Niclosamide induces miR-148a to inhibit PXR and sensitize colon cancer stem cells to chemotherapy

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https://doi.org/10.1016/j.stemcr.2022.02.005

SUMMARY

Tumor recurrence is often attributed to cancer stem cells (CSCs). We previously demonstrated that down-regulation of Pregnane X Receptor (PXR) decreases the chemoresistance of CSCs and prevents colorectal cancer recurrence. Currently, no PXR inhibitor is usable in clinic. Here, we identify miR-148a as a targetable element upstream of PXR signaling in CSCs, which when over-expressed decreases PXR expression and impairs tumor relapse after chemotherapeutic therapies in mouse tumor xenografts. We then develop a fluorescent reporter screen for miR-148a antagonists and identify the anti-helminthic drug niclosamide as an inducer of miR-148a expression. Consequently, niclosamide decreased PXR expression and CSC numbers in colorectal cancer patient-derived cell lines and synergized with chemotherapeutic agents to prevent CSC chemoresistance and tumor recurrence in vivo. Our study suggests that endogenous miRNA inducers is a viable strategy to down-regulate PXR and illuminates niclosamide as a neoadjuvant repurposing strategy to prevent tumor relapse in colon cancer.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide, responsible for 10% of cancer incidence and mortality despite diagnostic and therapeutic advances (Siegel et al., 2017). The principal CRC chemotherapeutic regimens are 5-fluorouracil, irinotecan, and/or oxaliplatin. The combination of these chemotherapeutic regimens with targeted therapies has further improved survival. However, treatment fails in 90% of cases once the disease becomes metastatic. Therapeutic resistance and tumor recurrence are thought to be caused by a subpopulation of tumor cells, termed cancer stem cells (CSCs) (Adorno-Cruz et al., 2015; Prud’homme, 2012). Unlike most cells in a tumor, CSCs can self-renew. CSCs strongly metabolize cytotoxic compounds, leading to their enrichment following treatment (Todaro et al., 2007), and their self-renewal abilities allow the subsequent initiation of tumor relapse (Dylla et al., 2008; Planque et al., 2016). Therefore, as well as targeting highly proliferative cancer cells, an effective CRC therapy must also efficiently eliminate colon CSCs.

We previously reported that CSCs drive post-treatment recurrence in colon cancer by overexpressing Pregnane X Receptor (PXR) protein (NR1I2) (Planque et al., 2016). PXR is a transcription factor that belongs to the nuclear hormone receptor superfamily (Lehmann et al., 1998). We demonstrated that PXR is needed for CSC self-renewal and the expression of key CSC-related chemoresistance genes (such as CYP3A4, ALDH1A1, and ABCG2). Furthermore, siRNA-based PXR knockdown increases chemosensitivity of CSCs, delaying post-chemotherapy tumor relapse (Planque et al., 2016). Accordingly, we (Planque et al., 2016) and others (Dong et al., 2017) have linked high levels of PXR expression with poor recurrence-free survival in colon cancer patients after chemotherapy. Taken together, these data point to PXR as a target to improve the efficiency of chemotherapy and counteract the selection and emergence of chemo-resistant CSCs and avoid post-treatment tumor recurrence.

The race is now on to translate these recent findings into a clinical therapeutic. However, although nuclear receptors usually respond to a specific set of high-affinity ligands, PXR is activated by a broad spectrum of low-affinity xenobiotics, and crystallographic studies revealed a ligand binding domain (LBD) with a large and conformable binding pocket that renders it extremely difficult to identify PXR antagonists using in silico or conventional medicinal chemistry approaches. Accordingly, only a few antagonists were described to date—SPA-70 (Lin et al., 2017), i-sulfophane (Zhou et al., 2007), or ketoconazole (Huang et al., 2007)—but their efficacy (Poulton et al., 2013) and safety remain inappropriate for clinical use (Fuchs et al., 2013). One of the means to down-regulate a protein of interest is the...
use of microRNA (miRNA) (Huang, 2017). miRNAs are small, noncoding RNAs that are approximately 20–25 nt in length. They regulate the expression of multiple target genes through sequence-specific hybridization to the 3’ untranslated region (UTR) of messenger RNAs, blocking their translation or causing their degradation. Indeed, in vitro experiments have previously identified that miR-148a-3p (miR-148a) directly participates in the post-transcriptional regulation of PXR in human hepatocytes (Rao et al., 2017; Takagi et al., 2008) and in oropharyngeal cancer cells (Reuter et al., 2019). In addition, it has been reported that miR-148a expression is lower in breast CSCs compared with non-CSCs (Boo et al., 2017). Moreover, the down-regulated expression of this miRNA is detected in various cancers, including gastric and colorectal, where, in contrast with PXR (Dong et al., 2017; Planque et al., 2016), high miR-148a expression is a good prognostic indicator in both early-stage (Igder et al., 2019) and treated metastatic CRC (Shivapurkar et al., 2014; Takahashi et al., 2012; Tsai et al., 2013).

