OLD TRICKS AND NEW TRENDS TO ADDRESS TUMORS: A MEDICINAL CHEMIST'S PERSPECTIVE

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SUNTO. – Molti sforzi nelle ultime decadi hanno permesso di ottenere nuovi principi attivi contro varie patologie tumorali, così da ridurre l'impatto oncologico in termini di disabilità e morti; ciononostante, molto resta da fare per aumentare la tollerabilità, la biodisponibilità e l'efficacia contro tumori aggressivi dei farmaci esistenti. Qui vengono descritti due approcci innovativi di ricerca, centrati sulla inibizione dell'interazione fra la RNA *binding protein* HuR e vari RNA messaggeri codificanti proto-oncogeni terapeuticamente importanti, e sull'attivazione del sistema immunitario del paziente contro cellule tumorali esprimenti il recettore PD-1 attraverso l'inibizione della sua interazione col ligando PD-L1. In entrambi i casi, due famiglie di *small molecules* biodisponibili e non tossiche sono state disegnate a partire da un prodotto naturale aspecifico (tanshinone, convertito in aza-tanshinoni sintetici – HuR), o da un peptidomimetico sintetico (arilossi bifenili convertiti in triazine disostituite – PD-1/PD-L1); dopo la sintesi e la caratterizzazione di alcune decine di analoghi, un *early lead* per ogni famiglia di composti è stato identificato e verrà ulteriormente profilato in ulteriori studi *in vivo*, mentre entrambi i progetti di *medicinal chemistry* proseguiranno allo scopo di identificare candidati preclinici ancora migliori.

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ABSTRACT. – Major efforts in the past decades have provided novel small molecule drugs and biologicals and innovative mechanisms against tumors, but the quest for safer, bioavailable and more effective active principles is still ongoing. This contribution focuses on innovative approaches to fulfill therapeutic goals in oncology; namely, the inhibition of the stabilizing interaction between the RNA-binding protein HuR and

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mRNAs of multiple oncogenes, and the unleashing of a strong immune reaction against cancer cells by antagonizing the PD-1 – PD-L1 interaction on the surface of cancer cells. "Classical" drug-like, bioavailable small molecules were either rationally designed from a naturally occurring template (tanshinones converted to aza-tanshinones – HuR), or from a synthetic peptidomimetic inhibitor (biphenyloxy aryls converted to disubstituted triazines – PD-L1); after their synthesis and activity profiling, early leads were identified to be further structurally optimized in the near future.

1. Background

In the last decades, many efforts were spent to understand the mechanism of cancer onset and progression at the molecular, biochemical and cellular level[1,2]. Tumor pathogenesis is now considered a multistep process in which normal cells progressively evolve to a neoplastic state through the acquisition of a set of hallmark capabilities (Hallmarks of Cancer) that enable them to become tumorigenic and, ultimately, malignant[3]:

- Genomic instability;
- Enhancement of cell survival;
- Reprogrammed cell metabolism;
- Invasion and metastasis;
- Elevation of local angiogenesis;
- Promotion of cell proliferation and tumorigenesis;
- Resistance to apoptosis;
- Evasion of immune recognition and tumor invasion promotion.

In this complex scenario, research is crucial for the development of effective anticancer agents exploiting different mechanisms of action, to be used in combination to improve the outcome of clinical treatments[4]. My presentation dealt with two "hot topics", representing significant modern avenues in anticancer research.

Human antigen R (HuR)[5] is an RNA-binding protein (RBP) member of the embryonic lethal abnormal vision (ELAV) family. As HuR is involved in the regulation of post-transcriptional gene expression for several mRNAs encoding for proteins relevant in oncogenesis and tumor progression, its altered expression or localization should lead to multiple pathologic phenotypes, including cancer[6]. Once more,

since multiple *in vitro* and *in vivo* studies have shown that HuR modulation impacts on the above-mentioned "Hallmarks of Cancer", its targeting is a promising strategy for a variety of cancers[7]. My research group, in collaboration with Dr. Arosio (SCITEC-CNR, medicinal chemistry), Prof. Provenzani (CIBIO, Trento University; biology and pharmacology) and Prof. Marinelli (Pharmacology Dept., Federico II – Naples University; computational and structural sciences), worked in the last decade to rationally design, synthesize and biologically profile novel small organic molecules as mRNA-HuR interaction disruptors and cytotoxic agents.

Despite immune surveillance elicited by the immune system, tumors can initiate a complex mechanism of immune evasion that leads to immune escape and, ultimately, to tumor progression[8,9]. In this scenario, cancer immunotherapy should activate the immune system to treat cancer, targeting the escape mechanism in general, and some of its specific components in particular[10]. The immune response is regulated by *stimulatory* and *inhibitory* immune checkpoints (ICs), which are in equilibrium to maintain self-tolerance and protect the host from tissue damage[11]. Among them, the **PD-1/PD-L1 axis** is one of the most exploited[12–14]. Monoclonal antibodies (mAbs) blocking PD-1/PD-L1 receptors have yielded clinical benefits in several tumor types, providing durable, long-term survival benefits and a better toxicity profile than conventional chemotherapy / targeted therapy[14]. To overcome the drawbacks of mAbs (*i.e.*, high costs, lack of oral bioavailability, poor tissue and tumor penetrating capabilities) my research group, in collaboration with Dr. Arosio, Prof. Marinelli and Prof. Sabbatino (Medicine Dept., Salerno University – biology, pharmacology), worked in the last decade to rationally design, synthesize and biologically profile novel small organic molecules as putative inhibitors of the PD-1/PD-L1 axis.

