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Journal:	*RSC Medicinal Chemistry*
Manuscript ID	MD-RES-09-2023-000540.R1
Article Type:	Research Article
Date Submitted by the Author:	02-Jan-2024

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Received 00th January 20xx,

Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Minimising the payload solvent exposed hydrophobic surface area optimises the antibody-drug conjugate properties

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Glucocorticoid receptor modulators (GRMs) are an established and successful compound class for the treatment of multiple diseases. In addition, they are an area of high interest as payloads for antibody-drug conjugate s(ADCs) in both immunology and oncology. Solving the crystal structure of compound 2, the GRM payload from ABBV-3373 and ABBV-154, in the ligand binding domain of the glucocorticoid receptor (GR) revealed key information to facilitate optimal ADC payload design. All four critical H-bonds between the oxygen functional groups on the hexadecahydro-1H-cyclopenta[a]phenanthrene ring system of the small molecule and protein were shown to be made (carbonyl at C3 to Gln570 and Arg611, hydroxyl at C11 to Leu563 and Asn564, carbonyl at C20 to Thr739, hydroxyl at C21 to Asn 564 and Thr739). In addition, an extra H-bond between the linker attachment site on **compound 2**, the aniline in the biaryl region, was observed. Confirmation of the stereochemistry of the acetal in **compound 2** as (R) was established. Finally, the importance of minimising the exposed hydrophobic surface area of a payload to reduce the negative impact on the properties of resulting ADCs, like aggregation, was rationalised by comparison of (R)-acetal **compound 2** and (S)-acetal **compound 3**.

Introduction

Antibody-drug conjugates (ADCs) are now an established therapeutic modality in the oncology field more than a dozen approved.[1] Of these oncology ADCs brentuximab vedotin[2] established key design elements for ADCs including a protease cleavable linker[3] to release the payload in the lysosome and the self-immolative group para-amino benzylic alcohol which attaches via a carbamate to a suitable basic nitrogen on the cytotoxic payload monomethyl auristatin E (MMAE).

Encouraged by this success in oncology other therapeutic areas pursued ADCs most notably in the immunology field. Probably the most successful class of immunology small molecule drugs are glucocorticoids which have more than 70 years of successful drug discovery activity.[4] Unfortunately, despite being highly functionalised glucocorticoids, exemplified by prednisolone[5] (Figure 1a) do not have a suitable nitrogen atom for linker attachment. Of the four oxygen functional groups the carbonyl at C3 via hydrazone attachment like in gemtuzumab ozogamicin[6] and the hydroxyl at C21 via ester attachment like in many steroid prodrugs[7] offer the best options for attachment.

To enable ADCs with a glucocorticoid payload, linker attachment to the C21 hydroxyl of dexamethasone[8, 9] (Figure 1b) has been explored using both ester[10] and carbonate.[11] However, for an immunology (iADC) the stable attachment of the payload to the antibody to avoid premature loss of the payload is critical. Concerned that the instability of esters and carbonates *in vivo* would result in the premature loss of payload a glucocorticoid with a suitable nitrogen to facilitate stable linker attachment was pursued. Comprehensive SAR studies identified the key structural features of glucocorticoids as the C3 carbonyl, C11 hydroxyl, C20 carbonyl and C21 hydroxyl[12, 13] and an ADC payload that retained these functional groups was desired. Ciclesonide[14] (Figure 1c) differs from dexamethasone in having an acetal at C16-C17 and is the C21 isobutyryl ester prodrug of the active desisobutyryl ciclesonide, more commonly known as des-ciclesonide (Figure 1d) with crystallographic studies on the small molecule reported.[15, 16]

