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Development of small molecule PET-tracers targeting PD-L1

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Introduction and Objective

Immune checkpoints are receptor-ligand systems and regulate immune responses by stimulation or inhibition. Programmed cell death protein 1 (PD-1) and its ligand Programmed cell death ligand 1 (PD-L1) function as an inhibitory checkpoint and upon binding negatively regulate immune responses, obstruct T cell signalling and proliferation, induce apoptosis and prevent excessive immune reactions and autoimmunity for self-tolerance.

Cancer cells may abuse this mechanism for immune evasion by overexpression of PD-L1 (Figure 1). PD-L1 expression is used as a predictive biomarker for anti-PD-1/PD-L1 immunotherapy in the clinic. The current method for quantifying PD-L1 expression by immunohistochemistry is confronted with intrinsic problems of invasive sampling (biopsy) as well as heterogeneous expression of PD-L1.

Positron emission tomography (PET) with PD-L1 selective tracers, as a non-invasive alternative to immunohistochemistry could improve *in vivo* quantification of PD-L1 for more accurate patient stratification and advanced anti-cancer therapy response.

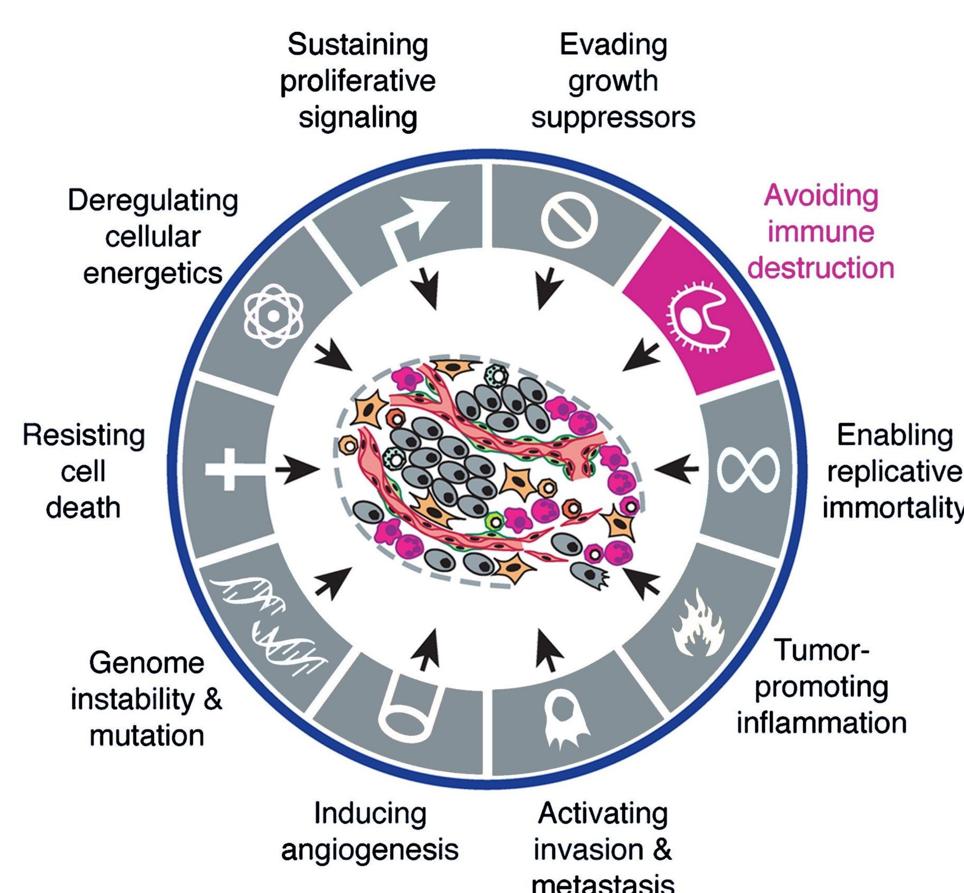

Figure 1: Avoiding immune destruction is a hallmark of cancer. Cells and tissues are constantly monitored by an ever-alert immune system and such immune surveillance is responsible for recognizing and eliminating early cancer cells and thus nascent tumors. Accordingly, solid tumors that do appear have somehow managed to avoid detection by the various arms of the immune system or have been able to limit the extent of immunological killing, thereby evading eradication. Adapted from Hanahan and Weinberg [1].