2. HuR

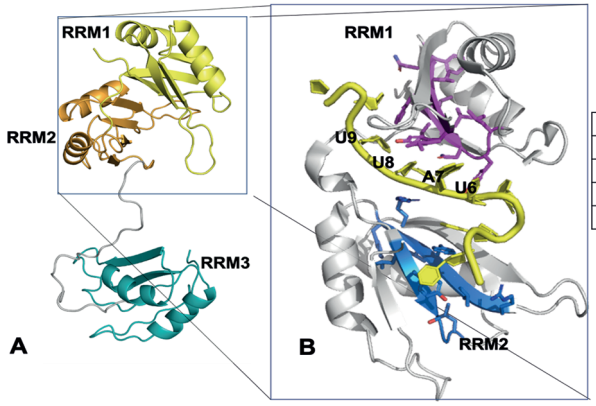
2.1. *Bio-structural background*

Human antigen R (ELAVL1, HuR) belongs to the ELAVL (Embryonic Lethal Abnormal Vision-like) family[1] that also includes HuB (or Hel-N1), HuC and HuD[15]. These proteins show three func-

tionally distinct RNA Recognition Motif (RRM) domains, which individually contribute to mRNA binding[16,17].

Characterization of the HuR-mRNA-binding elucidated the role for each RRM domain[16-20]. HuR RRM1 and RRM2 control the recognition of target mRNAs by binding to a 11-base AU-rich strand with nanomolar affinity. They bind to U-rich sequences (U-rich RNA and U-rich DNA), with higher affinity and preference for U-rich RNA sequences[21]. The structure of HuR complexed with a 11-mer RNA oligonucleotide derived from c-*fos* mRNA (PDB code 4ED5, *Fig. 1*[22]) shows a hydrophobic binding site, with key residues at the center of the β-platform (β1 and β3) in the RRM domain, and others located around the β-strands and in the RRM1-RRM2 linker. As to RNA, the RRM1 domain recognizes up to 5 consecutive uracils, while the inter-domain linker and RRM2 bind only to one or two nucleotides[16,23].

In the nucleus, HuR binds target pre-mRNA introns promoting splicing and alternative polyadenylation events[24,25]. Upon intrinsic or extrinsic stimuli, HuR acts as a shuttle, exporting associated-mature target mRNAs to the cytoplasm, where it mainly stabilizes and promotes the translation of such mRNAs. In so doing, HuR regulates the fate of thousands of coding and noncoding RNAs containing AU/UU-rich elements (AREs) sequences primarily located in their 3' untranslated regions (UTR), as briefly sketched in *Fig. 2*.



Domain	Sequence
RNP1_RRM1	$_{61}$LGYGFVNY$_{68}$
RNP2_RRM1	$_{22}$LIVNYL$_{27}$
RNP1_RRM2	$_{147}$RGVAFIRF$_{154}$
RNP2_RRM2	$_{108}$LYISGL$_{113}$

Fig. 1. A) Full length HuR model with RRM1, RRM2 and RRM3 in yellow, orange and cyan, respectively, and the linker between RRM2 and RRM3 in gray. B) Co-crystal structure of the tandem RRM1 and RRM2 HuR-mRNA$^{c\text{-}fos}$ complex (pdb code 4ED5); protein backbone in gray, main binding areas of RRM1 in magenta, of RRM2 in blue, mRNA$^{c\text{-}fos}$ in yellow[7].

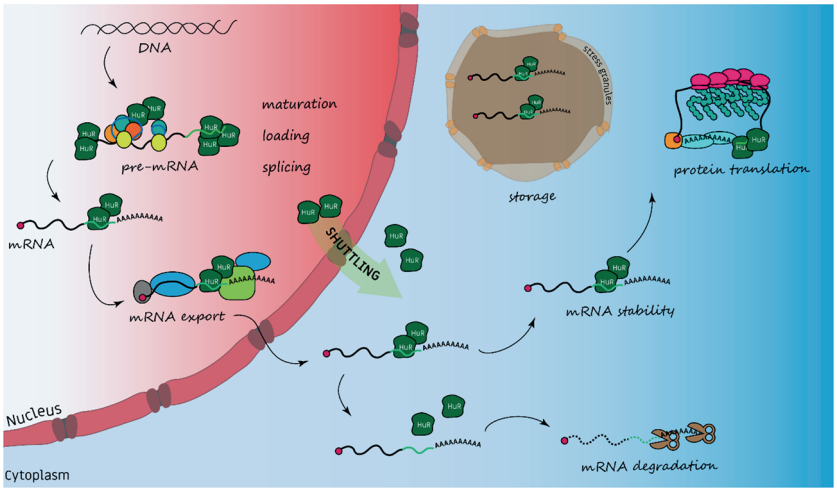


Fig. 2. Schematic representation of HuR functions within the cell.[7]

HuR is often overexpressed in human cancers, and is associated with tumor aggressiveness and worse prognosis. HuR plays a critical role in controlling most key cancer-associated traits, by regulating the expression of many genes[26,27]. Due to this, HuR has received considerable attention as a therapeutic target. Hundreds of *in vitro* and *in vivo* studies have shown that targeting HuR is a promising strategy, and its modulation has an impact on the "Hallmarks of Cancer"[7] – genomic instability, enhanced cell survival, reprogrammed cell metabolism, invasion and metastasis, increased proliferation and tumorigenesis, apoptosis resistance, immune evasion.

