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Biophysical and molecular characterization of HuR Inhibitors, CMLD-2 and Dihydrotanshinone I (DHTS), in Triple Negative Breast Cancer (TNBC)

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Running title: Exploring HuR inhibitors in TNBC: CMLD-2 and DHTS insights

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ABSTRACT

In the present study, we explored the oncogenic role of Human Antigen R (HuR), a post-transcriptional regulator implicated in cancer progression, metastasis, and therapy resistance. The present study evaluates the specificities and efficacies of two HuR inhibitors- CMLD2 and DHTS *in silico* and *in vitro*, in TNBCs. Apart from in-depth biophysical analysis, the cytotoxic effect of CMLD2 and DHTS was compared on the proliferation of TNBC cell lines [human: MDA-MB-231 & MDA-MB-468) and the 4T1[murine]. Western blotting was performed to access the differential modulation of HuR and its downstream targets i.e., MMP9 and β-catenin along with classical EMT markers viz. epithelial (E-cadherin), and mesenchymal (N-cadherin, and vimentin). Functional assays like wound healing, matrigel droplet invasion, and colony formation were performed and both drugs displayed substantial inhibitory activity on the invasiveness, migratory, and clonogenic potential of TNBC cells. However, at the level of regulation of molecular targets of HuR, CMLD2 shows much better specificity and reproducibility compared to DHTS which was validated using HuR-specific siRNA. In conclusion, both inhibitors successfully diminished the aggressive phenotype (i.e., migration, invasion, and colony formation capabilities) of TNBC cells. However, CMLD2 demonstrated greater specificity in inhibiting HuR and its downstream targets, compared to DHTS.

Abbreviations:

HuR: Human Antigen R

ELAVL1: Embryonic Lethal Abnormal Vision Like protein 1

TNBC: Triple Negative Breast Cancer

EMT: Epithelial-Mesenchymal Transition

RBP: RNA Binding Protein

MMP9: Matrix Metalloproteinase 9

DHTS: Dihydrotanshinone 1

RRM: RNA Recognition Motif 1

ARE: Adenine Uridine (AU) rich elements

PERK: Protein Kinase RNA-Like ER Kinase

elF2α: Eukaryotic Initiation Factor 2

ATF4: Activating transcription factor 4

AMPK: 5' AMP-activated protein kinase

AKT: Protein Kinase B

mTOR: mammalian target of rapamycin

CMLD2: 5,7-dimethoxy-8-(1-(4-methoxyphenyl)-3-oxo-3-(pyrrolidin-1-yl) propyl)-4-phenyl-2H-chromen-2-one

1. Introduction

Among all breast cancers, Triple-negative breast cancer (TNBC) is the most aggressive form and is linked with an unfavourable prognosis. Compared to the global incidence of around 15-20% of all breast cancers, the incidence rates are higher in India i.e., around 25-31% [1]. Among all the breast cancer subtypes, TNBCs are the most heterogeneous and aggressive type [2]. TNBC cells exhibit high invasiveness, and rapid metastasis to visceral organs like the lungs, brain, and liver; hence often associated with a patient's poor survival [3]. In the absence of hormone receptors [i.e., Estrogen (ER) & progesterone (PR)], and human epidermal growth factor 2 (HER2) in TNBCs, they are ineffective against the drugs that target these receptors. Overall, in the absence of any therapeutic marker, the primary treatment modality available for TNBC patients is surgical resection and chemotherapy. In this context, researchers actively seek out genes or proteins expressed differently in TNBCs, aiming to develop novel therapeutic approaches to combat this breast cancer subtype. Our laboratory investigations revealed that the RNA-binding protein i.e., Human Antigen R (HuR), could be one such putative drug target in TNBCs [4]. We recently reported a significant correlation between HuR protein expression and reduced survival in TNBC patients, indicating that HuR plays an oncogenic role in these cancers [4]. These findings also align with previous literature signifying that HuR as a prognostic factor in breast cancers [5–7]. HuR stabilises mRNAs of multiple key oncogenic proteins critical to TNBC oncogenesis and progression. Further, HuR contributes to chemo and radio therapy resistance in TNBCs by stabilising stress response mRNAs. Targeting HuR in this context impacts the protein expression of an array of oncogenic proteins and pathway drivers that promote the aggressiveness and therapy resistance shown by TNBCs. Furthermore, we also exhibited that Quercetin treatment significantly inhibits the migration and adhesion of TNBC cells via deregulating the HuR-β-catenin axis [4]. Like TNBCs, dysregulated HuR has also been implicated in multiple cancers [8–12]. Thus, targeting HuR is a prominent strategy in cancer therapy as it can act against the pro-survival activity of HuR, while also sensitizing cancer cells towards other therapeutics. HuR is a predominantly nuclear localised protein but in response to cell intrinsic or extrinsic stress, it translocates to the cytoplasm. Cytoplasmic localized HuR or “active form” is associated with its mRNA regulatory function. The HuR protein consists of three evolutionarily conserved RNA Recognition Motifs (RRMs). Among these, the N-terminal RRM1 and RRM2 are primarily responsible for recognizing and binding to target mRNAs that contain AU-Rich Elements (AREs) in their 3' Untranslated

Regions (UTRs), thereby stabilizing the associated mRNAs. The C-terminal RRM3 is essential for the dimerization of the protein [13]. Therapeutic targeting of HuR holds significance owing to its frequent overexpression in various cancer types. Three major approaches have been explored for developing HuR inhibitors [14]. The first one involves interfering with the ability of HuR to detect and bind to its target mRNAs with small molecules like CMLD2 (PubChem ID:16746438), quercetin (PubChem ID:5280343), DHTS (PubChem ID:11425923), KH3 (PubChem ID:45138018) etc. The second approach comprises the disruption of HuR dimerization and/or translocation to the cytoplasm (MS444 (PubChem ID:132904), SRI-42127 (PubChem ID:163415849), okecenone (PubChem ID:132015), and the third approach is the direct inhibition of HuR expression like specific siRNA and antisense oligonucleotide. The current study favours HuR-mRNA interaction inhibitors as they act precisely at the RRMs, thereby reducing off target effects compared to dimerization/translocation inhibitors that can influence broader nuclear export/import pathways or other nuclear functions of HuR. Further, small molecule inhibitors also have better cell permeability and are more manageable in terms of dose control, cost effectiveness and scalability compared to oligonucleotide therapies. While peptides also offer several advantages, such as high specificity and lower toxicity, they also pose challenges related to stability, bioavailability, and delivery, which can limit their practical application. On the other hand, small molecules provide better pharmacokinetic properties, including improved membrane permeability and metabolic stability, making them more suitable for drug development. In this study, we evaluated the effectiveness of two HuR inhibitors, CMLD2 (a coumarin-derived small molecule inhibitor of HuR) and DHTS (isolated from *S. miltiorrhiza* shown to bind to HuR) in TNBCs [15,16].

