low-dose group there were only small abnormalities in the blood test: GLO was increased which led to a decreased A/G ratio. In this study, the Severely toxic dose of 10% (STD_{10}) was determined to be 0.05 mg/kg.

AP3SH at 0.4 mg/kg has no significant effect on the central nervous and respiration systems in SD rats. AP3SH showed no chromosome aberration effect on mammalian cells, a low risk of genotoxicity in the Ames test, but could induce the formation of micronucleus of mice erythrocytes at 0.10-0.20 mg/kg dose level.

In conclusion, considering that systemic toxicity of drug delivery systems is mostly determined by small molecular payload, evaluation of AP3SH in several in vitro and in vivo toxicity models has been performed. It was found that MTD in single dose acute toxicity study in SD rats is more than 0.4 mg/kg, STD_{10} 0.6 mg/kg for repeated dose toxicity with weekly administration for 4 weeks, and STD_{10} 0.05 mg/kg for repeated dose toxicity with daily administration for 14 days. No significant effects on the central nervous and respiration systems as well as low risk of genotoxicity in chromosome aberration and Ames tests were observed. Therefore, AP3SH demonstrated an acceptable profile of toxicity and could be used as a cytotoxic payload in drug delivery systems.

Conclusion

9-Thioansamitocin-P3 (AP3SH) toxicity in rat animal models and in vitro assays were evaluated.

In single dose acute toxicity experiment AP3SH showed MTD >0.4 mg/kg.

AP3SH at 0.4 mg/kg didn't reveal any sing of toxicity towards respiratory or central nervous systems.

Declarations

Conflict of interest: Authors declare that they have no conflict of interest

Author contribution: All authors contributed equally

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