Considering the pathogenic functions of HuR, its inhibition *via* small-interfering RNAs or small molecules emerged as a putative therapeutic approach against multiple diseases. The modulation of either the expression, the translocation and the post-translational modification (PTM) profile of HuR, and its silencing are context-dependent and rely on the activation of specific factors[28,29]. Conversely, small molecules impairing the HuR-mRNA interaction are less context-dependent, although their potency may depend on the PTM profile of HuR[30,31]. Unpredictable side effects could be due to the ubiquitous expression of HuR, its pleiotropic, controversial functions, and the lethal phenotype connected with its ablation. The *in vivo* efficacy and tolerability of HuR-mRNA modulators must still be characterized[32] as, considering

the similarity of HuR with other ELAVL-like proteins, specificity remains a largely unknown issue.

2.2. *The tanshinone scaffold*

Tanshinones are a class of naturally occurring abietane diterpenes isolated in 1934 from *Salvia miltiorrhiza* (Danshen, or Tanshen in Chinese), a well-known herb in traditional Chinese medicine (TCM)[33]. Tanshen extracts from the dried root or rhizomes of *Salvia miltiorrhiza Bunge* were used in TCM and in other Asian countries as preventative or therapeutic remedies for coronary heart and vascular diseases, stroke, hyperlipidemia, endangiitis, arthritis, and hepatitis[34].

Prof. Provenzani (CIBIO, UniTN) validated an amplified luminescent proximity homogeneous assay (AlphaScreen) format, measuring the inhibition of an interaction between human HuR and the ARE domain of TNF- mRNA. After further validation with an RNA EMSA assay, dihydrotanshinone I (DHTS I) (**1**, *Fig. 3*) was identified as a nM inhibitor of recombinant HuR (rHuR)-mRNA complex formation endowed with cytotoxic activity[35]. A few other tanshinones (*i.e.*, cryptotanshinone **2**) were slightly less potent[36].

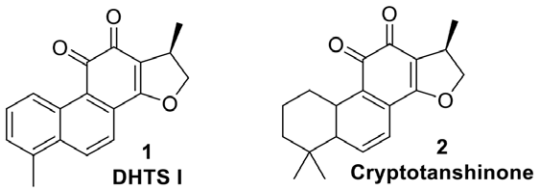


Fig. 3. Structure of selected, naturally occurring tanshinones **1** and **2**.

Thus, DHTS I was selected as a structural scaffold to be exploited for the identification of novel, synthetic, patentable HuR inhibitors. The computational work performed at UniNA-Federico II (Prof. Marinelli) showed that the whole A-B naphthalene ring system of natural tanshinones does not establish specific interactions in the HuR hinge pocket and could be neglected. The orto-quinone group (ring C, left, *Fig. 4*) is essential for activity and can be substituted on positions 6 and 7 (R'-R", left, *Fig. 4*) with groups endowed with varying bulkiness and electronic properties. As to ring D, the X heteroatom in the five-member ring could be replaced with other substituted N, or S, without

losing biological activity; and its 3 position (R, left, *Fig. 4*) could be decorated preferentially with substituted aryl rings.

2.3. *Synthetic aza-tanshinones*

An initial, computationally driven medicinal chemistry effort resulted in the synthesis of N-(phenylsulfonyl)-3-phenyl-5,6-dioxoindole **3** (right, *Fig. 4*), a dienophile/C/D ring-containing tanshinone analogue built around a chemical scaffold that we named "aza-tanshinone" (AT)[37].

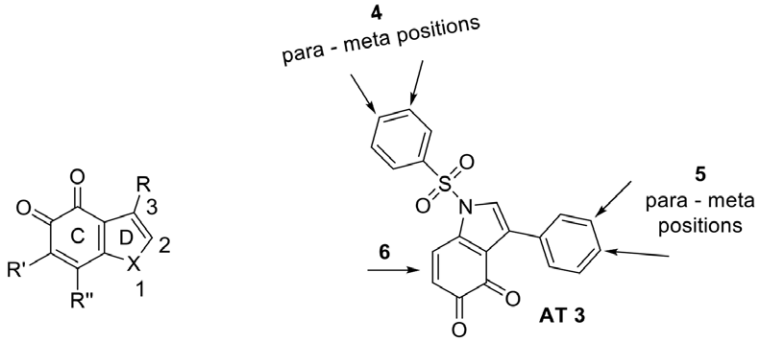
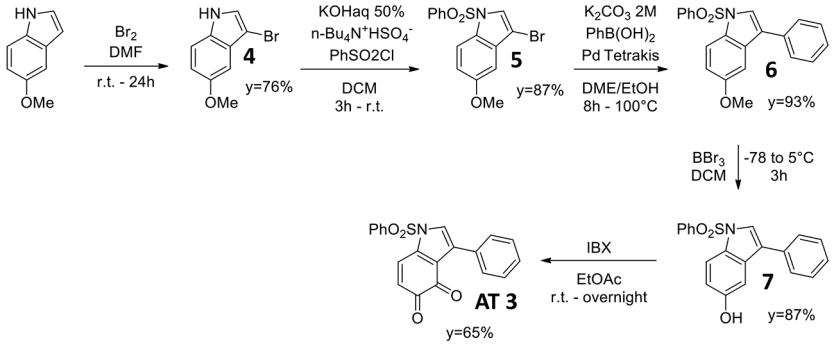


Fig. 4. Left, computationally suggested minimal bicyclic core scaffold for synthetic HuR-targeting tanshinone analogues; right, aza-tanshinone **3** and its main substitution patterns (**4** to **6**).