Structural information is a powerful tool for medicinal chemists and was used to guide the design of a glucocorticoid receptor modulator (GRM) ADC payload as there is a wealth of structural information of multiple steroidal small molecules bound in the ligand binding domain of the glucocorticoid receptor (GR).[17] Structures of both dexamethasone (RSC Protein Data Bank entry 4UDC) and des-ciclesonide (RSC Protein Data Bank entry 4UDD)bound in the ligand binding domain of the glucocorticoid receptor have been solved.[18] Modified analogues of des-ciclesonide that introduced a nitrogen from the cyclohexyl region of the compound were modelled and prioritised analogues synthesised and tested. This identified **compound 1** (Figure 2a) as a promising compound[19] with the (R)-stereochemistry of the acetal being confirmed by X-ray crystallography (Cambridge Structural Database entry ZAZTAZ).[20] Further SAR to add an attachment point for the dipeptide linker identified biaryl aniline **compound 2**[21] which advanced to the clinic as the payload on ABBV-3373.[22] Confirmation of the (R)-stereochemistry of the acetal in **compound 2** was achieved by solving the small molecule X-ray

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Figure 1. Marketed steroids a) Prednisolone, b) Dexamethasone, c) Ciclesonide (prodrug), d) Des-ciclesonide.

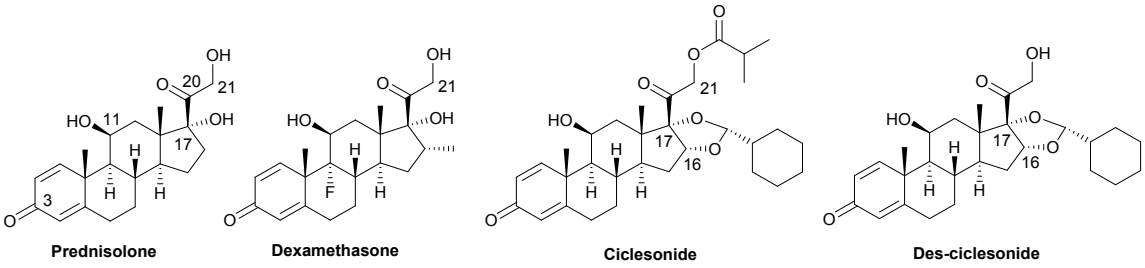
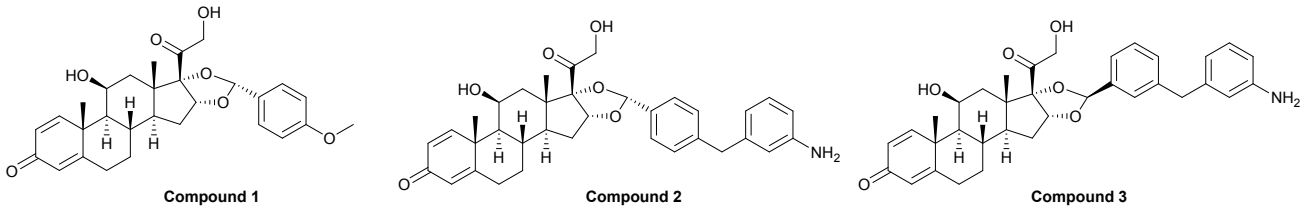


Figure 2. GRM compounds a) mono aryl anisole compound 1, b) biaryl aniline with (R)-acetal compound 2, c) biaryl aniline with (S)-acetal compound 3.



structure. (Cambridge Structural Database entry DIQQUT).[23] The crystal was a colourless prism with dimensions 0.20×0.18×0.12 mm^3 and the symmetry of the crystal structure was assigned the orthorhombic space group P2(1)2(1)2(1).

Three ADCs, ABBV-3373,[24] ABBV-154,[25, 26] and ABBV-319[27] from two therapeutic areas are in multiple clinical trials highlighting the importance of this class of GRM. As a result, the structure of **compound 2** bound in GR was solved to provide structural information to guide medicinal chemistry efforts, identify new H-bond interactions and confirm the (R)-acetal stereochemistry.

Materials and Methods

Protein expression and purification

Human glucocorticoid receptor (residues 528-777) with three mutations (V571M, F602S, C638D) was cloned into the expression vector pGEX4T1 with a thrombin-cleavable, N-terminal 8His-GST tag. The plasmid was transformed into *E. coli.* BL21(DE3), grown at 37 °C in TB medium to OD_{600} 2-3 in the presence of 20 µM **compound 2**, then protein expression induced with 0.5 mM IPTG at 18 °C for 16 hours.

