

RESEARCH ARTICLE

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Targeted protein degradation has emerged as a promising approach in drug discovery, utilizing small molecules like molecular glue degraders to harness the ubiquitin-proteasome pathway for selective degradation of disease-driving proteins. Based on results from proteomics screens we investigated the potential of niclosamide, an FDA-approved anthelmintic drug with a 50 year history in treating tapeworm infections, as a molecular glue degrader targeting the proto-oncogene cyclin D1. Proteomics screens in HCT116 colon carcinoma and KELLY neuroblastoma cells, found that niclosamide induces rapid cyclin D1 degradation through a mechanism involving the ubiquitin-proteasome pathway. A genetic CRISPR screen identified the E3 ligase CRL4^{AMBRA1} as a key player in this process. Structure-activity relationship studies highlighted critical features of niclosamide necessary for cyclin D1 degradation, demonstrating a correlation between mitochondrial membrane potential (MMP) disruption and cyclin D1 downregulation. Notably, various mitochondrial uncouplers and other compounds with similar drug sensitivity profiles share this correlation suggesting that MMP disruption can trigger cyclin D1 degradation, and that the cellular signal driving the degradation differs from previously described mechanism involving CRL4^{AMBRA1}. Our findings underscore the complexities of proteostatic mechanisms and the multitude of mechanisms that contribute to degrader drug action.

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Introduction

Small molecules that induce proximity to yield therapeutically beneficial ternary complexes of disease-relevant proteins are an emerging topic in drug discovery.^{1,2} The most frequent use of the concept has been in the field of targeted protein degradation (TPD), which has generated considerable clinical

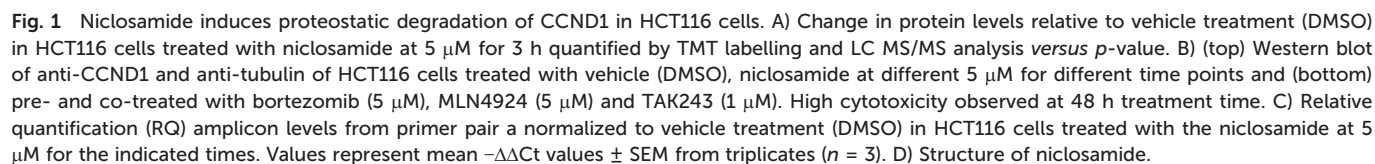
and commercial interest.^{3,4} The field of TPD gained considerable momentum after the mechanistic understanding of immunomodulatory drugs (IMiDs). They degrade the transcription factors (TFs) Ikaros and Aiolos and drive anti-myeloma activity in ubiquitin-dependent manner, which catalysed research into the development of molecules that can degrade disease-driving proteins.⁵ In the case of IMiDs such as lenalidomide (Revlimid), small molecules induce the complex formation between the E3 ligase adapter protein CRBN and the TFs leading to the ubiquitination and consequential degradation of the latter.^{6,7} Structural studies suggested that the molecules could be used as anchors to design bifunctional molecules, known as proteolysis targeting chimeras (PROTACs) that could engage CRBN and a protein of interest that would be degraded by the ubiquitin proteasome system (UPS).⁸ Whereas a rational design is possible for PROTACs, the discovery of small molecules which induce a novel interaction between an E3 ligase and a protein, so-called molecular glue degraders (MGDs) is more challenging as the binding partners are unknown *a priori*.^{9–11}

A typical workflow in the discovery process of a MGD begins with treating cells for a short duration with a compound, followed by global proteomics experiments. If a

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On the basis of global quantitative proteomics screening results, we investigated niclosamide as a potential molecular

Our proteomics screens in HCT116 colon carcinoma and KELLY neuroblastoma cells identified niclosamide as a drug that rapidly induced changes to the proteome (Fig. 1A and S1A†). Particularly, we became interested in its potential to degrade cyclin D1 *via* an apparent MGD mechanism. We treated HCT116 with niclosamide and checked the protein and transcript levels of cyclin D1 *via* immunoblotting and qPCR (Fig. 1B and C). In line with the proteomics results, we saw rapid protein degradation while the transcript levels remained unchanged. Co-treatment with E1 inhibitor TAK243, NEDD8 inhibitor MLN4924, and proteasome inhibitor bortezomib rescued protein levels, indicating the involvement of a cullin E3 ligase (Fig. 1B). These findings link niclosamide to cyclin D1 degradation through a UPS-dependent mechanism, potentially acting as an MGD.