In present work, our *in-silico* explorations corroborated that both CMLD2 and DHTS work by binding to HuR and preventing its RRMs from recognizing and binding to target mRNAs by competitive inhibition. Our biophysical and biochemical analysis attempt to explore and compare the specificity and efficacy of both the molecules in targeting HuR and its oncogenic activity.

2. Materials and methods

2.1 In silico docking and ligand-protein interaction prediction

The crystal structure of HuR in its closed confirmation containing two N-terminal RRMs (i.e. RRM1 and RRM2) along with RNA (PDB ID: 4ED5) was used for the molecular docking studies via Autodock 4.2.6, after the RNA and solvent removal followed by energy minimization using Pymol 3.1.3 and Swisspdbviewer respectively [17–19]. Similarly, the open configuration of the HuR containing RRM1 and RRM2 devoid of RNA (PDB ID: 4EGL) was also processed and used for the docking experiments with the CMLD2 and DHTS respectively using the same strategy [20].

The binding of the CMLD2 and DHTS was defined at the RNA binding crevice formed between the RRM1 and RRM2 [20]. The coordinates of CMLD2 and DHTS were obtained from the PubChem Database using the PubChem ID: 16746438 and 11425923 respectively and were subjected to energy minimization before docking [21]. The molecular docking of the CMLD2/DHTS in the defined binding site of the HuR was performed using Autodock 4.2.6 using the docking parameters as mentioned in the previous study [17,18]. From the top ten docked poses, the conformation with the lowest free energy binding within the largest cluster was selected as the optimal docked conformation for both CMLD2 and DHTS. Thereafter, the structural diagrams of RRM domains, RNA interacting residues, the difference between open and closed HuR configurations, and interactions within the HuR-CMLD2 and HuR-DHTS docked complexes in both open and closed configurations were constructed using Pymol 3.1.3 [22]. The architecture of the RRM1 and RRM2 was obtained from the PDBsum database using the HuR structure (PDB ID: 4ED5) [23]. Additionally, to understand the RNA interacting residues of the HuR, an RNA-protein interaction map was constructed using NucPlot [24].

2.2 ADMET analysis profiling of CMLD2 and DHTS

The SwissADME was used to assess the pharmacokinetic and physicochemical drug-likeliness properties (such as lipophilicity, H-bond donor, H-bond acceptor, etc) of both the HuR inhibitors CMLD2 and DHTS [25,26]. Additionally, the toxicology analysis was conducted using Osiris Property Explorer [27].

2.3 Binding site surface area and volume assessment

The binding site assessment (the depth, surface area, and volume of the binding pocket in the presence of the ligands) of both HuR-CMLD2 and HuR-DHTS best-docked complexes in both open and closed states (HuR PDB ID: 4EGL and 4ED5 respectively) was conducted using Dogsitescorer software [28].

2.4 Cell Lines

MDA-MB-231cells were procured from National Centre for Cell Science (Pune, India); while MDA-MB-468 (#CL-0290) and 4T1 (#CL-0007) were purchased from Elabscience Bionovation Inc. (TX, USA). All three cell lines were grown in DMEM (Gibco, NY, USA) complemented with 10% FBS, 5 U/mL penicillin, and 0.5 U/mL streptomycin, and 2 mM glutamine (all from Gibco, NY, USA). Cell lines were routinely screened for mycoplasma presence using an EZ-PCR kit (HaEmek, Israel).

2.5 Chemical Inhibitors

Two inhibitors were utilized for the present study. CMLD2 (Emperical formula: $C_{31}H_{31}NO_6$; Mol wt: 513.58) is a specific inhibitor of HuR having a coumarin core. DHTS (Empirical Formula: $C_{18}H_{14}O_3$; Mol wt: 290.447) derived from *Salvia miltiorrhiza*, is a compound commonly utilized in Chinese traditional medicine. For the present study, both CMLD2 (Cat No. 538339) & DHTS (Cat No. D0947) were procured from Sigma-Aldrich (MO, USA).

2.6 *MTT Assay*

For the MTT assay, TNBC cells were seeded at a final density of 3×10^4 cells/mLin a fresh DMEM medium supplemented with 10% FBS. A 100 µL aliquot of the cell suspension was added to each well of a 96-well tissue culture plate (Nunc, ThermoFisher Scientific, MA, USA), making it a total of 3000 cells/well. The cells were treated with varying concentrations of CMLD2 or DHTS for 48 h, along with untreated and vehicle treated cells were used as controls. At treatment completion, 10 µL of 5 mg/mL yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) solution was added to the wells. MTT is reduced by metabolically active cells resulting in intracellular purple formazan. Cell viability was assessed in a colorimetric fashion where the formazan crystals solubilized in

DMSO was quantified spectrophotometrically [29]. Following this, the IC_{50} and LC_{50} were calculated [30].

2.7 Quantitative real time PCR (qPCR)

Total RNA was isolated from cultured cells using the TRIzol™ Reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. A total of 1 μg of RNA was used to reverse transcribe cDNA using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) with a mix of anchored oligo(dT) and random hexamer primers as per manufacturer's protocol. The resulting cDNA was diluted 1:4 and used as a template for qPCR. Quantitative PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) on LightCycler 480 (Roche). Each reaction was carried out in a 15μL volume containing 1X SYBR Green Master Mix, 0.5 μM of each forward and reverse primer, and diluted cDNA. All reactions were run in technical triplicates. Cycling conditions were as follows: initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 55 °C for 30 seconds followed by 72 °C for 30 seconds. Amplification specificity was verified by melt curve analysis. Gene expression levels were normalized to two internal control genes, β-actin and GAPDH, and the relative expression was calculated using the comparative Ct method (2−ΔΔCt) [31].