Cell pellets were resuspended in lysis buffer (20 mM Tris, 300 mM NaCl, 5 mM Imidazole, 0.5 mM TCEP, pH 8.0) and lysed by processing once with an EmulsiFlex-C50 homogenizer (Avestin). Cellular debris was pelleted by centrifugation at 30,000 × g for 30 min. The supernatant was batch-bound to Talon resin, followed by washing the resin with the lysis buffer. The protein was eluted with elution buffer (20 mM Tris, 300 mM NaCl, 300 mM Imidazole, 0.5 mM TCEP, pH 8.0). Eluted protein was mixed with thrombin and dialyzed at 4 °C (20 mM Tris, 200 mM NaCl, 0.25 mM TCEP, pH 8.0) for 16 hours to remove the imidazole. The protein was further purified by size exclusion on a HiLoad Superdex 200 16/600 column equilibrated with 20 mM Tris, 150 mM NaCl, 0.5 mM TCEP, pH 8.0. After that, 5 molar folds of **compound 2** was added to the protein solution and the mixture was clarified with centrifugation and concentrated to 13 mg/mL. The 3-molar fold of co-activator peptide (KENALLRYLLDKDD) was added to the concentrated protein to form the final complex for crystallisation.

Crystallisation and data collection

The ternary complex of GR, **compound 2** and co-activator peptide was crystallised using sitting drop vapor diffusion method. More specifically, 100 nL of protein complex was mixed with 100 nL of crystallisation reagents and incubated over 80 uL of reservoir solutions of 24% (w/v) PEG400, 200 mM ammonium acetate, 100 mM sodium citrate pH 5.5 at 23 °C. The stacking thin plate crystals were initially found after 3 weeks, and they grew to their full size after 3 months. Plate crystals were separated, and flash frozen into liquid nitrogen using reservoir solutions as the cryo-protectant. Diffraction data were collected at a temperature of 100 K using IMCA-CAT beamline 17-ID at Argonne National Laboratory. Data collection statistics are summarised in Table 1.

Results and Discussion

The crystal structure of the GR ligand binding domain complex was determined with a co-activator peptide and the ADC payload **compound 2** to study the binding mode of the

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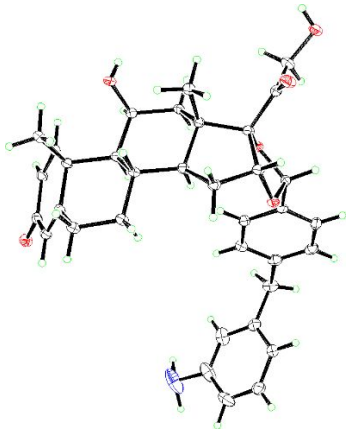

Figure 3. Small molecule X-ray of compound 2

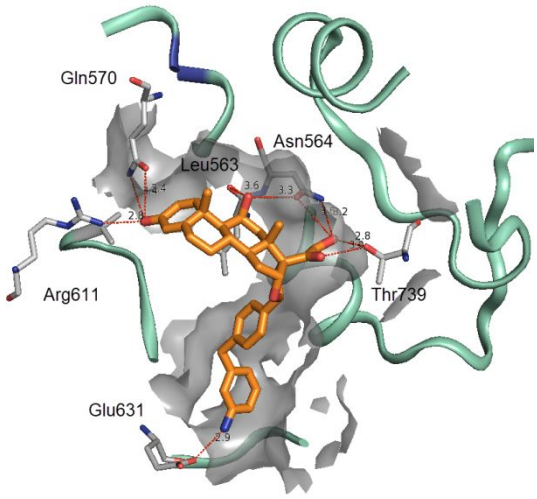

Figure 4. Structural pose of compound 2 (green) in the GR binding pocket with key residues highlighted.