Here, we demonstrate that miR-148a overexpression suppresses the expression of PXR and CSC chemoresistance both in vitro and in vivo. Because in vivo synthetic miRNA delivery remains an unsolved obstacle (Pecot et al., 2011), we developed a high-content cell-based fluorescent reporter system to identify niclosamide as a potent inhibitor of PXR expression via the induction of miR-148a. This strategy may be of therapeutic value in colon cancer and provides the basis for repurposing this anthelmintic drug as an adjuvant strategy to reduce CSC chemoresistance.

RESULTS

miR-148a level is inversely correlated with PXR expression in CRC cells and is down-regulated in CSCs

miR-148a-3p (miR-148a) has been reported to post-transcriptionally regulate PXR expression (Rao et al., 2017; Reuter et al., 2019; Takagi et al., 2008). This miRNA is positively correlated with the clinical outcome of treated-metastatic CRC patients, whereas the opposite is true for PXR (Dong et al., 2017; Planque et al., 2016; Takahashi et al., 2012; Tsai et al., 2013). We thus suspected that this miRNA would anti-correlate with the agency of PXR in CRC cells. Using RT-qPCR, we observed that PXR and miR-148a mRNAs were negatively correlated (r = −0.697, p = 0.0038) in a panel of CRC cell lines (SW620, HT29, LS174T, and HCT116) and patient-derived colon cancer cell lines established in our laboratory: from primary (CPP1, CPP6, CPP14, CPP24, CPP25, CPP35) and metastatic (CPP19, CPP30, CPP36) tumors or blood (circulating cancer cells, CTC44, Grillet et al., 2017) (Figure 1A). We also observed that the expression of PXR downstream target genes, such as ALDH1A1 (Planque et al., 2016) (Figure 1B), was inversely correlated with miR-148a expression (r = −0.677, p = 0.0055).

Because it has been reported that miR-148a expression is lower in spheroid-enriched breast CSCs compared with non-CSCs (Boo et al., 2017), we hypothesized that such a mechanism may contribute to the preferential expression of PXR in colon CSCs. We quantified miR-148a expression in CSCs that we had enriched from CRC cells by several approaches: isolation of cells with high ALDH (aldehyde dehydrogenase) enzymatic activity (considered as a robust CSC marker) (Ginestier et al., 2007), non-CSC depletion or reprogramming via chemotherapy treatment (Firi = 5-FU [fluorouracil] + SN-38) (Kreso et al., 2013; Todaro et al., 2007), and maintenance as colonospheres (Spheres) (Kanwar et al., 2010). We observed that expression of miR-148a was significantly lower in both CPP1 patient-derived CRC cells or in the metastatic CRC cell line (SW620) under conditions that enrich CSCs, i.e., ALDH-positive cells, Spheres, or after Firi treatment (Figures 1C and 1D), compared with non-CSCs, i.e., untreated (2D-UT), non-sorted (NS), or ALDH-negative cells. Moreover, as shown in Figures 1E and 1F, we observed a negative correlation between the expression of PXR mRNA or protein and miR-148a mRNAs in CPP1 cells according to their status: higher expression of PXR in CSCs (ALDHpos and Spheres) compared with non-CSC (ALDHneg and 2D-UT) conditions, and the opposite for miR-148a. We also observed that the expressions of PXR downstream target genes ALDH1A1 and ABCG2 were inversely correlated with miR-148a expression (Figures S1A and S1B). These results confirm that miR-148a level is negatively correlated with PXR expression in colorectal CSCs, suggesting that low miR-148a-3p expression may enable the preferential expression of PXR in these cells.

Overexpression of miR-148a represses the PXR expression and PXR signaling pathway