The synthetic route from commercial 5-methoxy indole, entailing bromination (**4**), sulfonamidation (**5**), Suzuki coupling (**6**), demethylation (**7**) and final oxidation to a-quinone AT **3** in overall good yields is depicted in *Scheme 1*.

AT **3** was biologically evaluated in a primary assay as a modulator of the HuR-mRNA interaction. In a cell-free assay, it resulted to be more potent than DHTS I **1**. According to a semi-quantitative analysis (Western Blot, *Fig. 5*), the formation of the fast-running HuR-bound mRNA spot (DMSO standard, second column) was completely prevented by AT **3** at low, 80 nM and 160 nM concentrations (last two columns on the right) with at least a two-fold potency increase with respect to DHTS I **1** (DT, third column, weak intensity reduction for HuR-bound mRNA).

The N^1-phenylsulfonamide group – originally intended as a protecting group to be removed – established relevant interactions with the hinge binding site on HuR. The computational model shown in *Fig. 6* shows the "best fit" of AT **3** (purple) together with DHTS I **1** (light blue) in the RRM1 HuR hinge binding site; the lipophilic part/phenyl sulfonamide does not directly establish molecular interactions with HuR, but its presence and bulkiness induces a binding-strengthening reorientation of AT **3** in the binding site.



Scheme 1. Synthesis of AT **3**.

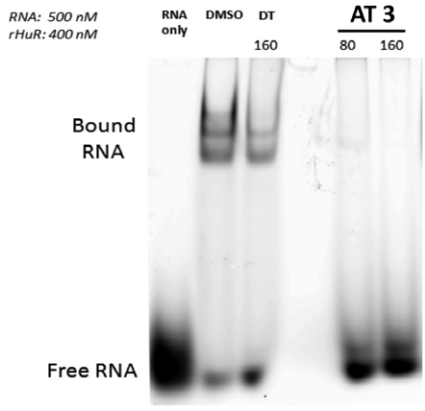


Fig. 5. Western blot, semi-quantitative analysis of the inhibition of HuR-mRNA interaction by DHTS I **1** and AT **3**.

AT **3** showed a decrease in potency (≈10 M scale) in cellular assays, indicating either stability, or cell permeability issues. Thus, additional synthetic efforts were deemed to be necessary in order to improve the bioavailability of ATs, and to explore their SAR; a first small array of ATs **8-10** was rationally designed and successfully synthesized (*Fig. 7*) by either adapting the synthesis of AT **3** (AT N-sulphonamides **8a-h**, substituted 3-aryl ATs **9a-e**), or by functionalizing AT **3** by Michael addition (7-substituted ATs **10a-k**, *Fig. 7*).

A preliminary characterization of AT analogues **8**-**10** did not identify a better prospect than parent AT **3**; in particular, several substitution patterns – *i.e.*, N-p-fluorosulphonamide AT **8c** and 7-(p-methoxyphenylthio) AT **10g** - showed comparable nanomolar potency as disruptors of the HuR-mRNA interaction. Conversely, the most significant hurdle limiting *in vivo* testing of AT derivatives was their extremely poor solubility in aqueous media.

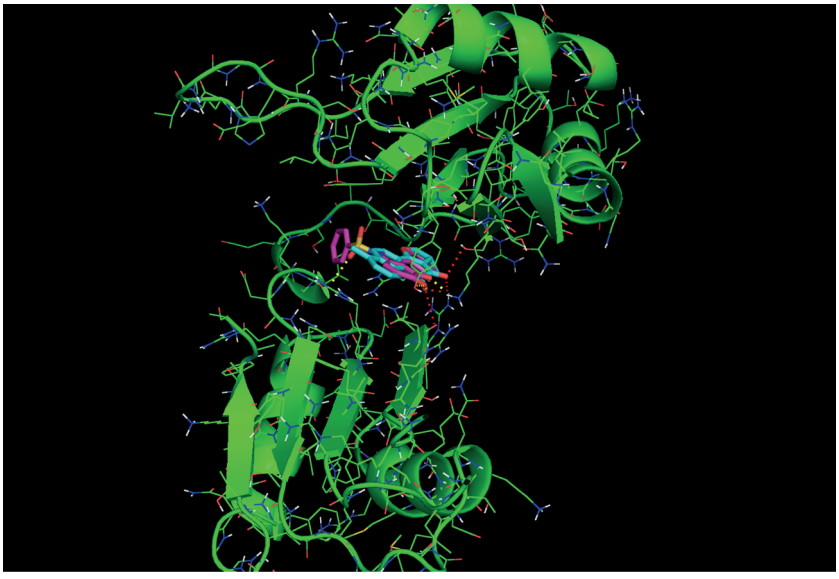


Fig. 6. Superimposition of AT **3** (purple) and DHTS I **1** (light blue) in the hinge binding site of HuR.

To this regard, another interesting hypothesis was tested through the synthesis of 3-(2',6'-substituted)aryl ATs **11a-d** (*Fig. 8*). We planned to investigate the effects of 2,6-disubstituted 3-phenyl rings on the pla-

narity of the whole AT system, hoping for a higher solubility dictated by an out-of-plane configuration assumed by the AT molecule due to the methyl clash with the neighboring quinone ring.