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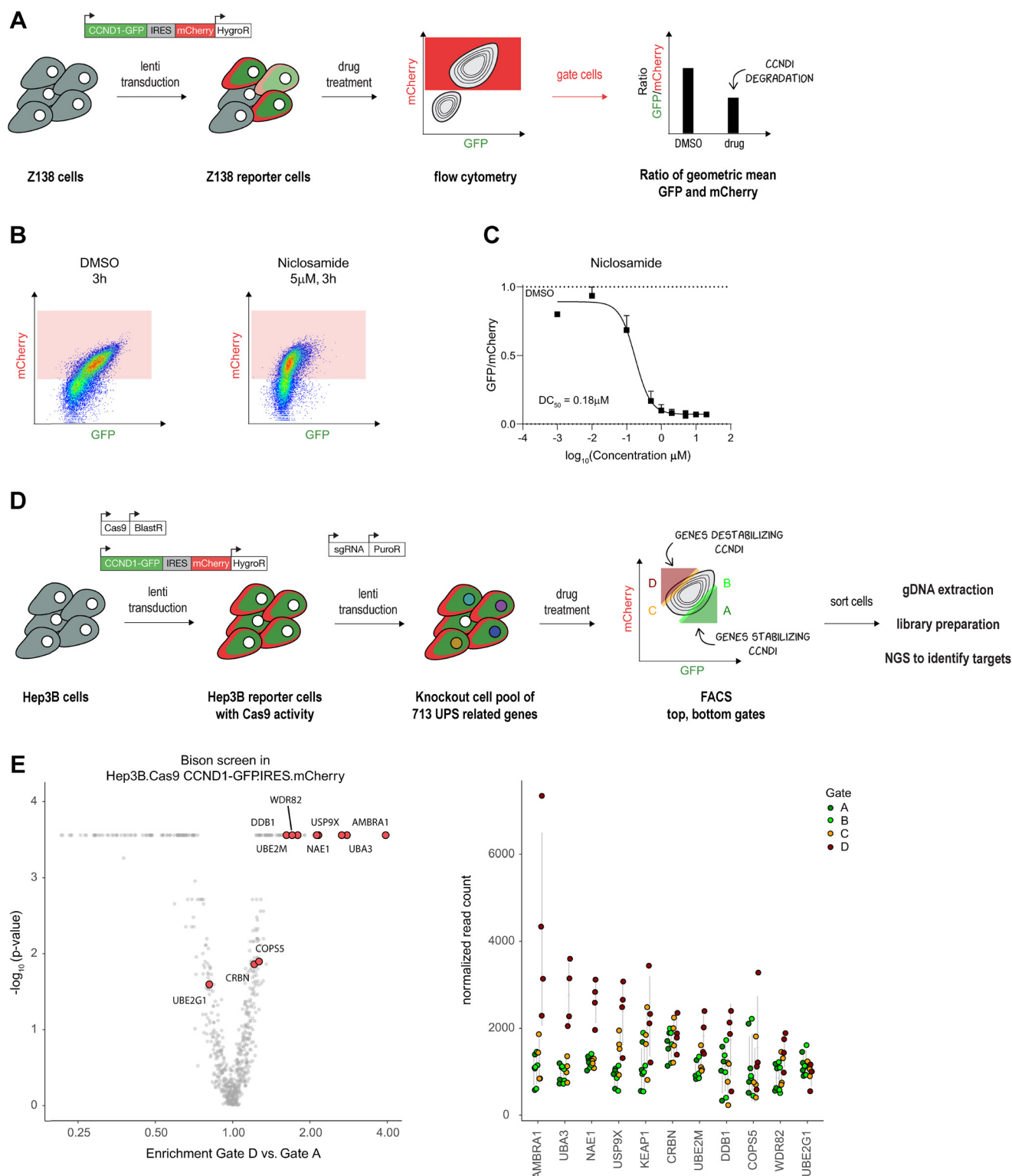


Fig. 2 CCND1 reporter cell lines capture degradation activity. A) Set-up towards establishing the fluorescence-based reporter system for quantifying CCND1 protein levels in Z138 cells. B) The population of Z138 reporter cells (Z138 CCND1-GFP.IRES.mCherry) gated by flow cytometry and used for quantification of CCND1. C) Dose response curve of CCND1 levels relative to vehicle (DMSO) treatment in the reporter cells treated with niclosamide for 3 h. Values represent the ratio of the geometric mean of GFP and mCherry values in the gated population. D) Workflow of setting up a CCND1 reporter in Hep3B cell with Cas9 activity and performing a FACS based genetic screen of members of the UPS which can rescue drug induced CCND1 degradation. E) Genes enriched in a FACS based genetic CRISPR screen (Bison library) rescuing niclosamide induced CCND1 degradation in Hep3B.Cas9.CCND1-GFP.IRES.mCherry reporter cells. The gates used are shown in D) and each contain 5% of the population shown. Gate D contains cells with gene KO's that stabilize CCND1, whereas gate A contains cells with gene KO's that destabilize CCND1.