Primer sequences used for qPCR are as follows:

β-actin

FP: 5'-*TCACCATGGATGATGATATCGC* **-3'**,

RP: 5'- *AATCCTTCTGACCCATGCC* **-3'**

GAPDH

FP: 5'- *CCATCACCATCTTCCAGGAG* **-3'**

RP: 5'- *TCTCCATGGTGGTGAAGACA* **-3'**

ELAVL1

FP: 5'- *CCCCAACCAGAACAAAAACG* **-3'**

RP: 5'-*ATGGGGGAGAACCTGAATCT* **-3'**

MMP9

FP: 5'- *CCCGGACCAAGGATACAGT* **-3'**

RP: 5'- *AGTGAAGCGGTACATAGGGT* **-3'**

c-Fos

FP: 5'-*GCCTCTCTTACTACCACTCACC*-**3'**

RP: 5'-*AGATGGCAGTGACCGTGG*-**3'**

2.8 Western Blotting

TNBC cells i.e. MDA-MB-468, MDA-MB-231, and 4T1 were treated with either CMLD2 or DHTS1 for 48 h, and processed as described earlier by us [32]. Primary antibodies used are: HuR (sc-5261) and c-Fos (sc-52, from Santa Cruz Biotechnology Inc., TX, USA); non-phospho (Active)-β-Catenin (19807, from Cell Signaling Tech, MA, USA); E-cadherin (610405), Vimentin (550513), and N-cadherin (610920, from BD, NJ, USA); MMP9 (ab A11147 from Abclonal, MA, USA), and to β -actin (A5441 from Sigma, MO, USA). Secondary goat anti-mouse (P0447)/rabbit (P0448) HRP-conjugated antibodies (from Dako, Glostrup, Denmark) were utilized. Protein bands were developed using Chemiluminescence HRP substrate (BioRad, CA, USA), and were imaged using the Chemi Doc™ imaging system from Bio-Rad (CA, USA).

2.9 Scratch Assay

The scratch assay was conducted as previously described [33]. Briefly, TNBC cells were grown to full confluence and the wound was created using a 200 µl micropipette tip. After PBS washing, a fresh culture medium containing vehicle, CMLD2, or DHTS, and the cells were incubated for 48 h (at 37°C). After the experiment, the wound area was calculated using ImageJ software (National Institutes of Health, USA).

2.10 Matrigel Droplet Invasion assay

Briefly, the MDA-MB-231 cells were mixed with the EHS matrix at a 1:1 ratio, and 10 µL droplets of the cell-Matrigel mixture were seeded (8000-10,000 cells/ droplet) on 24-well plates. After Matrigel solidification, media containing vehicle, CMLD2, or DHTS was added to the plates and incubated for next 48 h. After which the drug-free complete media was added and the cells were observed until they invaded out of Matrigel. Images were taken in Olympus CKX53, Tokyo, Japan, and the area of migration was calculated using ImageJ software (National Institute of Health, US).

2.11 *Clonogenicity Assay*

Briefly, TNBC cells were seeded in 6-well plates at a density of 300-500 cells/well. After incubation, the cells were treated with either CMLD2 or DHTS for 48 h. Upon completion of the treatment, the cells were allowed to grow in complete media for 10-14 days until colonies became visible. The colonies were then fixed using 4% formaldehyde and processed following the method previously described by us [34].

2.12 *3D Tumour Spheroid Formation Assay*

MDA-MB-468 and 4T1 cells were used for spheroids generation using the method previously described [35]. In short, a single-cell suspension of cells in the complete medium was plated onto ultralow-attachment plates (CORNING, MA, USA) and kept at 37°C with 5% CO_2. After 72 h, the spheroids were treated with vehicle, CMLD2, or DHTS for an additional 48 h. After the treatment, the media was discarded, and fresh media with Matrigel (EHS matrix, Cat # 356234, CORNING, MA, USA) were added. The spheroids were observed under a microscope (Olympus CKX53, Tokyo, Japan) after 3 to 4 days, to measure the extent of cell invasion.

2.13 *Statistical Analysis*

All the data are presented as the mean ± standard error of the mean. Statistical analysis and graph generation were performed using GraphPad Prism 8.0 software (CA, USA). A p-value of <0.05 was considered significant. Each experiment was performed at least three times.

3. Results and discussion

3.1 Both CMLD2 and DHTS could bind to HuR at the RNA-interacting interface

HuR recognizes and interacts with mRNA primarily via its two N terminal RRMs namely RRM1 and RRM2. These domains span from 18-94 and 107-186 amino acid residue positions that are connected by a 12-residue inter-domain linker (95-106 residues) **(Supplementary Fig. 1a)**. Both the RRM domains exhibit an αβ-sandwich with a characteristic β1-α1-β2- β3-α2-β4 topology which are connected by a distinctive 3_{10} helix inter-domain linker, which keeps these domains at a distance of >13 Å in the open/unbound configuration [20].

The structure of HuR switches between apo (open) and holo (closed) conformations characterized by inter-residue interactions between its RRM domains and an inter-domain flexibility provided by these residues in its RRM and linker domains. The open confirmation of HuR (PDB ID: 4EGL) is adopted when the protein is unbound to RNA and a closed confirmation (PDB ID: 4ED5) when bound. During, this event, a huge displacement of the RRM2 domain is observed as shown in **Fig. 1a**. The 11-nucleotide sequence from the 3' UTR of the c-Fos mRNA was used to elucidate HuR-mRNA interaction. The RNA binds between the positively charged clefts formed by N21, N25, Q29, K89 and K92 of RRM1; R97 and K104 of inter-domain linker; and N107, R147, R153, K182 of RRM2. Interestingly, a total of 21 HuR residues- N21, N25, Y26, Q29, L61, F65, K89, T90, K92, R97, S99, S100, K104, N107, Y109, R147, F151, R153, K182, A184, and A185 are known to interact with the RNA. Therefore, we have chosen this cleft for molecular docking studies (**Supplementary Fig. 1b**). Upon molecular docking, it was found that both CMLD2 and DHTS have the potential to interact with the RNA interacting interface of the HuR (PDB ID: 4ED5). The HuR-DHTS complex in the closed conformation showed that DHTS could interact with 11 amino acid residues of HuR such as I23, N25, Y26, Y63, K92, R97, I103, K104, I133, N134, and R153. Out of these 11 residues, 7 were known to interact with RNA (N25, Y26, K92, R97, K104, and R153) (**Fig. 1c)**. On the other hand, in the case of CMLD2, 13 residues such as I23, N25, Y26, L61, G62, Y63, F65, R97, I103, I133, N134, F151, and R153 were involved in interaction with CMLD2 where 7 residues are known to interact with RNA (N25, Y26, L61, F65, R97, F151 and R153) (**Fig. 1b)**. However, both CMLD2 and DHTS were able to interact with HuR in the closed conformation and exhibited good binding energy of -8.947 and -8.418 kcal/mol, respectively. To strengthen our findings, we compared binding pocket/cavity/site