Methods

- Ligand-based drug design approach (i): Commercially available small-molecule precursor candidates with known affinity towards PD-L1 were selected and potential PET-tracer candidates were synthesized by chemical modification of precursors applicable for radiosynthesis (i.e. methylation, fluoroethylation) (Figure 2A).
- *De novo* multi-step synthesis (ii): Novel compounds were synthesized using advantageous substructures identified by extensive literature research and ligand docking experiments utilizing the LigandScout software and a manually curated chemical database of bioactive molecules with drug-like properties (ChEMBL) for improved PD-L1 binding affinity and chemical properties (i.e. lipophilicity) (Figure 2B).
- Compound binding affinities (IC_{50}) were measured using a cell-free homogeneous time-resolved fluorescence (HTRF) assay[3], which is based on competitive binding of the compound of interest with PD-1 (40 nM) and endogenous PD-L1 (40 nM). Calculation was done with GraphPad Prism 8 software using the non-linear regression, variable slope (four parameters) curve fitting.
- Lipophilicity (log*D*) was determined according to an HPLC method[2]. Cytotoxicity was assessed by MTT assay using PD-L1 expressing CHO-K1 cells. In addition, compound stability was evaluated in HEPES buffer solution at pH 7.4 (0°C or room temperature).
- Radiolabelling of selected compounds and optimization of reaction conditions were performed on a fully automated GE TRACERlab FX C Pro synthesis module.