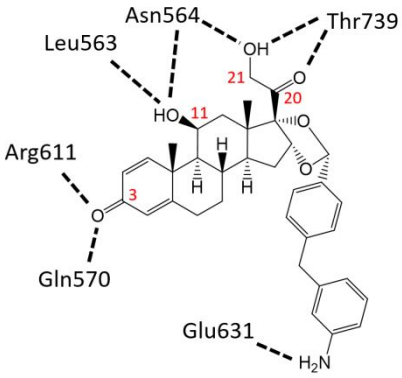

Figure 5. Compound 2 bound in GR with H-bonds shown by dotted lines.

Table 1. Summary of data collection and refinement statistics

	Crystallographic parameter	GR:Compound 2
Data collection	Space group	$P2_1$
Cell dimensions	*a*, *b*, *c* (Å)	38.51, 141.65, 47.90
	α, β, χ (°)	90.0, 93.46, 90.0
	Resolution (Å)	70.83 - 2.13 (2.33 - 2.13)*
	R_{pim}	0.064 (0.529)
	$I / \sigma I$	8.4 (1.5)
Completeness (%)	Spherical	56.7 (12.0)
	Elipsoidal	89.6 (60.6)
	Redundancy	3.4 (3.4)
	CC(1/2)	0.99 (0.53)
Refinement	Resolution (Å)	70.8 – 2.13
	No. reflections	16146 (802)
	R_{work} / R_{free} (%)	20.6 / 25.4
No. atoms	Protein	4033
	Ligand/ion	84
	Water	102
B-factors (Å ^{2})	Protein	43.32
	Ligand/ion	38.02
	Water	39.35
R.m.s. deviations	Bond lengths (Å)	0.008
	Bond angles (°)	0.89
Ramachandran values (%)	Favoured	98.1
	Allowed	3.7
	Outliers	0.2

*Parentheses refers to values in highest resolution shell

Table 2. Small molecule and protein interaction with H-bond distances.

Compound 2		Amino acid	Functional group	H-bond distance / Å
Functional group	Position			
Carbonyl	C3	Gln570	C=O	3.4
Carbonyl	C3	Gln570	NH_2	3.4
Carbonyl	C3	Arg611	NH_2	2.8
Hydroxyl	C11	Leu563	C=O	3.6
Hydroxyl	C11	Asn564	C=O	3.3
Carbonyl	C20	Thr739	C=O	3.0
Hydroxyl	C21	Asn564	C=O	3.6
Hydroxyl	C21	Asn564	NH_2	3.2
Hydroxyl	C21	Thr739	C=O	2.8
Aniline	Biaryl	Glu631	C=O	2.9

compound.[28] he molecular packing exhibited a dimer arrangement, where the monomers adopted similar conformations, and the co-activator peptide was visible as a short helix. The compound bound in the enclosed ligand pocket, where the four rings of the glucocorticoid scaffold formed the four expected H-bond interactions (Figure 4). The pose of **compound 2** core aligns closely with the common features of a smaller compound desisobutyryl ciclesonide (RSC Protein Data Bank entry 4UDD). By contrast, **compound 2** features a biaryl extension, where the linked diphenyl extends through the cavity towards the ligand entry portal.[29] The compound apparently destabilises the protein loop at the portal containing residues 633-637, which are disordered in the current structure, and the aniline is positioned at the opening and makes a H-bond interaction with Glu631. The stereochemistry of the acetal was confirmed as (R) and the V-shape of the compound is clearly visible. Distances for the ten H-bonds between **compound 2** and GR are listed in Table 2 and depicted in Figure 5.

ADCs on mouse anti-TNF with equivalent DAR were prepared with both **compound 2** and **compound 3** as the payload using the same linker enabling any differences in the properties of the two ADCs to be directly attributed to the payload. Both the aggregation level and hydrophobicity of the ADCs were shown to differ greatly. The mouse anti-TNF ADC with **compound 3** was more highly aggregated (4%) and had a longer retention time by hydrophobic interaction chromatography (HIC) of 4.51 minutes for the DAR4 peak. This contrasted with the mouse anti-TNF ADC with **compound 2** that had lower aggression (0.5%) and a shorter retention time by HIC of 4.28 minutes for the DAR4 peak.[30] Aggregation and retention time by HIC are both used as measurements of the hydrophobicity of an ADC. This data clearly showed that **compound 3** had a more negative impact on the hydrophobicity and drug-likeness properties of the ADC than **compound 2**.