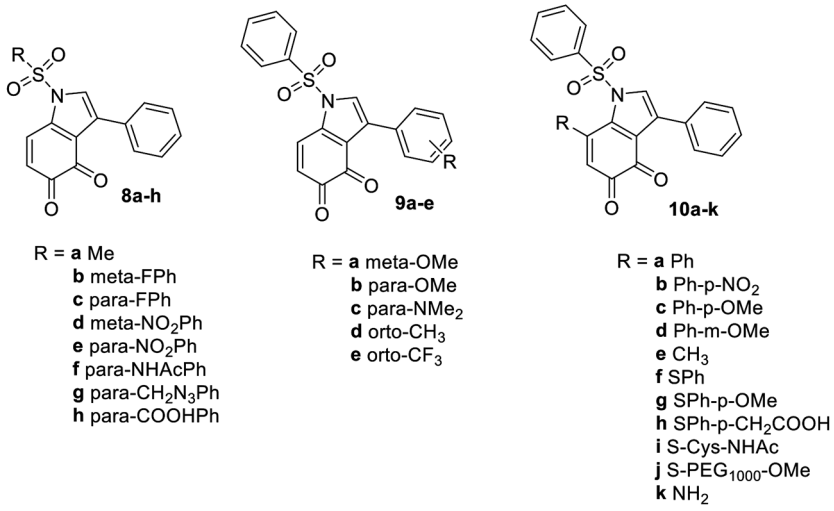


Fig. 7. Chemical structure of functionalized AT derivatives: AT N-sulphonamides **8a-h**, substituted 3-aryl ATs **9a-e** and. 7-substituted ATs **10a-k**.

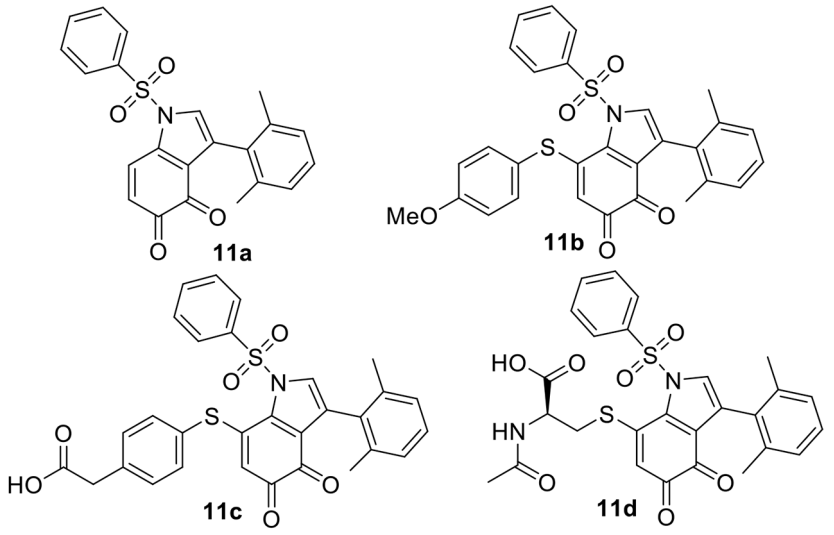


Fig. 8. Chemical structure of substituted 3-(2,6-dimethylphenyl) ATs **11a-d**.

The synthesis of 3-(2',6'-substituted)aryl ATs **11a-d** required significant optimization of the Suzuki coupling, due to the steric hindrance of bis-methylphenyl boronic acid; once successfully obtained, ATs **11a-d** were tested *in vitro* to confirm their preserved activity as disruptors of the mRNA-HuR interaction. Most potent analogues **11a** and **11b** were tested *in vivo*, and the significantly increased bioavailability of the latter enabled the determination of its anticancer and immunomodulating activity in mice models[38].

3. PD-1 / PD-L1

3.1. *Bio-structural background*

Programmed cell death protein 1 (PD-1) is a type I membrane protein of 268 amino acids, belonging to the extended cluster of differentiation 28 (CD28)/ cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) family of T_{reg} lymphocytes[39]. PD-1 binds to its PD-L1[40] and PD-L2[41] ligands, two B7 protein family members. PD-L1 is a 40kDa type 1 transmembrane protein, whose binding to PD-1 (K_d = 770 nM)[40] elicits an inhibitory signal that reduces the proliferation of antigen-specific T lymphocytes in lymph nodes, simultaneously reducing apoptosis in T_{reg} lymphocytes. PD-L1 is upregulated on macrophages, dendritic cells (DCs), T and B cells in response to multiple stimulations[42]. Structurally related PD-L2 shares PD-1 as a receptor (with a stronger K_d = 140 nM), but its expression is much more restricted, mainly to DCs and a few tumor lines[40].

Negative regulation of immune response to cancer cells by the PD-1/PD-L1 complex, and rescuing by cancer immunotherapy is shown in *Fig. 9*[43]. Antigen-presenting cells (DCs, macrophages, B cells) activate T cells and cause the expression of PD-1 on their surface, to prevent auto-immunity. Unfortunately, over-expression of PD-L1 on cancer cells negatively regulates T lymphocytes and allows tumor cells to escape the immune response. Cancer immunotherapy (either PD-1-directed monoclonal antibodies (mAbs) acting on T lymphocytes and PD-L1-directed mAbs acting on cancer cells) restores antitumor immunity.