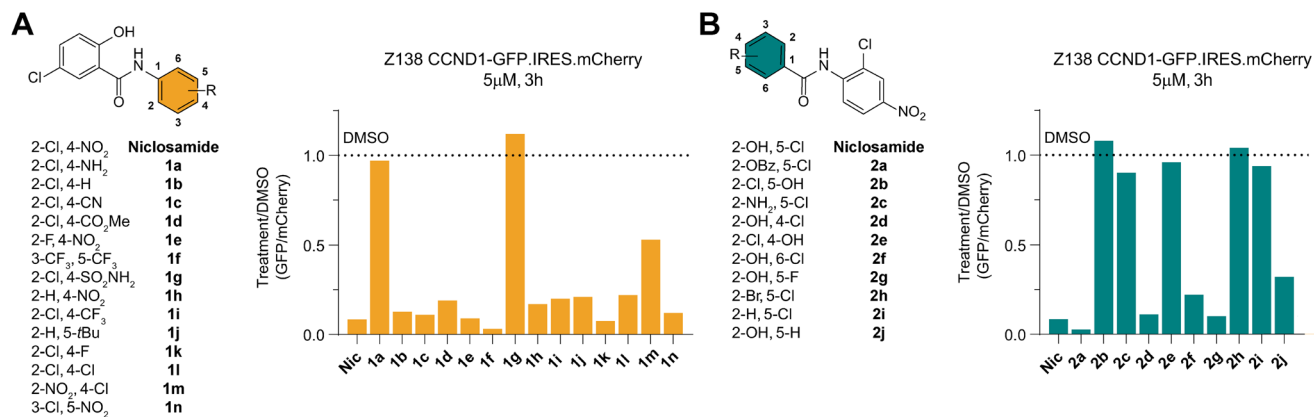


Fig. 3 Structure activity study of niclosamide towards CCND1 degradation. A) Molecules probing the nitroarene ring of niclosamide. Change in CCND1 levels relative to vehicle (DMSO) treatment in the reporter cells treated at a concentration of 5 μ M for 3 h. Values represent the ratio of the geometric mean of GFP and mCherry values in the gated population. B) As for A) but for molecules probing the salicylamide ring.

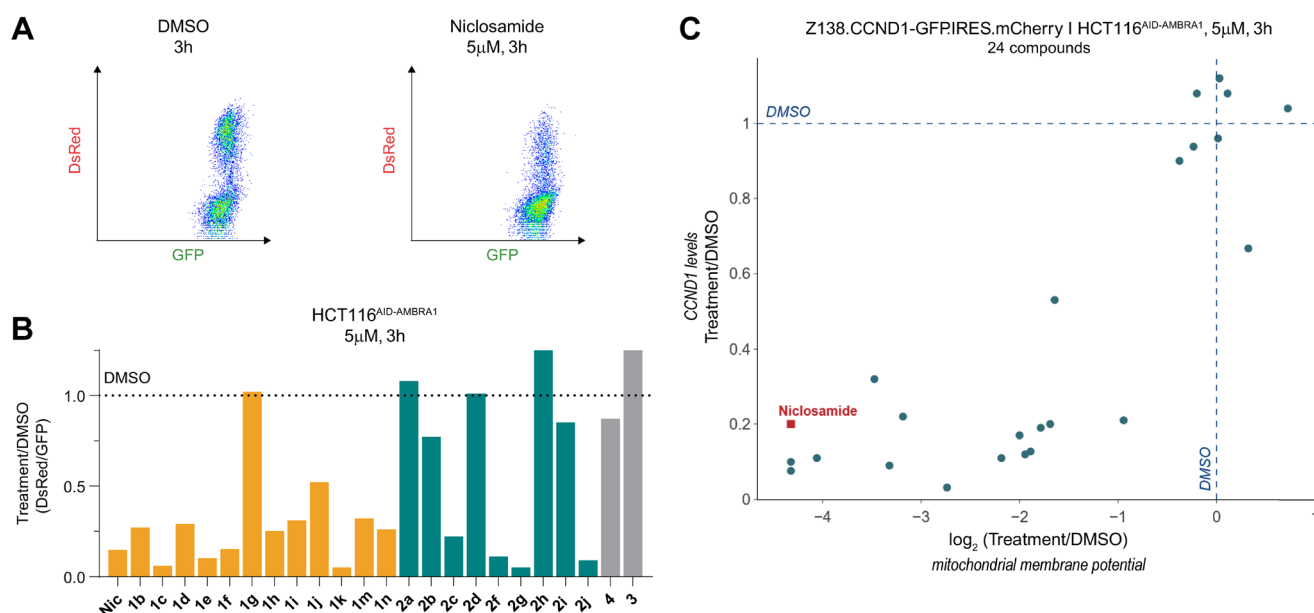
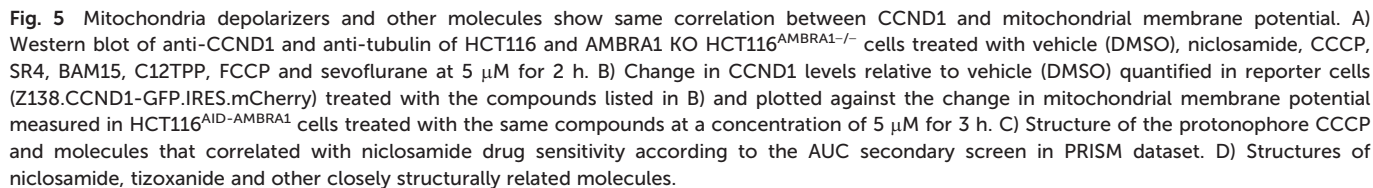