We then confirmed the causal relationship between miR-148a and PXR expression and/or activity. CRC cells were transfected with synthetic miRNA Mimic miR-148a-3p (miR-148a) or the mirVana microRNA Mimic Negative Control #1 (miCTRL). Two days after transfection, we observed a decrease of PXR and PXR target genes, such as ALDH1A1, FGF19 (Wang et al., 2011), or ABCG2 mRNAs in miR-148a-transfected cells compared with control cells (Figures 2A and S2A). DNMT1, a well-described miR-148a target gene (Yan et al., 2014), was used as positive control of miR-148a transfection. To monitor the effect of miR-148a on PXR protein expression and transcriptional activity, we then performed western blot analyses and gene reporter assays. First, we observed a decrease of PXR protein level in HT29 cells transfected with the
miR-148a-3p Mimic (miR-148a) compared with the miCTRL negative control (Figure 2B). In addition, we observed a significant decrease of PXR transcriptional activity (CYP3A4 promoter-driven luciferase, Drocourt et al., 2002) in miR-148a co-transfected HT29 or CPP1 cells compared with cells transfected with the miCTRL (Figure 2C). In accordance, the proportion of cells with high ALDH activity was also decreased in CRC cells transfected with the miR-148a-3p mimic compared with the miCTRL (Figures 2D and S2B) as a result of lower ALDH1A1 mRNA expression. Similar results were observed in HT29 cells infected with a lentivirus (pMIRNA) allowing the stable expression of miRNA and the copGFP (green fluorescent protein from the copepod Pontellina plumata) used for monitoring transfection efficiency and sorting. After purification and expansion of green fluorescent protein (GFP)-positive cells, we observed a down-regulation of PXR and PXR target gene mRNA expression (Figure S2C) and the proportion of cells with high ALDH activity (Figure S2D) in cells injected with a lentivirus encoding miR-148a (miR-148a/copGFP) compared with control cells (i.e., infected with the control copGFP lentivirus [CTRL/copGFP]).

**Overexpression of miR-148a represses the CSC phenotype**

To test the functional significance of miR-148a-mediated PXR down-regulation in CSCs, we transfected CRC cell lines with synthetic miRNA mimics.

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**Figure 1. miR-148a is inversely correlated with PXR in CRC and is down-regulated in CSCs**

(A and B) Inverse correlation between PXR or PXR target gene (ALDH1A1) (A) and miR-148a (B) expression in CRC cell lines purchased from ATCC (T84, SW620, HT29, LS174T, and HCT116, black squares) and patient-derived CRC cells established in the laboratory (primary [CPP1, CPP6, CPP14, CPP24, CPP25, CPP35]; metastatic [CPP19, CPP30, CPP36] or circulating cancer cells [CTC44], red squares) by RT-qPCR analyses (n = 2). The expression of PXR was normalized to RPLO ([60S acidic ribosomal protein P0]), while expression of miR-148a-3p was normalized to miR-103a-3p.

(C and D) miR-148a-3p expression in patient-derived CPP1 and SW620 colon cancer cells after cell sorting based on Aldefluor activity (ALDH-negative and -positive population) (C), or after 72 h of chemotherapy treatment (FIRI = 5 μM 5-FU + 50 nM SN38) or maintained as colonospheres (Spheres) (D). Data are expressed as mean ± SEM (n = 3). F.I., fold induction compared with non-sorted cells (NS) or untreated cells grown under adherent conditions (2D-UT).

(E and F) Negative correlation between PXR and miR-148a expression (data are expressed as mean ± SEM, n = 3) (E), and western blot analysis of PXR and ALDH1A1 protein expression levels (F) in CSCs (ALDH-positive cells [ALDHpos] and Spheres) and non-CSCs (NS cells, cells grown under adherent conditions [2D-UT] and ALDH-negative cells [ALDHneg]) from patient-derived CPP1 cells. *p < 0.05.
miR-148a significantly decreased CD44v6-positive cells (Figure 3A) and CD44v6 mRNA expression (Figure S3A) compared with cells transfected with the microRNA Mimic Negative Control (miCTRL). CD44v6 has been reported as a membrane marker of colorectal CSC (Ma et al., 2019; Todaro et al., 2014). Next, we tested the impact of the miRNA on the ability of colon cancer cells to form tumorspheres (Kanwar et al., 2010). As shown in Figures 3B and S3B, a reduced number of spheres was formed in CRC cells transfected with miR-148a mimic compared with cells transfected with the negative miCTRL. We noted that this inhibitory effect of miR-148a was not restricted to the primary spheres alone but also in the following generations (Figures S3C and S3D), suggesting that miRNA-148a reduces CSC self-renewal ability. Moreover, restoration of PXR expression via the use of a PXR cDNA construct devoid of the 3’UTR miR-148a-targeting sequence (Takagi et al., 2008) significantly reversed the miR-148a inhibitory effects on ALDH-positive cells (Figure 3C), sphere-forming ability (Figure 3D), and PXR target gene expression (Figure 3E) initially observed in HT29 miR-148a/copGFP cells. Indeed, these rescue experiments are evidence that miR-148a-insensitive PXR expression is sufficient to reverse...
miR-148a-mediated inhibition of CSC markers (ALDH1A1, CYP3A4) and self-renewal, supporting the hypothesis that PXR down-regulation is involved in miR-148a effects.