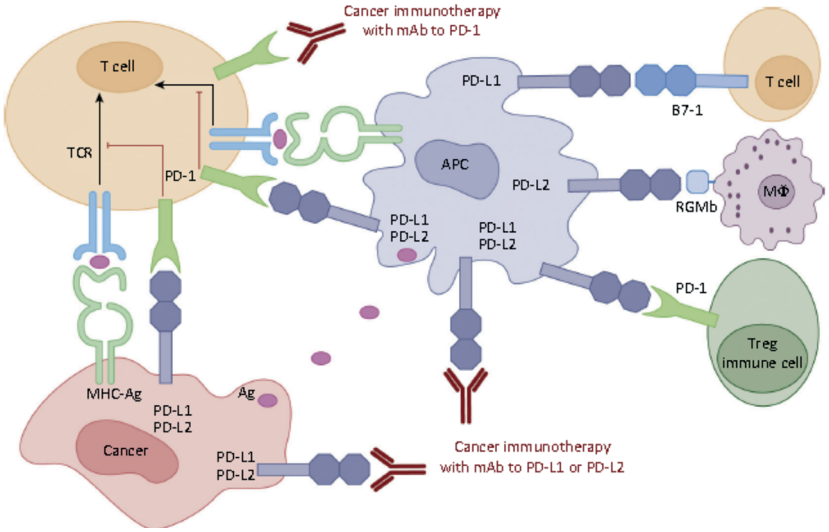


Fig. 9. Antitumor immunity: negative regulation by PD-1 – PD-L1, rescuing by cancer immunotherapy[43].

Several mAbs were clinically developed to block the PD-1/PD-L1 interaction by binding to and inhibiting PD-1 (Nivolumab, Pembrolizumab, Dostarlimab and Cemiplimab) or PD-L1 (Atezolizumab, Avelumab and Durvalumab)[44]. mAbs are expensive, show limited stability, poor tissue and/or tumor penetrance, may deplete immune cells and can cause immune-related adverse events; responsiveness of patients to mAbs is limited to ≈20%–40%, and most mAb-driven immunotherapies fail due to partially unknown reasons.

Small molecules are orally bioavailable, less expensive and their dosing regimen is more flexible; thus, complementing mAbs with small molecules might have great clinical impact. The structure of a homogeneous human PD-1 – human PD-L1 complex (h/h[45]) were reported and compared, to give a structural model to guide the rational design of small molecule inhibitors. Interaction hot spots, to model small molecule PD-1 – PD-L1 inhibitors, were not found for the PD-L1 – interacting surface of human PD-1; three hot spots were identified on the PD-1 – interacting surface of human PD-L1[45].

3.2. *The 1,3,5-triazine scaffold*

Bristol Myers Squibb reported the first non-peptidic, biphenyl-containing PD-L1 binders[46], centered on an aryl (phenyl, pyridyl) tri-or tetra-substituted core; a few relevant examples (**12a-d**) are shown in *Fig. 10*. In particular, **12d** (BMS-202) is a strong PD-1/PD-L1 inhibitor (IC_{50} = 18 nM, homogeneous time-resolved fluorescence/ HTRF assay) that binds to PD-L1, increases its thermal stability by 12.4°C, can dissociate pre-formed PD-1/PD-L1 complexes[46], shows good PK and is active in multiple mouse tumor models[47].

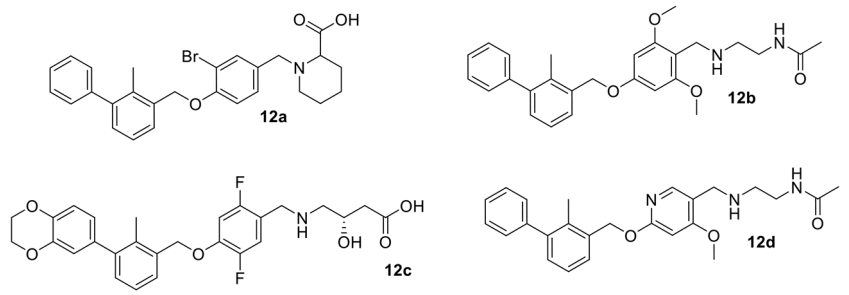


Fig. 10. BMS binders of PD-L1: biphenyl-containing compounds **12a-d**.

The X-ray complex between **12d** and PD-L1 shows how the small molecule induces PD-L1 dimerization by binding at the interface between two PD-L1 monomers, preventing the interaction with PD-1 to form and stabilize a PD-L1 homodimer (*Fig. 11*)[46]. The two PD-L1 units are not arranged in a completely symmetric conformation, and define a hydrophobic tunnel where a ligand fits to stabilize the dimer.

We focused on 1,3,5-triazines as privileged, flexible scaffolds in medicinal chemistry[48], knowing that a biphenyl ether substituent is the main driving group for PD-L1 surface binding[49]; an oppositely oriented meta 1,4-diamino-acetyl group, as in **12d**, is needed for PD-L1 interaction; and that a *meta* substitutions on the central core seem to be acceptable[50]. The resulting synthetic plan to 1,3-disubstituted (from dichlorotriazine, DCT, bottom) and 1,3,5-trisubstituted triazines (from trichlotoriazine, TCT, middle), compared with **12d**, is shown in *Scheme 2*.

