AWARD NUMBER: W81XWH-16-1-0411

TITLE: Targeting Mitochondrial Inhibitors for Metastatic Castrate-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Dr. Samuel Denmeade

CONTRACTING ORGANIZATION:

Johns Hopkins University Baltimore, MD 21218

REPORT DATE: Sept 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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Table of Contents

Page

1. Introduction	3
2. Keywords	3
3. Accomplishments	3-7
4. Impact	8
5. Changes/Problems	8
6. Products	8
7. Participants & Other Collaborating Organizations	9
8. Special Reporting Requirements	10
9. Appendices	10

Introduction

The overarching challenge and focus area for this Partnering PI-Idea Development Award proposal is to rapidly develop novel therapeutic agents and validate these in pre-clinical studies needed to initiate clinical development of these agents for metastatic castrate resistant prostate cancer (mCRPC). The hypothesis of the present proposal is that an innovative and effective therapeutic approach is possible by covalently coupling niclosamide and 7 hydroxy- β -Lapachone (70H β -Lap) analog lipophilic mitochondria toxins (MT) to human serum albumin (HSA) via a PSA specific peptide linker sequence to systemically deliver these novel agents via the blood so that these cell penetrant MTs are restrictively released only via enzymatically active PSA within extracellular fluid (ECF) at sites of mCRPC. The advantage of ECF hydrolysis is that only a fraction of cancer cells need to secrete PSA since its enzymatic activity amplifies the level of liberated cell penetrant MTs within the ECF shared by all cells within the metastatic site overcoming the problem of tumor cell heterogeneity by inducing a substantial "bystander effect".

Keywords

Metastatic castration resistant prostate cancer, mitochondria toxins, human serum albumin, PSA-activated prodrugs

Accomplishments

• What were the major goals of the project?

Specific Aim 1. Synthesis of HSA-coupled PSA cleavable niclosamide payload-1. **Specific Aim 2.** Synthesis of HSA-coupled PSA cleavable 7OH- β -Lapachone Payload-2 **Specific Aim 3.** Evaluate each of HSA-couple PSA cleavable MT payloads for: 1) efficiency of PSA cleavage; 2) *in vitro* therapeutic efficacy as monotherapy vs. combinational therapy against a series of human mCRPC cell lines, 3) *in vivo* therapeutic efficacy vs. host toxicity as monotherapy vs. combinational therapy against a series of human mCRPC xenografts growing both subcutaneously and within the tibia; and 4) plasma vs. tissue biodistribution.

• What was accomplished under these goals?

During the second year of support we have made significant progress with regards to the major tasks outlined in the statement of work. Boc-protected N-methyl ethylene diamine (EDA) was coupled to leucylproline (LP) dipeptide and Boc removed to produce a compound which was coupled to the critical aromatic hydroxyl group of niclosamide via a EDA-Carbamate linkage to produce LP-EDAC-niclosamide. The authenticity of this compound was documented by ¹H and ¹³C NMR and mass spectrometry. Coupling of LP-EDAC to the hydroxyl of niclosamide prevents its anion formation thereby blocking its protonophoric ability. The LP dipeptide was chosen because when the N-terminal of L is covalently linked to the C-terminal Q in the PSA peptide to produce HSSKLQ//LP-EDAC-niclosamide, PSA efficiently hydrolyzes the peptide between Q//L releasing LP-EDAC-niclosamide. Once liberated, LP-EDAC-niclosamide is an excellent substrate for C-terminal cleavage between P and the EDAC linker by either dipeptidyl peptidase IV [DPPIV] whose expression is upregulated on the plasma membrane of prostate cancer cells, or by fibroblast activation protein (FAP) expressed by tumor infiltrating CAF/MSCs within the cancer microenvironment, as we have documented previously. Once, LP-EDAC-niclosamide is hydrolyzed, the liberated EDAC coupled intermediate undergoes spontaneous self-cleavage liberating niclosamide, During the first year of support we synthesized LP-(EDAC)-Niclosamide in good yield using ethylene diamine

derivatives as the self-cleaving linkers (SCL) and LP as dipeptide substrate for DPPIV and scaled up the synthesis of LP-(SCL)-Niclosamide (payload 1) to obtain gram quantities.