Overexpression of miR-148a impairs CSC chemoresistance and delays post-treatment recurrence in mouse xenograft models

Because PXR drives the expression of a large network of genes instrumental for CSC chemoresistance (including ALDH1A1, ABCG2, and CYP3A4; Planque et al., 2016), we assessed the impact of miR-148a overexpression on the survival of ALDH-positive patient-derived CRC cells treated in vitro with a cocktail of chemotherapy drugs for CRC (Firi = 5-FU + SN38). As shown in Figure 4A, transfection of ALDH-positive sorted cells with the miR-148a mimic significantly decreased their survival after 72 h of treatment with Firi (4-fold decrease of EC50 [half maximal effective concentration], p < 0.05), compared with cells transfected with a control mimic. In addition, as shown in Figure 4B, while the proportion of ALDH-positive cells was significantly increased after chemotherapy treatment in cells transfected with a control mimic, this enrichment was severely impaired in miR-148a-transfected cells, suggesting that miR-148a expression sensitizes CSCs to chemotherapy. To confirm these results in vivo, we generated tumor xenografts by subcutaneously injecting
sphere-derived HT29-miR-148a/copGFP or control HT29-CTRL/copGFP cells into nude mice (n = 18 mice/group). Once tumors reached a volume of 100 mm³, mice were randomized and received either vehicle (PBS, n = 6 mice/group) or FRI cocktail (50 mg/kg 5-FU and 25 mg/kg irinotecan, n = 12 mice/group) twice a week for 4 weeks from day 1 to 29 (Figure 4C). First, we observed a better chemotherapy treatment response in miR-148a/copGFP xenografts compared with the control group. Four days after halting treatment (day 33), six mice from the PBS- and FRI-treated groups were sacrificed for subsequent analysis, while tumor growth was monitored in the remaining FRI-treated mice. As shown in Figure 4C, tumor recurred rapidly in the control group, but not in the miR-148a/copGFP group, suggesting that miR-148a overexpression impairs tumor relapse after chemotherapy. Analysis of GFP-positive tumor cells collected at day 33 showed that, although the proportion of ALDH-positive cells was significantly increased in FRI-treated compared with vehicle-treated CTRL/copGFP tumors, this enrichment was impaired in tumors derived from the miR-148a/copGFP group (Figure 4D). We also tested the self-renewal ability...
therapy is to achieve efficient systemic delivery in whole organisms. We first observed that sphere formation was strongly decreased in cells collected from the F1R1-treated miR-148a/copGFP tumors (Figure 4E). In agreement, when challenged in vivo for tumor initiation in subcutaneous xenograft assays (200 or 2,000 cells/mouse, n = 5 per condition), we observed (Table S1) that the frequency of CSCs in the residual F1R1-treated tumors was ~5 times lower in miR-148a-derived tumors cells than in CTRL tumor cells (p = 0.006). These results clearly indicate that miR-148a overexpression may represent an efficient strategy to decrease CSC chemoresistance and prevent post-treatment recurrence.

High-content screening identifies niclosamide as a pharmacological inducer of endogenous miR-148a expression

