Antibody-drug conjugates (ADCs) are designed to bind tumor-associated antigens and deliver cytotoxic payloads to antigen-positive cells. Some ADCs also have non-targeting cells (including antigen-negative cells) by a mechanism referred to as the bystander effect. This effect can be beneficial when the antigen is heterogeneous among cells in a solid tumor, but it can also increase off-target toxicity of ADCs. Herein, we report on a unique pharmacological property of the Dolaflexin platform which provides a controlled bystander effect that retains the benefits of the bystander effect with respect to anti-tumor cytotoxicity but reduces the off-target toxicity.

The controlled bystander effect termed “DolaLock,” achieved through design of a payload, astaurin F-hydroxypropylamine (AF-HPA), that is membrane-permeable and capable of bystander killing but is further stabilized to membrane impermeable astaurin F (Af). This payload of the bystanders ‘locks’ the highly potent AF in the cell. Using Dolaflexin-based ADCs, we investigated the extent of intracellular AF-HPA and AF release, tumor cell retention and bystander activities in vitro and in vivo. We observed both membrane-permeable and - impermeable species within cells. Co-culture assays employing 3D xenografted human ovarian cancer cells confirmed the cell permeability and bystander killing capabilities of AF-HPA released from a Dolaflexin-based ADC. Bystander studies of Dolaflexin-based ADCs revealed time-dependent concentrations of AF-HPA and AF as well as a significant accumulation of AF in xenografted tumor cells, consistent with the DolaLock mechanism. A decrease in AF formation was seen in migrating resistant tumor studies which demonstrates that AF is in contrast to HPA, not a P-glycoprotein (Pgp) substrate. This property may offer additional benefit in Pgp-expressing tumors.

In summary, we have shown that the proprietary AF payload used in the Dolaflexin platform allows for a controlled bystander effect which likely contributes to the enhanced efficacy and lack of neuropathy we have observed with Dolaflexin-based ADCs in nonclinical models.

**Discussion and Conclusions**

- The controlled bystander effect, termed “DolaLock”, was achieved with a proprietary astaurin derivative by incorporating a hydroxylastaurin (AF-HPA) to allow for intracellular metabolism to the corresponding astaurin (AF) which is highly potent and can cause the bystander killing observed in co-culture assays.
- AF is highly potent when formed intracellularly but has limited cell permeability.
- Treatment of N87 cells with AF-HPA resulted in the intracellular formation and retention of AF.
- Multi-drug resistance transporter studies demonstrated the AF, in contrast to AF-HPA, is not a P-glycoprotein substrate. This was confirmed in a P-glycoprotein positive cell line, where AF continued to accumulate while AF-HPA was effused.
- Bio-distribution studies of a Dolaflexin-based ADC revealed the in vivo formation of AF-HPA and AF and significant bystander effect in xenografted tumors, consistent with the DolaLock mechanism.

- The proprietary astaurin payload, AF-HPA, used in the Dolaflexin platform, results in a controlled bystander effect which contributes to the enhanced efficacy and lack of neuropathy observed with Dolaflexin-based ADCs in nonclinical models.

**References**