scoring analysis by Dogsitescorer for both the CMLD2-HuR and DHTS-HuR complexes. It was found DHTS binding cavity was 16.88 Å deep and displayed a surface area and volume of 1160.94 $Å^2$ and 785.92 $Å^3$ (**Table 1**). Being a bigger molecule, CMLD2 displayed a 21.11 Å deep binding pocket with a higher binding pocket surface area as well as a volume of 1624.51 $Å^2$ and 1392.64 $Å^3$, highlighting that CMLD2 could block the RNA-binding site of HuR more efficiently than DHTS. Interestingly, both the ligands were found to interact with HuR in open conformation (PDB ID: 4EGL) as well. A total of 8 HuR residues- T20, Y68, V69, N107, N134, S135, R136, and F151 were found to be involved in interaction with the DHTS where only 2 RNA interacting residues (N107 and F151). On the other hand, CMLD2 was found to be interacting with 11 HuR residues - R19, T20, N21, R97, N107, Q119, N134, S135, R136, V137, F151 of which 4 were RNA interacting residues (N21, R97, N107, F151) **(Table 2)**. The Dogsitescorer analysis showed that CMLD2 possess a bigger binding pocket with surface area and volume of 1079.83 $Å^2$ and 877.06 $Å^3$ as compared to 1005.06 $Å^2$ and 835.58 $Å^3$ (**Table 1**). However, the depth of the cavity formed by these ligands on HuR in an open configuration was almost similar.

Our in-silico studies showed that both DHTS and CMLD2 can interact with the RNA interacting interface of the HuR in both open and closed configurations. However, CMLD2 was found to be more efficient due to its ability to form a larger binding pocket and higher binding energy by interacting with more HuR residues which are meant to interact with RNA. Further the drug-likeliness properties of the ligands via ADMET analysis revealed CMLD2 (513.58 Da) has a greater number of aromatic heavy atoms (22 atoms) as compared to lower molecular weight (273.3 Da) DHTS (10 atoms) and also had 6 H-bond acceptors as compared to 3 of DHTS **(Supplementary Fig. 2)**. A total of 9 rotatable bonds were also present in the case of CMLD2, indicating the possibility of attaining multiple configurations during molecular docking. The ESOL LogS values of CMLD2 and DHTS were found to be -5.34 and -4.69 respectively, highlighting better solubility of DHTS as compared to CMLD2. The lesser solubility of CMLD2 can be potentially addressed by using complexing agents like cyclodextrins that can improve the aqueous solubility, bioavailability and stability [36]. Other cargo delivery agents like Viral Like Particles (VLPs) can also be used to encapsulate the compound to ensure its precise cargo delivery [37].

The in-silico toxicity analysis predicted that DHTS has no mutagenic property but might possess tumorigenic, irritant, and reproductive effects risks. On the other hand, the CMLD2 was found to be a better drug candidate as it showed no predicted mutagenic as well

as tumorigenic risks. Additionally, the Boiled-egg diagram indicated that DHTS could penetrate the Blood-brain barrier, whereas the CMLD2 did not possess such property (**Supplementary Fig. 2)**. Next, to functionally evaluate the HuR inhibition capacity of both the CMLD2 and DHTS, various cell-based assays were conducted.

3.2 CMLD2 and DHTS demonstrate variations in their antiproliferative effectiveness on TNBC cells

To evaluate the inhibitory activity of CMLD2 and DHTS on TNBC cell lines MTT assay was performed. For the experiments, two TNBC cell lines viz. MDA-MB-231 (mesenchymal stem-like), MDA-MB-468 (basal-like 1), and one murine metastatic TNBC cell line- 4T1 were used. CMLD2 and DHTS both displayed a dose-dependent inhibition of proliferation and survival in all three cell lines with both drugs exhibiting a slightly weaker effectiveness in MDA-MB-231 cells compared to the other two. Among the two drugs, the natural compound DHTS exhibited greater cytotoxic activity across all three cell lines than the HuR-specific CMLD2 at comparable concentrations. The half maximal inhibitory concentrations (IC_{50}) for CMLD2 were 46.85 μM, 24.14 μM, and 23.21 μM for MDA-MB-231, MDA-MB-468, and 4T1 respectively (**Fig. 2a**), while for DHTS, the values were 2.229 μM, 1.711 μM and 1.422 μM at 48 h of treatment (**Fig. 2b**). Sublethal treatment dosages well below IC_{50} were decided upon for further experiments to evaluate signaling changes, differential protein expression, and adaptive responses of the cells to the drugs. For MDA-MB-231, the treatment dose was determined to be 20 μM for CMLD2 and 1.5 μM for DHTS, while for MDA-MB-468 and 4T1 the treatment doses were decided at 15 μM CMLD2 and 0.7 μM DHTS respectively. In the above *in vitro* experiments, DHTS demonstrated a better inhibition of cancer cell proliferation in TNBC cell lines. However, protein expression analysis of downstream targets of HuR (i.e., MMP9 and β-catenin) revealed the differences in the specificities of both drugs. Among the two, CMLD2 on all the tested molecular markers (i.e., HuR-downstream targets and EMT markers) was consistent and mimicked the results obtained using HuR-specific siRNA (**Supplementary Fig. 3a**).