Among the first triazines made, 2,4-disubstituted compound **13** was particularly promising. Its synthesis in good overall yield is reported in *Scheme 3*.

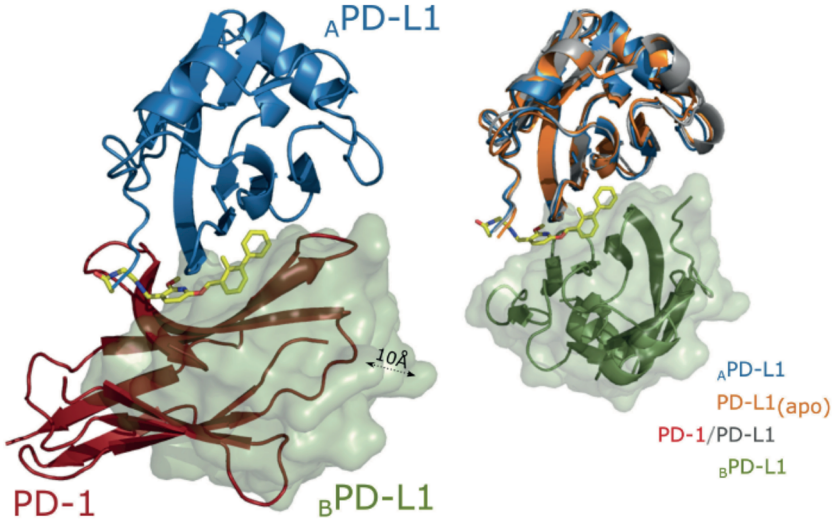
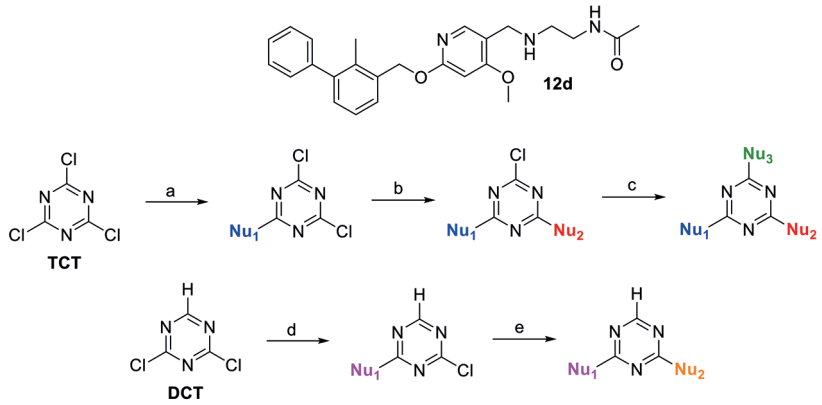
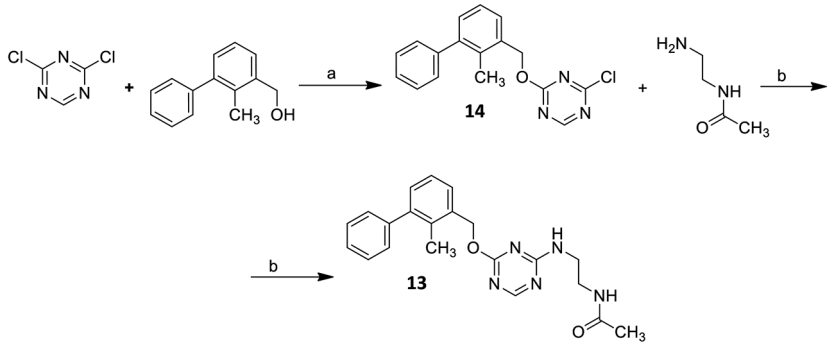


Fig. 11. Hetero PD-1/PD-L1 *vs.* homo-PD-L1 complexes: influence of compound **12d** (yellow)[65].



Scheme 2. General scheme to 2,4,6-tri- (top) and 2,4-disubstituted (bottom) 1,3,5-triazines. a) Nu_1, -20°C to 0°C, DIPEA, up to 30 minutes; b) Nu_2, -20°C to rt, DIPEA, up to 5h; c) Nu_3, 70°C, DIPEA, up to 8h; d) Nu_1, -20°C to rt, DIPEA, up to 5h; e) Nu_2, 70°C, DIPEA, up to 6h.

Similar synthetic pathways were executed to yield an array of di- and tri-substituted triazines **15**-**18**, whose affinity for PD-L1 measured through Homogeneous Time Resolved Fluorescence (HTRF)[52] is reported in *Tab. 1*. They include modifications on the third triazine position (**15a-e**, top/green, *Fig.12*); on the polar side chain (**16a-k**, right/red); on both the third triazine position and the polar side chain (**17a,b**, top-right/green-red); and on the biphenyl ether substituent (**18a,b**, left/blue, *Fig.12*).



Scheme 3. a) DIPEA, DCM, 0°C to rt, 3days, **70%**; b) N-acetylethylenediamine, DIPEA, CH_3CN, 70°C, 16 hrs, **72%**[51].