One of the major challenges of synthetic miRNA-based therapy is to achieve efficient systemic delivery in whole organisms. An optional strategy to the direct administration of synthetic miR-148a could reside in the identification of pharmacological miR-148a inducers. To identify such compounds, we developed an imaging-based high-content screening assay using a lentiviral miR-148a-3p ratio-metric fluorescent sensor. The sensor features a bidirectional promoter to express simultaneously a GFP from Aequorea coerulescens (GFP) and the dsRed1 red fluorescent protein (Magill et al., 2010). In the 3' UTR of the GFP transcript, we incorporated two miR-148a-3p recognition elements that correspond to the mature miR-148a sequence (Figure 5A). Thus, the level of GFP reflects the activity of the miR-148a, while the dsRed1 signal is used to normalize and minimize the effects of variable expression levels and nonspecific transcriptional regulation, providing a ratio-metric measure (GFP/dsRed). We generated a CPP1 patient-derived cancer cell line stably expressing the miR-148a-3p sensor construct. As shown in Figures 5A and S4A, we observed a significant decrease of GFP/dsRed ratio when cells were transfected with miR-148a mimic compared with mirVana microRNA Mimic Negative Control #1 (miCTRL), thus validating our assay. In addition, using fluorescence-activated cell sorting (FACS), we confirmed the preferential expression of miR-148a-3p in dsRed*GFP* compared with dsRed*GFP* cells (Figure 5B), and inversely a lower expression of miR-148a target gene mRNAs: DNMT1 and PXR (Figure 5C). These data confirm not only that our miR-148a-3p ratio-metric fluorescent sensor provides accurate and quantitative in-cell measurement of miR-148a expression but also proves directly the inverse correlation between miR-148a-3p and PXR expression in sorted cells. We next used this functional tool to screen the Prestwick Chemical Library, which contains 1,280 molecules with known bioavailability and safety in humans. Cells were plated in 384 wells and then treated with 5 μM of each compound for 48 h, followed by fixation, DAPI labeling of nuclei, and image acquisitions and analysis with a high-content screen automated microscope. We identified 10 compounds that significantly reduced the GFP/dsRed ratio (Z score < −2 and p < 0.05; Figure 5D; Table S2). We then performed RT-qPCR to measure their effects on endogenous miR-148a expression and miR-148a target gene expression. As shown in Figure S4B, only niclosamide significantly increased miR-148a expression in CPP1 cells compared with vehicle treatment. Accordingly, we observed a decrease in both DNMT1 and PXR mRNA expression in niclosamide-treated cells (Figure S4C). Indeed, niclosamide-mediated induction of miR-148a (Figure 5E) and PXR mRNA inhibition (Figures S4D and S4E) were confirmed on several CRC cells. In agreement, we observed a significant decrease of PXR protein level after 5 μM niclosamide treatment (Figure 5F). Amazingly, this drug was already pulled out of the Prestwick Library as a potential CRC metastasis blocker (Sack et al., 2011), as well as a potential inhibitor of both WNT/β-Catenin (Osada et al., 2011) and NOTCH (Wang et al., 2009) pathways. To verify the direct involvement of miR-148a on niclosamide-mediated down-regulation of PXR, we used antagonir-148a, i.e., a chemically modified single-stranded RNA molecule designed to specifically bind to and inhibit endogenous miR-148a-3p molecule (anti-miR miRNA inhibitor). Cells were first transfected with the antagonist to block specifically miR-148a-3p activity (anti-miR-148a) or a negative control (anti-miCTRL miRNA Inhibitor Negative Control #1 [anti-miCTRL]) prior to niclosamide treatment. As shown in Figures 5G, S4E, and S4F, while niclosamide significantly down-regulated PXR mRNA and protein levels in cells transfected with the control sequence (anti-miCTRL), it has a negligible effect in cells transfected with the miR-148a inhibitor. These results demonstrate that the upregulation of miR-148a is crucial for niclosamide-mediated inhibition of PXR expression. In contrast, the miR-148a antagonir was unable to antagonize the inhibitory effect of niclosamide on both WNT (LGR5, CCND1, JAG1) and NOTCH (HES1, SURVIVIN) target gene expression (Figure 5G), suggesting a miR-148a-independent pathway(s) in those effects. Overall, these results demonstrate that, although further studies are needed to decipher how niclosamide induces miR-148a, its inhibitory effect on PXR expression directly involves miR-148a expression.

Niclosamide inhibits PXR activity and the CSC phenotype

Having established the induction of miR-148a and the inhibition of PXR expression by niclosamide in CRC cells,
we then tested its impact on the colon CSC phenotype. As shown in Figures 6A and 6B, niclosamide decreased PXR and DNMT1 mRNA expression in LS174T and CPP1 cells in a dose-dependent manner. Accordingly, the expressions of ALDH1A1 mRNA and ALDH-positive cells were also reduced after niclosamide treatment in CPP1 (Figure 6C) or CPP14, CPP36, and CTC44 cells (Figures S5A and SSB). In addition, as shown in Figure SSC, the inhibitory effect of niclosamide onto the ALDH-positive cells requires miR-148a induction because this inhibitory effect was abolished in a cell line stably transfected with a PXR cDNA construct devoid of the 3′UTR miR-148a-targeting sequence (LS174T-PXR) (Planque et al., 2016). Finally, as observed with the miR-148a mimic, niclosamide treatment significantly reduced the CD44v6 mRNA and CD44v6-positive populations (Figures 6D and S5D), as well as sphere-forming CRC cells (Figures 6E and 6F).
formation in a dose-dependent manner, with almost a complete suppression at the highest dose (5 μM) in all CRC cell lines tested.

**Niclosamide prevents chemotherapy-induced enrichment of CSCs and delays post-treatment recurrence in mouse xenograft models**