Having observed the V-shape of (R)-acetal **compound 2** in both the small molecule crystal structure and the protein crystal structure of **compound 2** bound in GR it was rationalised that this minimised the solvent exposed surface area compared to (S)-acetal **compound 3** and resulted in the lower aggregation of ADCs with **compound 2** as their payload. Energy minimised conformations of both **compound 2** and **compound 3** were generated in Chem3D and depicted in Figure 6 with their solvent exposed surface shown as a wire mesh coloured by atom.

Using 3D Methods in Pipeline Biovia three surface areas were calculated on the energy minimised conformations of **compound 2** and **compound 3**. For calculation of the polar solvent accessible surface area N, O, P, S are considered as polar atoms along with any hydrogens attached to them and any atom with a formal charge.

The calculated data (Table 3) supported the hypothesis of ADC aggregation being impacted by the exposed hydrophobic surface area of a payload. While the polar solvent accessible surface area, the area capable of enabling aqueous solubility, for **compound 2** and **compound 3** was similar (216.5 and 224.3 Å respectively) the solvent accessible surface areas differed significantly. **Compound 2** had a solvent accessible surface area of 718.9 Å, almost 100 Å less than that of **compound 3** (814.7 Å). Similarly, the solvent accessible volume of **compound 2** at 646.4 Å was substantially lower than for **compound 3** (736.1 Å). Considering this data it is clear that while both compounds have similar solubility driving polar areas **compound 2** has less hydrophobic surface area to solvate than **compound 3**. This significant finding identifies that a key parameter for ADC payload design is to maximise the exposed hydrophilic surface and probably more importantly to minimise the exposed hydrophobic surface of payload.

Table 3. Solvent accessible surface area for (R)-acetal compound 2 and (S)-acetal compound 3.

ID	Polar Solvent Accessible Surface Area / Å	Solvent Accessible Surface Area / Å	Solvent Accessible Volume / Å
2	216.5	718.9	646.4
3	224.3	814.7	736.1

Identification of the H-bond interaction made between aniline and Glu631 provided a second important guide for ADC payload design. That is, introduction on nitrogen to the payload should not just be considered as a location to attach the linker. Moreover, whenever possible SAR and structural information should be used to propose possible additional interactions to drive both the potency and selectivity of the payload for its target.

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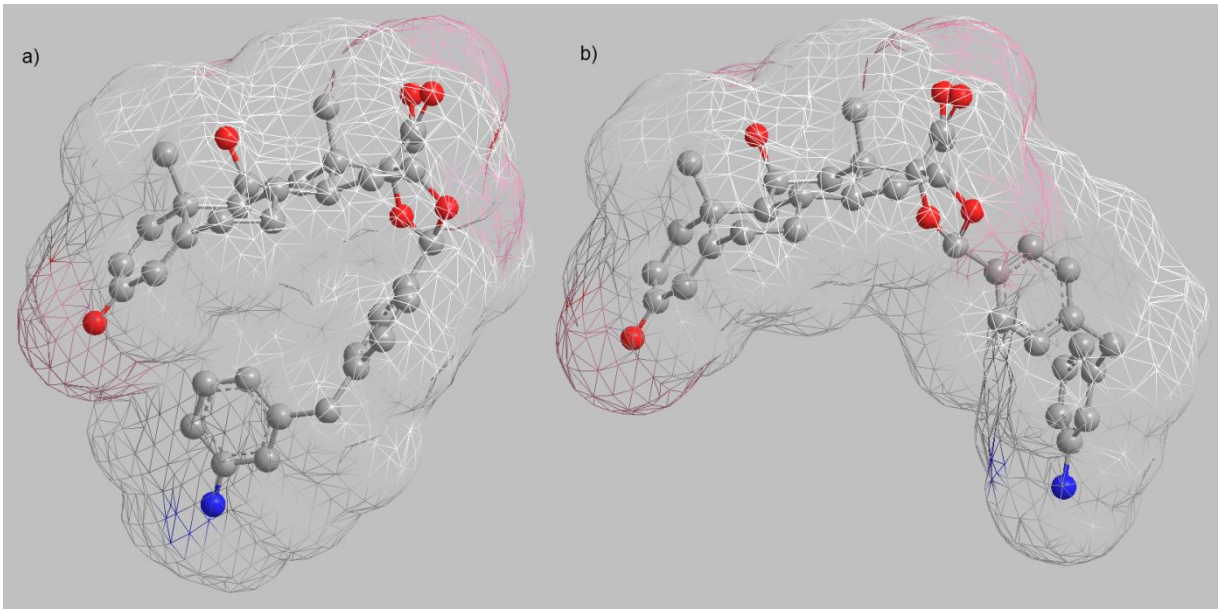