3.3 CMLD2 exhibits greater specificity in inhibiting HuR compared to DHTS

To examine whether the cytotoxicity exhibited by the drugs viz. CMLD2 and DHTS were through their inhibitory activity on HuR, we subsequently evaluated HuR protein expression along with its downstream targets i.e., MMP9 and β-catenin. Both MMP9 and β-

catenin are established downstream targets of HuR. The 3' UTR region of the MMP9 mRNA has 4 AREs of which HuR was demonstrated to have a strong constitutive binding to 3' of the AREs and have a stabilizing effect [38,39]. β-Catenin is also established as a bona fide target of HuR. di Silanes et al. using REMSA and REMSA supershift assay demonstrated that endogenous HuR binds to β-catenin mRNA transcript [40]. Additionally, Chou et al, established that β-catenin translation was regulated in mammary carcinoma in a HuR-dependent manner [41]. Hence in our present experiments, the MMP9 and β-catenin protein expression were monitored in response to HuR inhibition by CMLD2 and DHTS. Our experiments showed that treatment with CMLD2 resulted in a significant downregulation of HuR protein with a concomitant downregulated expression of HuR target proteins MMP9 and β-catenin (**Fig. 3a**). Though the mode of action of CMLD2 is competitive inhibition by disrupting HuR-mRNA interaction, the observed downregulation in HuR protein might be attributed to the autoregulatory activity of HuR wherein HuR might exhibit autoregulatory activity by binding to and stabilizing its mRNA [42,43]. The decrease in protein expression levels of HuR, MMP9, and β-catenin was consistent across all three cell lines. However, in DHTS-treated TNBC cells, though DHTS elicited a downregulation in HuR in all three cell lines, it failed to show consistency for the downstream targets (**Fig. 3b**). The densitometric quantification of MMP9 and β-catenin largely showed a statistical insignificance among biological replicates.

3.4 CMLD2 treatment demonstrates superior effects on EMT proteins compared to DHTS

As HuR as well as its downstream effectors i.e., MMP9 and β-catenin are well-established mediators of epithelial-to-mesenchymal transition (EMT) thereby supporting the migration and invasion of cancer cells [44–46], we next investigated the protein expression profiles of the EMT markers after treatment with CMLD2 and DHTS. CMLD2-treated cells expressed reduced N-cadherin and vimentin in MDA-MB-231 cells (**Fig. 4a**). Here we would like to mention that MDA-MB-231 cells lack E-cadherin protein expression because of the hypermethylation of its gene promoter [47], and parental MDA-MB-468 cells are vimentin negative [48]. A marked decrease was observed in E-cadherin and N-cadherin in CMLD2-treated MDA-MB-468 and 4T1(**Fig. 4a**). However, no significant change in vimentin was seen in the murine cell line 4T1. In DHTS-treated cells (**Fig. 4b**), an overall upregulation in E-cadherin and N-cadherin expression was observed in both MDA-MB468 and 4T1 (not statistically significant), when compared among bio-replicates. Significant downregulation in

Vimentin expression was observed in 4T1 in response to DHTS treatment. In MDA-MB-231, N-cadherin showed a statistically significant downregulation, however, Vimentin levels remained unchanged in DHTS-treated cells (**Fig. 4b**). In the present study, a suboptimal dose of DHTS was chosen, based on the observed downregulation of HuR expression. At these suboptimal treatment doses, DHTS did not significantly inhibit the assayed EMT markers, whereas CMLD2 substantially downregulated E-cadherin, N-cadherin, and Vimentin. It is important to note that the mRNAs of all three EMT markers examined in the present study have previously been reported to be regulated by HuR [49–51]. In line with these findings, siRNA knockdown of HuR resulted in the downregulation of all three EMT markers (**Supplementary Fig. 3a**). Our findings showed that, despite the molecular alterations, both CMLD2 and DHTS significantly inhibited the migration and invasion of TNBC cells, indicating that DHTS may exert its effects through HuR-independent mechanisms. Based on our results, we hypothesize that the cytotoxicity exhibited by DHTS might not largely be due to its modulation of HuR. While DHTS is a disruptor of HuR-mRNA binding, the compound has also been reported to be an inhibitor of Erp57 and to activate breast cancer apoptosis in a PERK-elF2α-ATF4 dependent manner [52]. In hepatocellular carcinoma, DHTS has also been shown to have an anti-proliferative effect by influencing the AMPK/AKT/mTOR pathway and MAP Kinase signaling pathway [53]. The combined action of HuR-dependent and HuR-independent mechanisms of DHTS action might explain the efficacy of DHTS as an anticancer molecule. The HuR-independent activity of DHTS can be a reason for the inconsistencies obtained at the molecular level when compared among DHTS-treated biological replicates in the present study, which was also validated by our biophysical data.

3.5 Both CMLD2 and DHTS significantly inhibit migration, invasion, and clonal potential of TNBC cells

Next, we examined the physiological effects of altered EMT proteins after treatment with HuR inhibitors through cell migration assays (scratch assays). As EMT pushes the cancer cells towards a mesenchymal phenotype, we hypothesized that the reduction in the EMT-associated proteins i.e., E-cadherin, N- cadherin and vimentin (after HuR inhibition) would compromise the migratory capability of the TNBC. Our results demonstrated that the control cells exhibited dynamic cell migration with the monolayer wound almost completely healing within 48 h. As expected, both CMLD2 and DHTS-treated cells inhibited the cell migration in all three cell lines resulting in incomplete wound closure of cells (**Fig. 5a, b**). Of the three

cell lines, MDA-MB-468 is more epithelial, hence the difference in migration rate was observed to be more prominent in MDA-MB-468 for both CMLD2 and DHTS. Overall, despite CMLD2 exhibiting superior inhibition of EMT markers at the molecular level compared to DHTS, the rate of monolayer closure appeared comparable in both treatments.