Subsequently, we developed and validated a solid phase synthesis of a resin bound Lys-Pro-Lys-Pro-Lys peptide, Figure 1. The ε-amino group of lysine on the solid resin (i.e. denoted as gray in Figure 1). is protected with 1-(4,4-Dimethyl-2,6 dioxocyclohexylidene) ethyl (Dde). Fmoc-Pro-OH and Fmoc-Lys-(Dde)-OH [N-Fmoc-N'-[1-(4,4-Dimethyl-2,6 dioxocyclohexylidene) ethyl]-D-lysine] was then used for subsequent couplings (steps ad). This was followed by the coupling of N-succinimidyl-PEG-3-monobromomaleimide to the α -amino group of lysine (step f). The Dde was removed with hydrazine in solid phase and the free ε -amino group was reacted with N,N-Disuccinimidyl carbonate to convert it to N-succeinimidyl carbamate (steps g-h). This activated carbamate was reacted with the free primary amine terminal of histidine, H in HSSKLQ//LP-EDAC-niclosamide with the free ε-amino group of lysine (K) Dde protected to form a urea conjugate



Fig. 7

(a) Fmoc-Pro-OH, HATU, DIEAP, DMF; (b) 20% piperidine, DMF (c) Fmoc-Lys-(Dde)-OH, HATU, DIEAP, DMF; (d) 20% piperidine, DMF (e)Repeat steps a-d (1 more cycle); (f) N-succinimidyI-PEG-3monobromomaleimide, DIEAP, DMF; (g) 5% hydrazine, DMF; (h) N,N¹-Disuccinimidyl carbonate, DMF, DIEA (i) Payload with primary amine, DMF, DIEAP; (j) final deprotection with 5% hydrazine , DMF and cleavage, 1% TFA, DCM; (k) Human serum albumin, pH 6.6-7.0

(step i). Next, the Dde group protecting the ε -amino of lysine was removed and the entire compound cleaved off the resin (j). This was purified by LC using LCMS analysis. The purified product was then coupled to the cysteine 34 sulfhydryl of HSA via the monobromomaleimide to form a succinimide-thioether ring (STR).

Unfortunately, we found that this STR linkage, however, undergoes significant spontaneous cleavage by a retro-Michael reaction under physiologic conditions, Figure 2. When this reaction occurs in-vivo, it results in dose-limiting systemic toxicity as the therapeutic agent is spontaneously released to circulate in the blood forming adduct with other sulfhydryl containing species like gluthathione, cysteine etc. generating a systemically toxic molecule TM, Figure 2. In contrast, opening of the succinimidethioether ring (succinimidyl hydrolosis) to form succinamic acid thioether results in forming a stable drug-protein-conjugate (SDPC) with a very long half-life of up to 2 years, **Figure 2.** Thus, if succinimide-thioether that is formed upon conjugation of the sulfhydryl

group of HSA to maleimide is hydrolysed to the succinamic acid thioethers to form the SDPC, this eliminates the problem of poor in-vivo stability of



group of HSA to maleimide is hydrolysed to the succinamic acid thioethers to form the SDPC, this eliminates the problem of poor in-vivo stability of the protein-drug conjugate. Further investigations documented that the electron withdrawing inductive effect of the N-substituent on the nitrogen of the succinimide-thioether plays a major mechanistic role in determining the rate of the succinimidy hydrolysis to succinamic acid thioethers. Thus, in the studies performed in the **second year** of the support, synthetic pathways were developed to uses 2-fluoro-5-maleimidobenzoic acid (compound 3 in **Figure 3**) for stably link compounds to the Cys-34 of albumin since it possesses the required electron withdrawing inductive ability needed by combining the resonance effect of the aromatic ring and the electron withdrawing property of fluorine at para position to the nitrogen of the succinimide thioether ring.

To validate this approach, a chemically modified analogue of the naturally occurring sesquiterpene γ -lactone, thapsigargin, 8-O-(12-aminododecanoyl)-8-O-debutanoyl thapsigargin (12ADT), **Figure 3**, was chosen as the initial cytotoxic drug stably coupled to albumin. This is because 12ADT is acid stable and contains a primary amine allowing its



covalent coupling via formation of a peptide bond. 12ADT is a potent inhibitor (IC₅₀ 10 nM) of their endoplasmic reticulum (ER) calcium ATPase (ie, SERCA 2b) pumps and thus induces depletion of the high (ie, >500 μ M) Ca⁺² in the ER, inducing both an ER stress response and a "capacitance entrance" of extracellular Ca⁺² in human mCRPCs producing a sustained increase in intracellular Ca⁺² (Ca_i) to >1 μ M over the next 18–36 h. The combination of ER stress and a sustained elevation of Ca_i, eventually results in the apoptotic death of mCRPC with an LD₅₀ of <50 nM. An additional factor is that 12ADT's potent killing ability is retained when coupled to single amino-acids. Using LC-MS, we documented that by 2-fluoro-5-maleimidobenzoic acid coupling to the sulfhydryl of HSA was stable for more than a month at 37⁰ in human plasma.

Based upon this validation, in the **second year** of support, we developed a solid phase synthesis on a resin (i.e. denoted as gray in **Figure 4**) for coupling HSSKLQ//LP-EDACniclosamide to HSA as described in **Figure 4&5**, by using N-succinimidyl-PEG-3-2fluoro-5-maleimidobenzoic acid for the coupling to the α -amino group of lysine as described in step f of **Figure 1**, whose epsilon nitrogen was converted to a trivalent azide.