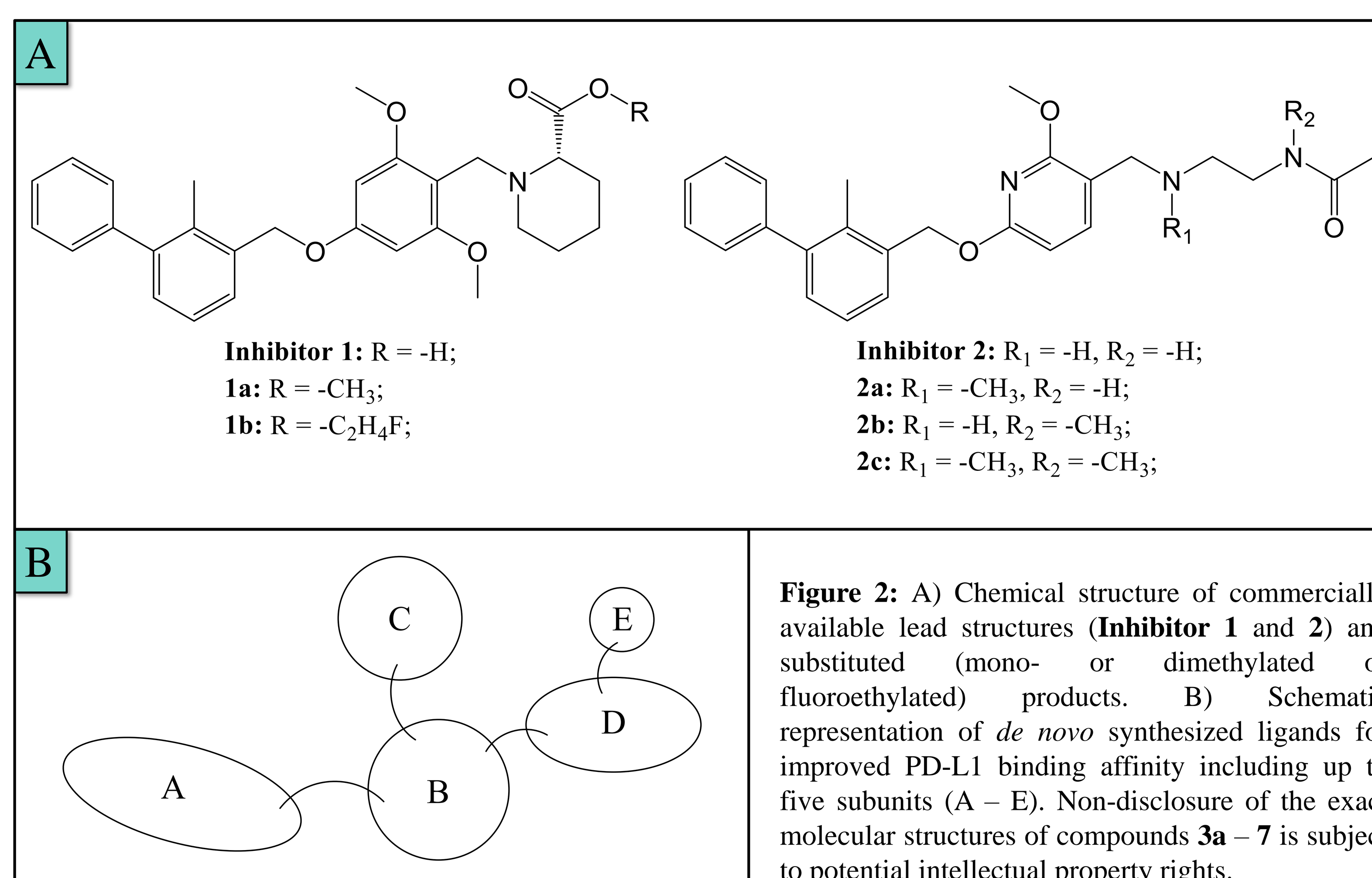

Figure 2: A) Chemical structure of commercially available lead structures (**Inhibitor 1** and **2**) and substituted (mono- or dimethylated or fluoroethylated) products. B) Schematic representation of *de novo* synthesized ligands for improved PD-L1 binding affinity including up to five subunits (A – E). Non-disclosure of the exact molecular structures of compounds **3a** – **7** is subject to potential intellectual property rights.

Results

- Five small-molecule ligands were synthesized based on two commercially available precursors with known affinity[4] by O- and N-methylation as well as O-fluoroethylation (i) (Table 1). Lead structures and products showed low binding affinities (>100 nM). In general, substitution led to a decrease of binding affinity.
- Seven potential PD-L1 targeting compounds were successfully synthesized by multi-step *de novo* synthesis (ii) (Table 1). Methylated (**3a** – **5**) and fluorinated compounds (**6** and **7**) demonstrated exceptionally low IC_{50} values as low as 6.2 and 11.5 nM, respectively.
- The measured log*D* values were in line with the general perception that methylation and fluoroethylation increases lipophilicity. Ligands demonstrated rather high log*D* values ranging from (i) 4.1 to 5.0 and (ii) 3.4 to over 5.75. It should be noted that higher log*D* values may lead to additional undesired non-specific binding.
- The radiolabelling of four products was successfully established and optimizations regarding reaction temperature, reaction time and precursor amount resulted in radiochemical conversions of up to (i) 49% (**1a**), 54% (**2a**), 56% (**2b**) and (ii) 67% (**4a**) as determined by radio-HPLC with regard to the activity of the [^{11}C]methylating agent.

Table 1: Evaluated binding affinities (IC_{50} values), measured HPLC log*D* values, optimized radiochemical conversions (RCC), toxicological EC_{50} values and in-solution stability followed for 20 days at 0°C of lead structures and newly synthesized molecules.

Substance	IC_{50} [nM]	µHPLC log$P_{OW}^{pH\ 7.4}$	RCC [%]	Cytotoxicity EC_{50} [µM]	In-solution stability [%]
Inhibitor 1	202 ± 27	3.16 ± 0.16	-	52.3 ± 11.7	96
1a	5758 ± 613	4.90 ± 0.27	49	-	86
1b	1155 ± 339	5.02 ± 0.28	-	-	91
Inhibitor 2	101 ± 10	3.88 ± 0.12	-	24.9 ± 4.1	99
2a	430 ± 62	4.13 ± 0.16	54	-	99
2b	524 ± 67	3.97 ± 0.13	56	-	95
2c	1305 ± 185	4.28 ± 0.18	-	-	99
3a	6.2 ± 0.6	3.36 ± 0.04	-	-	-
3b	30.2	4.88 ± 0.26	-	-	-
4a	10.2 ± 0.2	4.93 ± 0.27	67	-	-
4b	18.7	5.09 ± 0.29	0	-	-
5	16.4	4.54 ± 0.21	-	-	-
6	1292	>5.75	-	-	-
7	11.5	5.21 ± 0.31	-	-	-

Conclusion

Novel *de novo* synthesized molecules were superior in terms of binding affinities over the ligand-based drug design approach. The radiolabelling with carbon-11 and fluorine-18 as well as the applicability of these promising compounds as potential PET-tracers for improved PD-L1 quantification are subject to ongoing research.

Acknowledgments

Thanks to Univ.-Prof. Mag. Dr. Thierry Langer for the access to the LigandScout software, to Dr. Katharina Pallitsch and her research group for the NMR and mass spectrometric measurements, and to the MIC research cluster. This project is supported within the COMET framework by the Austrian Federal Government through the FFG and the provinces of Styria and Vienna.

References

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