To further confirm the results, a Matrigel droplet invasion assay was conducted with the invasive MDA-MB-231 cells. In this assay, cell invasion was evaluated in a 3D environment by plating a mixture of cells and EHS-matrix in a droplet (*as described in Materials and Methods*). In both treatment groups (CMLD2 and DHTS), the control cells began to invade out of the Matrigel drop by day 3, whereas the treated cells required more than 4 days. By day 7, a significant difference in invasion was observed in both CMLD2 and DHTS-treated cells. The invading front perimeter was reduced by approximately 60% in the CMLD2-treated cells, and about 50% in the DHTS-treated cells (**Fig. 5c**). These results suggest that while DHTS has a similar effect to CMLD2 in inhibiting the migratory and invasive potential of TNBC cells, its influence on HuR downstream targets and EMT markers is less pronounced compared to CMLD2. To further assess the impact of HuR inhibition on the long-term survival and replicative capacity of cells, colony formation assays were conducted on the TNBC cell lines for 12 days following treatment with CMLD2 and DHTS for 48 h. The stained colonies showed that DHTS treatment significantly outperformed CMLD2 in suppressing the clonogenic potential of MDA-MB-468, MDA-MB-231, and 4T1 cells (**Fig. 6a, b**). These findings align with the cell proliferation assays, where DHTS demonstrated greater cytotoxicity than CMLD2.

3.6 CMLD2 and DHTS treatment resulted in the inhibition of MDA-MB-468 and 4T1-derived 3D culture

Multicellular 3D tumour spheroid formation assay, using Matrigel, was used to assess the anchorage-independent growth of cells. The relevance of tumour spheroids in examining drug efficacies lies in the ability of spheroids to roughly mimic avascular tumour regions while also establishing key physiologically relevant factors like cell-cell communication, nutrient, and drug penetration gradient. Here, spheroids of MDA-MB-468 and 4T1 were formed and one-time treatment of vehicle, CMLD2, or DHTS was given 48 h before the addition of Matrigel. Following this, the growth and invasion of the cells into Matrigel were monitored for 7 days. CMLD2 treatment resulted in a complete disintegration of the pre-formed spheroids, while DHTS-treated spheroids demonstrated a significant decrease in the spheroid

size compared to the control (**Fig. 7a, b**). The differential modulation of E-cadherin by the two compounds merits deliberation. MDA-MB-231, owing to its hypermethylated E-cadherin status, lacks protein expression. However, in MDA-MB-468 and 4T1 cells, a "dual role" of E cadherin has been reported, with the protein expression being associated with an oncogenic role in both cell lines [54,55]. Additionally, as previously mentioned, HuR positively regulates E-cadherin. Both siHuR and CMLD2, downregulated E-cadherin in these cell lines. Conversely, DHTS upregulated E-cadherin. Prior research by Fan et al. (2022) on U-2OS cells demonstrated that DHTS improved cell-cell adhesion by upregulating E-cadherin at the mRNA and protein levels [56]. We have also reported the upregulation of E-cadherin by DHTS at doses close to IC_{50} previously, as well [57]. This differential modulation of E-cadherin by the two drugs may explain the discrepancies observed in the 3D-spheroid assay outcomes. The downregulation of E-cadherin likely contributes to the disintegration of spheroids with CMLD2 treatment, while the upregulation of E-cadherin might explain why DHTS treatment preserves the morphology of pre-formed spheroids, despite inhibiting their proliferation.

Interestingly, it is to be noted that while DHTS stabilizes the closed conformation, its inhibitory activity on HuR is non-uniform. Ribonucleoprotein-immunoprecipitation followed by microarray studies performed by Lal et al., [16] revealed that mRNAs with higher U/AU rich elements were enriched rather than depleted suggesting that the inhibitory activity of DHTS was more prominent only for mRNAs with lower U/AU densities in their UTRs. Hence the relevance of DHTS as an inhibitor of HuR is specific only to mRNA transcripts with lower HuR binding affinities. Based on our investigations, the other reason for the superior performance of CMLD2 can be the size of the molecule being approximately twice as that of DHTS. The larger size might contribute to its specificity by reducing the off-target interactions as opposed to DHTS whose off-target effects might interfere with its ability to consistently inhibit HuR. Overall, on the basis of our results, we advocate that CMLD as superior inhibitor of HuR over DHTS.

3.7 Disruption of c-Fos RNA Binding to HuR by CMLD2 and DHTS in TNBCs

Previous studies have revealed the crystal structure of HuR interacting with the 11-nucleotide sequence from the 3' UTR of c-Fos mRNA. Our in-silico analysis demonstrated that both CMLD2 and DHTS binds to the RNA-interacting interface of HuR in both its open and

closed conformations. To experimentally validate the inhibition of RNA binding by CMLD2 and DHTS, we monitored c-Fos mRNA expression in MDA-MB-231 cells. Following treatment for 48 hours at sub-optimal doses of 20 μM CMLD2 and 1.5 μM DHTS, c-Fos mRNA levels were quantified and found to be reduced to approximately exactly 50% compared to the control group (n=3) (**Fig. 8a**). To further validate these findings, c-Fos protein levels were examined in both MDA-MB-231 and MDA-MB-468 cell lines. Sub-optimal treatment of both CMLD2 and DHTS led to a significant reduction in the c-Fos protein levels (**Fig. 8b**). However, CMLD2 treatment exhibited a more pronounced decrease in c-Fos protein levels compared to that of DHTS treatment, underscoring its efficacy, in line with our previous experiments (**Fig. 8b**). Additionally, HuR silencing in both cell lines resulted in a reduction in c-Fos protein levels, further validating our findings. These results confirm that both CMLD2 and DHTS interact with the RNA-binding interface of HuR, leading to destabilization of c-Fos mRNA, ultimately causing a downregulation of c-Fos levels. Finally, to evaluate the concentration-dependent effect of CMLD2, MDA-M-231 cells were treated with 10, 20, 30 and 40 μM doses, followed by the quantification of c-Fos protein levels in the whole cell lysates. Interestingly, an inverse correlation was observed between CMLD2 concentration and c-Fos levels, with increasing concentration resulting in progressive decline (**Supplementary Fig. 3b**).

Together, the findings bolster our hypothesis, demonstrating that CMLD2 effectively disrupts HuR-RNA binding and suppresses subsequent c-Fos expression.