Fig. 4 Niclosamide induced CCND1 degradation correlates with mitochondria depolarization. A) Mitochondrial membrane potential (MMP) quantified using flow cytometry in HCT116^{AID-AMBRA1} cells stained with JC-1. B) Change in MMP relative to vehicle (DMSO) treatment in HCT116^{AID-AMBRA1} cells treated at a concentration of 5 μ M for 3 h. Values represent the ratio of the geometric mean of GFP and DsRed values of all the measures cells. C) Change in CCND1 levels relative to vehicle (DMSO) quantified in reporter cells (Z138.CCND1-GFP.IRES.mCherry) treated with the compounds listed in B) and plotted against the change in MMP measured in HCT116^{AID-AMBRA1} cells treated with the same compounds at a concentration of 5 μ M for 3 h.

with niclosamide in publicly available drug sensitivity datasets (PRISM, CTRP, accessible on <http://depmap.org>) exhibited a similar phenotype. Among the top correlates were inhibitors of the anti-apoptotic proteins Bcl-2 and Mcl-1 located on mitochondria, as well as a telomerase inhibitor (BIBR15322) and molecules like KHS101, which targets the cell growth-promoting TACC3, and tyrphostin A9, a tyrosine kinase receptor PDGFR inhibitor. Notably, the correlation between cyclin D1 degradation and mitochondrial depolarization was maintained among the active molecules (Fig. 5B).

While some molecules arguably contained acidic protons, this was not a common structural feature across all compounds (Fig. 5C). Notably, compounds like tyrphostin A9 and KHS-101, whose reported targets do not suggest disruption of mitochondrial function, have been shown to induce cytotoxicity through this mechanism.^{30,31} Interestingly, aside from the lack of niclosamide induced cyclin D1 degradation in HCT116^{AMBRA1-/-} cells, there was no increase in phosphorylation of threonine 286,^{23,32-34} a key PTM for the nuclear export and ubiquitin-mediated degradation (Fig. S3D†).



It is worth noting that the ionophore nanchangmycin, an antibiotic known to disrupt endocytic uptake of viruses and

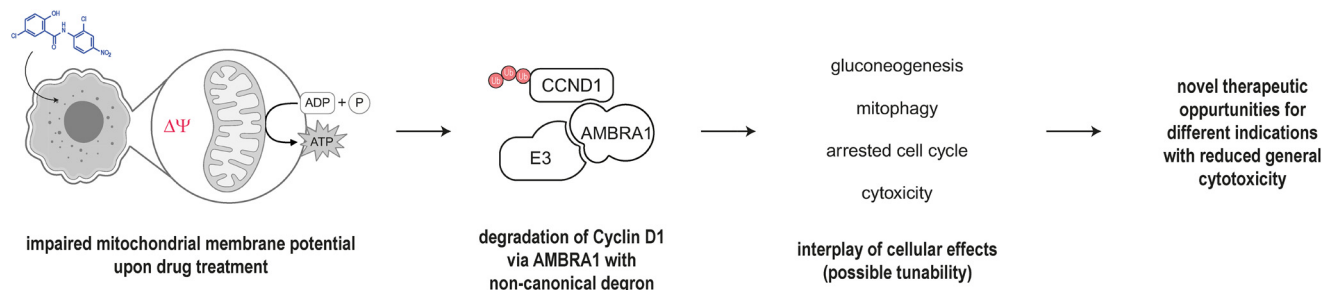


Fig. 6 Polypharmacology of niclosamide.