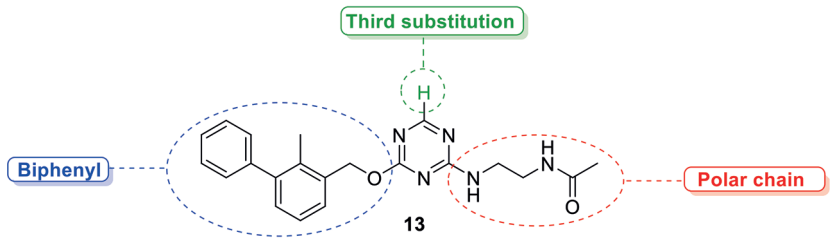
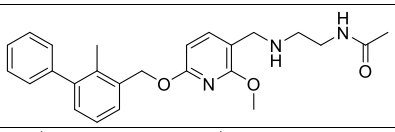
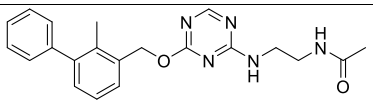
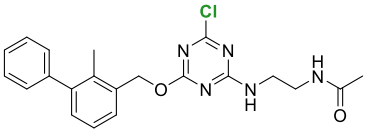
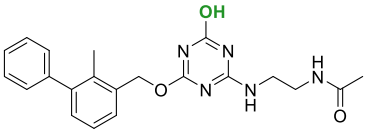
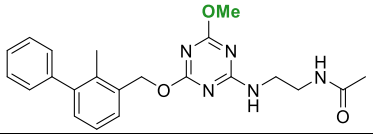
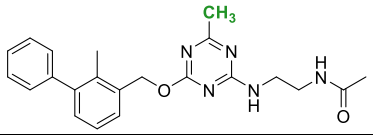
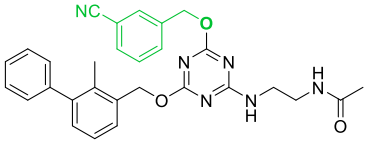
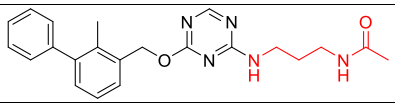
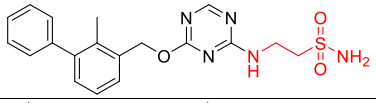
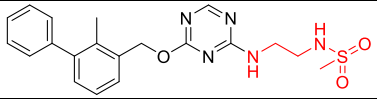
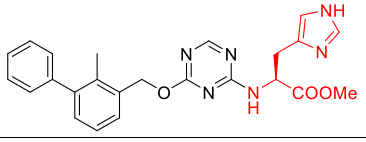


Fig. 12. Modification patterns for the synthesis of di- and tri-substituted triazines **15-18**.

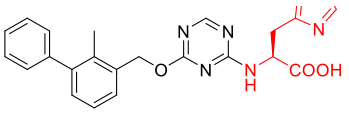
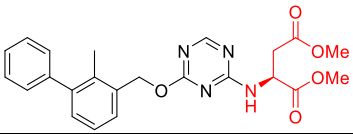
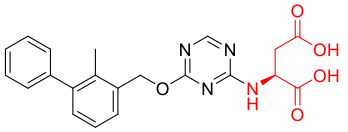
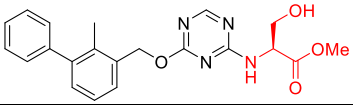
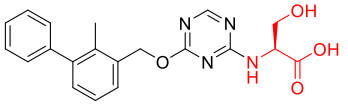
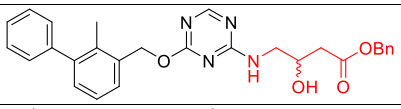
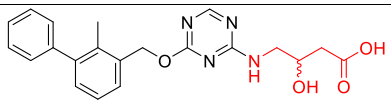
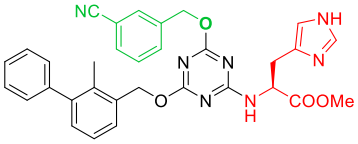
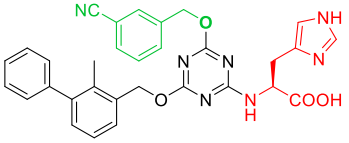
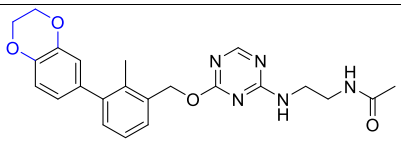
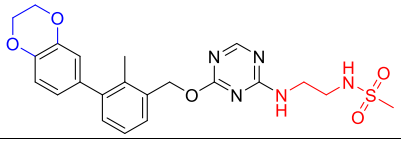
While most analogues resulted to be inactive, and left-modified **18a,b** are currently being profiled, a few substitution patterns (*i.e.*, chloro **15a**, elongated **16a** and sulfonamides **16b,c**) yielded promising activity results. Further computer-assisted modifications are planned, as will be a "scaffold hopping" approach entailing the replacement of 1,3,5 triazines with alternative core structures, and the "pseudo-dimerization" of triazine **13**, according to literature[53].

Tab. 1. HTRF assay results on 1,3,5-triazines 15-18.

Code	Structure	IC_{50}
12d		
13		115±24 nM
15 a		315±16 nM
15b		>10 μM
15c		1.532±0.075 μM
15d		>10 μM
15e		>10 μM
16a		
16b		551±132 nM
16c		241±58 nM
16d		4.196±0.68 μM

To be continued on next page

Tab. 1. Continued from previous page.

16e		>10 μM
16f		>10 μM
16g		>10 μM
16h		692±37 nM
16i		>10 μM
16j		>10 μM
16k		3.39±0.146 μM
17a		>10 μM
1b		>10 μM
18a		Under investigation
18b		Under investigation

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