4. Conclusions

In this study, we evaluated the biophysical and molecular specificity of two HuR inhibitors i.e., CMLD2 and DHTS, in TNBC cells. Both inhibitors bind to the RRM1 and RRM2 motifs of HuR, thereby disrupting HuR-mRNA interactions. Our biophysical analysis indicated that compared to DHTS, CMLD2 has a larger binding pocket, higher binding energy, and a greater number of aromatic heavy atoms, which might enhance its ability to bind more effectively to the HuR protein. Cellular analysis further revealed that while both inhibitors effectively reduced the migration, invasion, and colony formation abilities of TNBC cells, CMLD2 demonstrated greater specificity in inhibiting HuR, its downstream targets MMP9 and β-catenin, and EMT markers compared to DHTS. Overall, the results provide sufficient evidence that CMLD2 exhibits greater specificity than DHTS in targeting the HuR protein and its associated signaling pathway. All these findings were validated by demonstrating significant reduction of c-Fos mRNA and protein levels following sub-optimal treatment of CMLD2 and DHTS, demonstrating the robustness of our hypothesis. Furthermore, the concentration dependent analysis of CMLD2 confirmed its effectiveness in disrupting HuR-c-Fos mRNA binding, ultimately leading to downregulation of c-Fos protein levels.

Further research is crucial to uncover the molecular mechanisms through which DHTS suppresses the physiological traits of TNBC cells independently of HuR inhibition. Being a natural compound, dihydrotanshinone (DHTS) is typically extracted from *Salviae miltiorrhizae* using organic solvents such as petroleum ether and ethyl acetate, as reported in previous chromatographic studies [58]. To enhance sustainability, an eco-friendly extraction protocol can be optimised by employing Response Surface Methodology (RSM), which has proven effective in maximizing yield while minimizing the environmental impact [59,60]. Given the medical significance of *Salviae miltiorrhizae*, integrative systems biology approaches, leveraging both transcriptomic and metabolomic datasets, can support targeted metabolic engineering strategies aimed at enhancing the biosynthesis of bioactive constituents like tanshinones [61,62].

Meanwhile, studies on cyclodextrin-based encapsulation and viral-like particle conjugation have shown potential in improving the delivery efficiency and bioavailability of CMLD2, offering promising directions for enhanced therapeutic applications. Moreover, continued investigation into these molecular pathways is vital to further elucidate DHTS's

complex role in cancer cell regulation. Gaining deeper insight into these mechanisms could enable the development of targeted therapies, providing new opportunities for advancing TNBC treatment and intervention.

CRediT authorship contribution statement

Arundhathi J. R. Dev: Data Curation, Methodology, Investigation, Formal analysis, Validation, Visualization, Writing–original draft, Writing–review & editing. **Lakshay Malhotra:** Data Curation, Formal analysis, Software, Validation, Visualization, Writing–original draft, Writing–review & editing. **Akanksha Kashyap:** Formal analysis, Methodology, Validation. **Sheikh Mohammad Umar:** Formal analysis, Investigation, Validation. **Meetu Rathee:** Data Curation, Investigation, Validation. **Swarnava Samanta:** Data Curation, Formal analysis. **Rahul Kharkwal:** Data Curation, Formal analysis.
Debarka Sengupta: Data Curation, Formal analysis, Resources, Supervision. **Chandra Prakash Prasad:** Conceptualization, Data Curation, Formal analysis, Funding acquisition, Investigation, Project Administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interests

All authors declare no competing interests that could have influenced the work reported in this article.

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Data availability statement

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figure Legends

Fig. 1. Evaluating the interaction of HuR inhibitors with the open and closed forms of HuR. a) Conformational change in the N-terminal RRMs of HuR from open to closed conformation upon binding to mRNA. The 11 nucleotide HuR binding motif of c-Fos mRNA 3' UTR was used to study the changes in protein structure upon interaction with mRNA **b)** Docking of CMLD2 in the binding pocket formed by RRM1 and RRM2 of HuR in the closed (PDB ID: 4ED5) and open conformation (PDB ID: 4EGL) elucidating the key amino acid residues involved in HuR-CMLD2 interaction. **c)** Docking of DHTS to HuR N-Terminal RRMs similar to **b).**

Fig. 2. Effect of HuR inhibitors i.e., CMLD2 and DHTS1 on TNBC cell proliferation. a) TNBC cell lines (MDA-MB-231, MDA-MB-468, and 4T1) were exposed to varying concentrations of CMLD2 (5-50 μM) for 48 h. The error bars indicate the standard error of the mean (n = 5). **b)** TNBC cell lines (MDA-MB-231, MDA-MB-468, and 4T1) were exposed to different concentrations of DHTS (0.5-10 μM) for 48 h. The error bars represent the standard error of the mean (n = 5).

Fig. 3. CMLD2 inhibits HuR and its downstream targets i.e., MMP9 and β-catenin better than DHTS. a) *Upper panel*- Representative Western blot for HuR protein and its downstream targets (MMP9 and β-catenin) in TNBC cell lines (MDA-MB-231, MDA-MB-468, and 4T1) treated with a sub-optimal dose of CMLD2 i.e., 20 μM for MDA-MB-231 and 15 μM for MDA-MB-468 and 4T1 for 48 h. *Lower panel*- Quantification of the HuR, MMP9, and β-catenin protein bands was performed by calculating the integrated densitometric values and normalizing them to β-actin levels. All error bars indicate the standard error of the mean (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **b)** *Upper panel*- Representative Western blot for HuR protein and its downstream targets (MMP9 and β-catenin) in TNBC cell lines (MDA-MB-231, MDA-MB-468, and 4T1) treated with a sub-optimal dose of DHTS i.e. 1.5 μM for MDA-MB-231 and 0.7 μM for MDA-MB-468 and 4T1 for 48 h. *Lower panel*- Quantification of the HuR, MMP9, and β-catenin protein bands was performed by calculating the integrated densitometric values and normalizing them to β-actin levels. All error bars indicate the standard error of the mean (n = 3). *$p < 0.05$; **$p < 0.01$; ns= non-significant.

Fig. 4. The HuR inhibitor CMLD2 modulates EMT markers more effectively than DHTS. **a)** *Upper panel*- Representative Western blot for EMT proteins in TNBC cell lines

(MDA-MB-231, MDA-MB-468, and 4T1) treated with a sub-optimal dose of CMLD2 i.e., 20 μM for MDA-MB-231 and 15 μM for MDA-MB-468 and 4T1 for 48 h. *Lower panel*- Quantification of the E-cadherin, N-cadherin, and Vimentin protein bands was performed by calculating the integrated densitometric values and normalizing them to β-actin levels. All error bars represent the standard error of the mean (n = 3). *p < 0.05; **p < 0.01; ns= non-significant. **b)** *Upper panel*- Representative Western blot for EMT proteins in TNBC cell lines (MDA-MB-231, MDA-MB-468, and 4T1) treated with a sub-optimal dose of DHTS i.e. 1.5 μM for MDA-MB-231 and 0.7 μM for MDA-MB-468 and 4T1 for 48 h. *Lower panel*- Quantification of the E-cadherin, N-cadherin, and Vimentin protein bands was performed by calculating the integrated densitometric values and normalizing them to β-actin levels. All error bars represent the standard error of the mean (n = 3). **p < 0.01; ns= non-significant.