increase cytosolic Ca^{2+} concentration,^{35–37} showed hyperpolarization and low cyclin D1 levels (Fig. S3C†). The prodrug nitazoxanide of the anti-helminthic tizoxanide, on the other hand, has been reported to depolarize mitochondria.^{38,39} Due to structural similarities with niclosamide, we tested molecular variants of tizoxanide. Under our assay conditions, Tizoxanide and its analogue **7a** induced slight mitochondrial hyperpolarization, while **7b** led to pronounced depolarization. The molecular variants suggest that the nitro group on the thiazole may influence how the molecule affects MMP but more strikingly, despite structural similarities to niclosamide and sign of depolarisation, neither of the molecules altered cyclin D1 levels (Fig. S3C†). These observations indicate that disrupting MMP is necessary but not sufficient to induce cyclin D1 degradation.

Conclusion

The rapid downregulation of cyclin D1 upon niclosamide treatment and its recovery with UPS inhibition presented a potential case for a MGD mode of action, with our targeted genetic screens identifying the endogenous ligase CRL4^{AMBRA1} responsible for cyclin D1 degradation. Given the short half-life of cyclin D1 (ref. 32) we briefly considered a mechanism involving post-transcriptional down-regulation through reduced translation; yet finding that niclosamide-mediated degradation is rescued by knock-out or degradation of AMBRA1 renders this unlikely. Our findings firmly placed AMBRA1 in a pathway mediating cyclin D1 degradation following niclosamide induced membrane depolarization. Additionally, we found that molecules with cytotoxic profiles similar to niclosamide also exhibited both degradation and depolarization phenotypes. Yet the absence of cyclin D1 degradation by tizoxanide and related molecules suggested that the disruption of MMP alone was also not sufficient to induce degradation. Given the diversity of chemical structures inducing downregulation, it is unlikely that niclosamide acts directly as the MGD involved in such an interaction. Given the observed polypharmacology of niclosamide we believe that the poor bioavailability of the orally administered drug is key in the lack of toxicity in humans as opposed to tapeworms where the suggested

mechanism is indeed the uncoupling of oxidative phosphorylation.^{26,33,34}

Cyclin D1, in addition to its cell cycle regulatory role, has been directly implicated in glucose metabolism.^{40–42} AMBRA1, in turn, regulates cyclin D1 levels and plays a crucial role in mitophagy induced *via* MMP disruption by translocating to the mitochondria and amongst others interacting with LC3 or parkin.^{43–45} A cell with a compromised MMP, as triggered by a protonophore, is forced to increasingly rely on gluconeogenesis to meet ATP demands anaerobically. Loss of hepatic cyclin D1 similarly leads to increased gluconeogenesis illustrating the direct link between cyclin D1 and mitochondrial integrity. Instead of a direct MGD mechanism, we hypothesize that niclosamide, and other molecules described herein, exploit the physiological circuitry linking MMP and AMBRA1 to induce the degradation of cyclin D1 (Fig. 6). Other examples for small molecule degraders indirectly impacting protein levels by supercharging endogenous pathways have recently been described.⁴⁶ While the majority of mitochondrial depolarizers are toxic and unsuitable as therapeutics, we show that clinically approved drugs can impact the mitochondrial potential and believe that within this window of MMP disruption, derivatives can be made that exhibit superior degradation abilities for cyclin D1 with potentially less unselective toxicity. This in turn would potentially open new potential therapeutic avenues for cyclin D1 degradation while mitigating broad toxicity should niclosamide prove a viable option in future clinical trials.

Data availability

The proteomics data supporting this article have been included as part of the ESI.† This study was carried out using publicly available data from <http://depmap.org>. All codes and data analysis pipelines have been published and are stated in the corresponding experimental section.

Conflicts of interest

N. H. T. is a founder and shareholder of Zenith Therapeutics as well a consultant to Ridgeline Discovery and Red Ridge Bio. B. L. E. has received research funding from Novartis and Calico. He has received consulting fees from Abbvie. He is a





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