Figure 6. Solvent exposed surface area shown as a wire mesh coloured by atom for energy minimised conformations of a) (R)-acetal compound 2, b) (S)-acetal compound 3.

Conclusion

Steroids are a major call of pharmaceutical in multiple therapeutic areas. Following the clinical success of oncology ADCs with cytotoxic payloads, the use of GRM as an ADC payload is an area of high interest. While extensive guidelines for small molecule design exist, there is a dearth for ADC payloads. For example, following conjugation to an antibody the payload has a disproportionately large impact on the properties of the resulting ADC.

The crystal structure of **compound 2** bound in GR showed that all four of the oxygen functional groups on the hexadecahydro-1H-cyclopenta[a]phenanthrene ring system of the small molecule made their predicted H-bond interactions with the GR protein:

1. Carbonyl at C3 to Gln570.
2. Carbonyl at C3 to Arg611.
3. Hydroxyl at C11 to Leu563.
4. Hydroxyl at C11 to Asn564.
5. Carbonyl at C20 to Thr739.
6. Hydroxyl at C21 to Asn564.
7. Hydroxyl at C21 to Thr739.

In addition, a new H-bond interaction between the aniline in the biaryl region of **compound 2** and Glu631 was observed. Typically, a nitrogen is incorporated to a compound of interest as an ADC payload to facilitate dipeptide linker attachment. However, identification of this new interaction provides a significant guide to ADC payload design. Incorporation of nitrogen should not just be seen as a method for linker attachment, but that SAR and structural information must be used to enhance payload design and incorporated additional interactions with the target to drive both potency and selectivity of new analogues.

One of the goals of this crystallisation was to confirm the stereochemistry of the acetal as the (R)-isomer. Not only was this stereochemistry verified as (R) it also confirmed the importance of minimising the hydrophobic surface area of a payload. Inspection of Figures 3, 4 , 5 and 6 immediately emphasises the V-shape of **compound 2** which dramatically reduces the exposed hydrophobic surface area thereby reducing impact on the drug-like properties of resulting ADCs. As such a key design parameter for ADC payloads is proposed that is to maximise the exposed hydrophilic surface and probably more importantly to minimise the exposed hydrophobic surface of payload.

Abbreviations

ADC	Antibody-drug conjugate.
BrAc	Bromoacetamide.
DAR	Drug to antibody ratio.
H-bond	Hydrogen bond.
GR	Glucocorticoid receptor.
GRM	Glucocorticoid receptor modulator.
HIC	Hydrophobic interaction chromatography.

MMAE	Monomethyl auristatin E.
PDB	Protein data bank.
SAR	Structure-activity relationship.
TNF	Tumour necrosis factor.

Funding sources

Authors ADH, HZ, WQ, and RJ are employees of AbbVie. KL was an employee of AbbVie at the time of the study. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Use of the IMCA-CAT beamline 17-ID (or 17-BM) at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with Hauptman-Woodward Medical Research Institute (no additional funding to disclose). Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357 (no additional funding to disclose).

PDB CODES

Figure 3 **Compound 2** in ligand binding domain of GR - PDB code xxxx. Structure for GR with compound **2** has been deposited in the RSC Protein Data Bank. Authors will release the atomic coordinates and experimental data upon article publication.

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