Fig. 5. Impact of HuR inhibitors CMLD2 and DHTS1 on migration and invasion of TNBC cells (MDA-MB-231, MDA-MB-468, and 4T1). Wound-healing was performed using TNBC cells treated with either **a)** CMLD2, or **b)** DHTS alone for 48 h. Graphs were generated showing the relative migration of TNBC cells treated with vehicle (DMSO). All bars represent the standard error of the mean (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001; ns= non-significant. **c)** Invasion analysis was performed on MDA-MB-231 cells using Matrigel droplet invasion assay as described in the Materials and Method section. The relative migration was calculated by measuring the area of migration in CMLD2 or DHTS-treated cells, compared with control. The graphs were generated showing the relative migration ability of TNBC cells treated either with 20 μM CMLD2 or 1.5 μM DHTS compared with controls. All error bars indicate the standard error of the mean (n = 3). **p < 0.01.

Fig. 6. Examining the impact of HuR inhibitors CMLD2 and DHTS1 on colony formation potential in TNBC (MDA-MB-231, MDA-MB-468, and 4T1) cells. The colony-forming potential was examined by a clonogenicity assay (*as described in the Materials and Method section*). **a)** *Upper panel*- Representative CFU images of TNBC cell lines treated with a sub-optimal dose of CMLD2 i.e., 20 μM for MDA-MB-231 and 15 μM for MDA-MB-468 and 4T1 for 48 h. *Lower panel*- Graphs were made showing the relative colony formation ability of TNBC cells treated with CMLD2, compared with controls. All error bars indicate the standard error of the mean (n = 3). *p < 0.05. **b)** *Upper panel*- Representative CFU images of TNBC cell lines treated with a sub-optimal dose of DHTS i.e. 1.5 μM for MDA-MB-231 and 0.7 μM for MDA-MB-468 and 4T1 for 48 h. *Lower panel*- Graphs were

made showing the relative colony formation ability of TNBC cells treated with DHTS, compared with controls. All error bars indicate the standard error of the mean (n = 3). *p < 0.05; **p < 0.01.

Fig. 7. Impact of HuR inhibitors CMLD2 and DHTS1 on 3D spheroids of TNBC cells. a) MDA-MB-468 and 4T1 TNBC cells were treated with 60 μM CMLD2 for the assay (*as described in the Materials and Method section*). However, CMLD2 disintegrated pre-formed spheroids. **b)** MDA-MB-468 and 4T1 TNBC cells were treated with 3 μM DHTS for the assay. After the treatment, the area of the spheroids was calculated, and graphs were plotted showing relative spheroid size between the treated and controls. All error bars represent the standard error of the mean (n = 3). **p < 0.01.

Fig. 8. Validation studies for the *in-silico* data corroborating HuR and c-Fos mRNA interaction. a) qPCR data showing the decreased mRNA expression of ELAVL1, MMP9 and c-Fos in MDA-MB-231 in response to CMLD2 and DHTS treatments *p< 0.05; **p< 0.01. b) Decreased protein expression of c-Fos upon treatment with CMLD2, DHTS and HuR specific siRNA (25 nM) in the cell lines MDA-MB-468 and MDA-MB-231. All bars represent the standard error of the mean (n = 3). *p< 0.05; **p< 0.01. Transient transfection of HuR siRNA (sc-35619, Santa Cruz Biotechnology) at 25 nM final concentration was performed using Lipofectamine® 3000 (Thermo Fisher Scientific) according to the manufacturer's protocol. All bars represent the standard error of the mean (n = 3).

Table 1

BINDING POCKET COMPARISON		
	CMLD2	**DHTS1**
CLOSED CONFORMATION (4ED5)		

DOCKED COMPLEX		
DEPTH (Å)	21.11	16.88
SURFACE ($Å^2$)	1624.51	1160.94
VOLUME ($Å^3$)	1392.64	785.92
DONORS	26	21
ACCEPTORS	21	18
OPEN CONFORMATION (4EGL)		
DOCKED COMPLEX		
DEPTH (Å)	17.42	17.18
SURFACE ($Å^2$)	1079.83	1005.06
VOLUME ($Å^3$)	877.06	835.58
DONORS	20	18

ACCEPTORS	13	14

Table 2: List of HuR residues found interacting with DHTS/CMLD-2 in apo (open) and closed (holo) configuration

S.No.	Residues	DHTS-HuR (apo)	DHTS-HuR (holo)	CMLD2-HuR (apo)	CMLD2-HuR (holo)
1.	Arg 19	-	-	Yes	-
2.	Thr 20	Yes	-	Yes	-
3.	Asn 21	-	-	Yes	-
4.	Ile 23	-	Yes	-	Yes
5.	Asn 25	-	Yes	-	Yes
6.	Tyr 26	-	Yes	-	Yes
7.	Leu 61	-	-	-	Yes
8.	Gly 62	-	-	-	Yes
9.	Tyr 63	-	Yes	-	Yes
10.	Phe 65	-	-	-	Yes
11.	Tyr 68	Yes	-	-	-
12.	Val 69	Yes	-	-	-
13.	Lys 92	-	Yes	-	-
14.	Arg 97	-	Yes	Yes	Yes
15.	Ile 103	-	Yes	-	Yes
16.	Lys 104	-	Yes	-	-
17.	Asn 107	Yes	-	Yes	-
18.	Gln 119	-	-	Yes	-
19.	Ile 133	-	Yes	-	Yes
20.	Asn 134	Yes	Yes	Yes	Yes
21.	Ser 135	Yes	-	Yes	-
22.	Arg 136	Yes	-	Yes	-
23.	Val 137	-	-	Yes	-
24.	Phe 151	Yes	-	Yes	Yes
25.	Arg 153	-	Yes	-	Yes

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