We tested the impact of niclosamide on CSC chemoresistance and post-treatment relapse in our preclinical mouse xenograft models. We subcutaneously xenografted 15,000 CPP1 cells from spheroids and allowed tumor volume to reach 100 mm³ (day 0). Mice were then exposed to the following treatment regimens: PBS (n = 5 mice, CTRL), FIRI alone (n = 10 mice, 50 mg/kg 5-FU and 25 mg/kg irinotecan, twice a week, i.e., Tuesdays and Fridays), niclosamide alone (n = 5 mice, NICLO: 20 mg/kg, 5 days/week, i.e., Mondays to Fridays), or combined niclosamide + FIRI (n = 10 mice, NICLO + FIRI) (Figure 7A). First, we observed that tumor growth was slightly slower in the NICLO group than in the CTRL group. Importantly, we did not observe any clinical signs of toxicity during these 4 weeks of treatment (body weight, diarrhea, skin ulcers, hyper/hypoactivity, changes in motor activity). Six hours after the last treatment (day 29), six FIRI-treated mice per group (+/- NICLO) were sacrificed for subsequent analyses, while tumor growth and post-treatment relapse were monitored in the remaining mice. Tumor relapse was clearly noticeable from 2.5 weeks post-treatment (day 46), especially in the group of mice treated with FIRI alone. However, combination of niclosamide treatment with FIRI significantly...
delayed tumor relapse compared with mice treated with chemotherapy alone, leading to a significant reduction of tumor volume and weight at day 57 (Figure 7B). We then analyzed the reservoir of CSCs from tumors collected after the chemotherapy (day 29). After tumor dissociation, live tumor cells (7AAD−) were purified using a cell sorter, pooled, and then challenged in vitro for their ability to form spheres and in vivo for tumor initiation in a secondary xenograft assay. As shown in Figure 7C, sphere formation was strongly reduced in cells isolated from the NICLO + FIRI-treated tumors with a 3-fold reduction of CSC frequency quantified using ELDA (Hu and Smyth, 2009) compared with cells isolated from FIRI-treated tumors. In agreement, secondary injection of 250 cells/mouse (Figure 7D) isolated from the niclosamide + FIRI-treated tumors produced fewer tumors than cells recovered from FIRI-treated mice, demonstrating that niclosamide co-treatment decreased the proportion of tumorigenic cells after chemotherapy treatment. Indeed, both RNAscope (Figure 7E) and RT-qPCR (Figure 7F) analyses confirmed that PXR mRNA expression was significantly reduced in tumors resected from mice co-treated with niclosamide plus chemotherapy compared with mice treated with chemotherapy alone.
with niclosamide and FIRI compared with FIRI-treated tumors. Moreover, we observed that the increase in PXR and ALDH1A1 mRNA expression after FIRI treatment was severely hampered when mice were pretreated with niclosamide, suggesting that niclosamide-mediated PXR down-regulation decreases the preferential survival of CSCs during chemotherapy. These results were confirmed in vitro on several patient-derived cells (CPP1, CPP14, CPP36, and CTC44). Although the proportion of ALDH-positive cells was significantly increased after chemotherapy (Firi = 5-FU + SN38) treatment, this enrichment was abolished when cells were pretreated with 5 μM niclosamide (Figure S6A). In agreement, RT-qPCR revealed that the increase in PXR and ALDH1A1 mRNA expression after Firi treatment was also severely hampered when cells were pretreated with niclosamide (Figure S6B). Taken together, these results indicate that repurposing niclosamide as an adjuvant therapy combined with chemotherapy decreases the pool of chemoresistant CSCs, delaying tumor relapse.

DISCUSSION

Although considerable progress has been made in its diagnosis and treatment, metastatic CRC remains characterized by high incidence, low sensitivity to therapy, high risk of developing resistance to chemotherapy, treatment failure, and a low 5-year survival rate (Siegel et al., 2017), making CRC a medical challenge and necessitating the discovery or development of effective treatment strategies. Accumulating studies demonstrated that CSCs, a cancer cell subpopulation with unlimited capacity for self-renewal, differentiation, and tumorigenesis, is one of the reasons for relapse and metastasis (Adomo-Cruz et al., 2015; Dylla et al., 2008; Planque et al., 2016; Prud’homme, 2012; Todaro et al., 2007). In a previous study, we demonstrated that relapse-prone CSCs are characterized by high PXR expression and activity, and this nuclear receptor drives the expression of a large network of genes that are instrumental for CSC chemoresistance (Planque et al., 2016). Thus, decreasing PXR expression/activity using pharmacological inhibitors may represent a promising strategy to improve the efficiency of conventional chemotherapy in CRC patients through the sensitization of CSCs. To inhibit the action of PXR, we developed here an alternative and innovative strategy to those focusing on small-molecule antagonists based on the epigenetic modulation of PXR by a miRNA.

We are the first to demonstrate that miR-148a-3p expression is down-regulated in colon CSCs (Figure 1) and confirmed that its upregulation regulates negatively PXR expression and activity (Figure 2). In addition, we observed that miR-148a significantly affects colon CSC phenotype (Figure 3) by reducing Aldefluor activity, CD44v6 expression, and self-renewal. These results confirm the observation previously reported on a colon cancer cell line (SW480) showing that miR-148a suppresses the expression of stem cell markers and increases their chemo-sensitivity (Shi et al., 2019). These observations agree with previous reports showing the inhibitory effect of miR-148a on key CSC signaling pathways, such as WNT/β-CATENIN (Peng et al., 2017) or STAT3 (He and Xue, 2017). Furthermore, we observed that overexpression of miR-148a-3p decreased CSCs chemoresistance and significantly delayed tumor relapse after chemotherapy treatment in mouse xenograft models (Figure 4). In accordance with our in vivo data, it has also been shown that miR-148a overexpression inhibited the growth of ovarian OVCAR3 xenograft tumors in nude mice (Zhu et al., 2019).