Figure 4



Figure 5

During the **second year**, we developed a synthetic route for producing 7OH- β -Lapachone (7OH- β -Lap) as follows. The hydroxyl group of 5-hydroxy-1,4-naphthoquinone (obtained from Sigma), compound **1** in **Figure 6**, is protected with triisopropylsilyl chloride (TIPS). This is followed by reaction with dimethyl amine (DMF). The pure amino quinone formed, compound **2**, is then treated with concentration hydrochloric acid to form hydroxyl group in position 3. This is followed by reaction with 1-bromo-3-methylbut-2-ene to obtain compound 3. This product is then treated with dilute sulfuric acid for acid catalyzed cyclization. This is then followed by deprotection of the TIPS with TBAF to obtain compound **4**, the desired product.



a) TIPSCI, Imidazole, DMF; b) NHMe₂; c) conc. HCl; d) 1-bromo-3-methylbut-2ene, NaI, Et_{3N} , DMF; e) dil H₂SO₄; f) TBAF, THF

Figure 6

• What opportunities for training and professional development has the project provided?

Nothing to Report

• How were the results disseminated to communities of interest?

Peer-reviewed publications as follows:

- Akinboye ES, Rogers OC, Isaacs JT. 2-fluoro-5-maleimidobenzoic acid-linked albumin drug (MAD) delivery for selective systemic targeting of metastatic prostate cancer. Prostate. 2018 Jun;78(9):655-663. doi: 10.1002/pros.23494. Epub 2018 Mar 24. PubMed PMID: 29572902.
- Akinboye ES, Brennen WN, Denmeade SR, Isaacs JT. Albumin-linked prostate-specific antigen-activated thapsigargin- and niclosamide-based molecular grenades targeting the microenvironment in metastatic castration-resistant prostate cancer. Asian J Urol. 2019 Jan;6(1): 99-108. <u>https://doi.org/10.1016/j.ajur.2018.11.004</u>. Epub 2019 Nov 28.

• What do you plan to do during the next reporting period to accomplish the goals?

Using the same approach outlined in Figure 4 &5, the 7OH- β -Lapachone-HSA-coupled PSA activated payload 2 will be synthesized. This payload 2, as well as the niclosamide -HSA-couple PSA activated payload 1 will be evaluated for: 1) efficiency of PSA hydrolysis; 2) in vitro therapeutic efficacy as monotherapy vs. combinational therapy against a series of human mCRPC cell lines, 3) in vivo therapeutic efficacy vs. host toxicity as monotherapy vs. combinational therapy against a series of human mCRPC xenografts; and 4) plasma vs. tissue biodistribution.

Impact

• What was the impact on the development of the principal discipline(s) of the project? Nothing to Report Yet

• What was the impact on other disciplines?

Nothing to Report Yet

• What was the impact on technology transfer?

Nothing to Report Yet

• What was the impact on society beyond science and technology? Nothing to Report Yet

Changes/Problems

Nothing to Report

Products

- Akinboye ES, Rogers OC, Isaacs JT. 2-fluoro-5-maleimidobenzoic acid-linked albumin drug (MAD) delivery for selective systemic targeting of metastatic prostate cancer. Prostate. 2018 Jun;78(9):655-663. doi: 10.1002/pros.23494. Epub 2018 Mar 24. PubMed PMID: 29572902.
- Akinboye ES, Brennen WN, Denmeade SR, Isaacs JT. Albumin-linked prostate-specific antigen-activated thapsigargin- and niclosamide-based molecular grenades targeting the microenvironment in metastatic castration-resistant prostate cancer. Asian J Urol. 2019 Jan;6(1): 99-108. <u>https://doi.org/10.1016/j.ajur.2018.11.004</u>. Epub 2019 Nov 28.

Participants & Other Collaborating Organizations

Name:	John Isaacs
Project Role:	Principal Investigator
Nearest person month worked:	3
Contribution to Project:	Dr. Isaacs has coordinated/ supervised all aspects of this project on a daily basis.
Funding Support:	Not applicable

Name:	Samuel Denmeade
Project Role:	Partnering Principal Investigator
Nearest person month worked:	1
Contribution to Project:	Dr. Denmeade is a collaborator in coordinating supervising all aspects of this project, including the collection and analyzes of the data. He mentors Dr. Akinboye.
Funding Support:	Not applicable

Name:	Susan Dalrymple
Project Role:	Sr. Research Specialist
Nearest person month worked:	6
Contribution to Project:	Ms. Dalrymple performs all routine quality assurance testing on cell lines used in this study. In addition, she has coordinated all aspects of the animal studies.
Funding Support:	Not applicable

Name:	Emmanuel Akinboye
Project Role:	Postdoctoral Fellow
Nearest person month worked:	12
Contribution to Project:	Dr. Akinboye synthesizes all of the compounds proposed in this application. He uses 1H and 13C NMR and mass spectrometry for the quality assurance for each compound. He also performs the mass spect analysis in the drug biodistribution studies.
Funding Support:	Not applicable

Special Reporting Requirements Partnering PI will submit his separate progress report.

Appendices None