In vivo delivery of miRNA has unsolved technical issues and so far, they are inappropriate for therapeutic use due to their poor pharmacokinetic properties in vivo (Pecot et al., 2011). Therefore, one possible alternative could be the identification of small molecules acting as endogenous miR-148a inducer or activator. To identify such compounds, we developed a microscopy-based high-content screening assay based on a lentiviral dual-green/red fluorescence miR-148a-3p sensor strategy. The screening of 1,280 molecules already approved by US Food and Drug Administration (FDA; Chemical Prestwick Library) allowed us to identify niclosamide as a putative miR-148a activator. The advantage of focusing on such drugs is that their safety profile is well understood and clinically manageable, meaning that they could proceed to clinical trials reasonably quickly. For instance, niclosamide is an oral chlorinated salicylanilide anthelmintic agent that inhibits mitochondrial oxidative phosphorylation in the cells of parasitic worms that has been used for decades for the treatment of tapeworms (Li et al., 2014).

As a consequence of endogenous miR-148a upregulation, we observed that niclosamide treatment decreases the expression of miR-148a target genes, such as DNMT1 and PXR, in a miR-148a-dependent manner (Figures 5 and 6). In addition, niclosamide treatment inhibited CSC features by reducing the expression of key CSC markers, such as Aldefluor activity or CD44v6 and the sphere-forming ability of patient-derived CRC cell lines and circulating tumor cells (Figure 6). These observations confirm previous studies reporting that niclosamide attenuates the tumor-initiating and survival potential of colon CSCs (Arend et al., 2016). Moreover, niclosamide has been reported to decrease the Aldefluor-positive population in breast (Londoño-Joshi et al., 2014) and melanoma (Zhou et al., 2017) cancer cells, and reduced the frequency of both CD44+/CD24- high CSC cell population and mammosphere formation in breast cancer cell lines (Liu et al., 2016). Finally, we report that niclosamide co-treatment potentized frontline
chemotherapeutic agents’ efficiency in targeting colon CSCs (Figure 7). Notably, we demonstrated using in vitro and in vivo (mouse xenografts) experiments that niclosamide exhibits remarkable synergism with CRC mainstream anticancer drugs because it sensitizes colon CSCs to chemotherapy drugs, decreasing chemoresistance-related gene expression, and delays tumor relapse after treatment cessation. Our preclinical data strongly suggest that niclosamide could be repurposed as neoadjuvant strategy to potentiate chemotherapeutic drugs for treating CRC, through miR-148a induction and the subsequent inhibition of its target genes involved in CSC self-renewal and chemoresistance.

These preclinical data warrant further validation and translation toward clinical trials to confirm their effectiveness in patients. Notably, niclosamide is orally administered to helminthiasis patients, while in our study we used intraperitoneal administration. However, this route of administration has already been successfully used by others, leading to a significant inhibition of tumor growth in human ovarian (Shangguan et al., 2020) and breast cancers transplanted tumor models (Liu et al., 2016). Osada et al. (2011) found that oral administration of niclosamide (200 mg/kg body weight) resulted in sufficient distribution of the drug into murine xenographs. In patients, the serum concentrations of niclosamide are 0.25–6.0 μg/mL (Andrews et al., 1982), corresponding to 0.76–18.35 μM. These concentrations correspond well to the active concentration range for miR-148a induction observed in our study (5 μM). Importantly, a phase I clinical trial is currently ongoing to evaluate the maximum tolerated dose of niclosamide in CRC patients (ClinicalTrials.gov: NCT02687009) and breast cancers transplanted tumor models (Burock et al., 2018). Thus, based on these ongoing studies and on our preclinical work, we believe that additional trials could be designed to investigate clinical opportunities offered by niclosamide repurposing as adjuvant strategy, as miR-148a inducer to decrease PXR expression and CSC chemoresistance during chemotherapy in metastatic CRC patients.

**EXPERIMENTAL PROCEDURES**

See supplemental information for more details.

**Staining for flow cytometry analysis and FACS**

CD44-allophycocyanin (APC) (559,942; BD Pharmingen), CD44 v6-APC (clone REA706, 130-111-238; Macs Miltenyi), or CD44 v6-phycocerythrin (PE) (FAB3660P; R&D) antibodies (1/100) were incubated with 100,000 cells in PBS containing 5% FBS for 20 min at 4°C. The Aldefluor assay (Stem Cell Technologies) or ALDHRed assay (Millipore) was performed according to the manufacturer’s instructions. ALDH-positive and ALDH-negative cells were identified by comparing the same sample with and without the ALDH inhibitor diethylaminobenzaldehyde (DEAB).

Stainings were analyzed using the MACSQUANT (Miltenyi) analyzer. Cells were sorted using a FACSAria II (BD) and analyzed using Flowing software (v 2.5.1; http://flowingsoftware.btk.fi/).

**High-content screening**

We performed an automated screening of all 1,280 FDA-approved drugs in the Prestwick Chemical Library at 5 μM in black clear-bottom 384-well plates using the CPPI-miR-148a sensor cellular model. This screening was performed with four replicates of each compound, for a total of sixteen 384-well plates using a Tecan EVO200 robotic liquid handling system (Tecan Trading AG). Image analysis and quantifications of output parameters were obtained with High Content Screen Studio Cell Analysis software (v3.0; Thermo Scientific). Image analysis allowed first detecting and counting cells in wells with DAPI nuclear labeling. Then we quantified a cell-by-cell GFP/dsRed1 signal ratio into enlarged nuclear region (region of interest [ROI] + 2 pixels), the specific readout for our screening. “Hits” were identified using the robust Z score statistical method on GFP/dsRed1 signal ratio (cf. screening data normalization section in supplemental information).

**Xenograft transplantation and combination of niclosamide and chemotherapy treatment**

All experiments were performed according to the European Union (Council directive 86/609/EEC) and institutional/local guidelines on laboratory animal usage. Animal protocols were approved by the French ethical committee for animal testing (authorization referral #1627). All efforts were directed at minimizing animal discomfort and to reduce the number of animals used (3R rule). Cells in suspension were injected subcutaneously into the right flanks on NOD/SCID mice (Charles River) in a 1:1 mixture of Matrigel and DMEM in a final volume of 100 μL. Tumor volume ([length x width²]/2) was measured with a caliper. Randomization and treatment started once tumor volume reached 100 mm³, and mice were sacrificed when tumors reached 1,500 mm³.

**Statistical analysis**

For each experiment, data are shown as mean ± SEM of n independent experiments (n = number of independent experiments). GraphPad Prism7 software was used for data analysis. The Mann-Whitney test was used to analyze the difference between two groups of quantitative variables with alpha value set at 5%: *p < 0.05, **p < 0.005, ***p < 0.001.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.02.005.

**AUTHOR CONTRIBUTIONS**

C.P., J.M.P., and J.P. designed the research; C.P., J.M.P., and J.P. designed part of the experiments; L.B., O.B., E.M., G.L.G., A.G., E.P., M.B., B.B., J.P., and C.P. performed the experiments; L.B., O.B., E.M., G.L.G., A.G., E.P., M.B., C.H.-K., B.B., J.P., J.M.P., and C.P. drews et al., 1982), corresponding to 0.76–18.35 μM. These concentrations of niclosamide are 0.25–6.0 μg/mL (Andrews et al., 1982), corresponding to 0.76–18.35 μM. Important, a phase I clinical trial is currently ongoing to evaluate the maximum tolerated dose of niclosamide in CRC patients (ClinicalTrials.gov: NCT02687009) and a phase II trial (NIKOLO) to investigate the safety and efficacy of orally applied niclosamide in metastatic CRC patients with progressive disease after chemotherapy (Burock et al., 2018). Thus, based on these ongoing studies and on our preclinical work, we believe that additional trials could be designed to investigate clinical opportunities offered by niclosamide repurposing as adjuvant strategy, as miR-148a inducer to decrease PXR expression and CSC chemoresistance during chemotherapy in metastatic CRC patients.

**EXPERIMENTAL PROCEDURES**

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analyzed the data; J.E.B. and M.P. provided reagents/analytic tools; F.H., J.P., J.M.P., and C.P. wrote the manuscript.

CONFLICTS OF INTERESTS

The authors declare no competing interests.

ACKNOWLEDGMENTS

We acknowledge the contribution of iExplore animal facility (IGE, Montpellier) and the AniRA lentivectors production facility from the CELPHEDIA Infrastructure and SFR Biosciences (UMS3444/CNRS, US8/INSERM, ENS de Lyon, UCB). We thank C. Dupray (IRBM, Montpellier) from the Montpellier RIO Imaging platform for flow cytometry experiments and Jérôme Torrisani (INSERM UMR 1037, University of Toulouse III, France) for the gift of MIR148a-copGFP and pMIRNA1-copGFP. This work was supported by grants from the INCa, Cancéropôle GSO, Association pour la Recherche contre le Cancer, Key Initiative Muse “Biomarkers and Therapy,” GEFLUC, and SIRIC of Montpellier (France).

Received: March 30, 2021
Revised: February 4, 2022
Accepted: February 7, 2022
Published: March 10, 2022

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