Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing

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MicroRNAs (miRNAs) are small noncoding RNAs that inhibit gene expression at the posttranscriptional level. They are synthesized in the nucleus by RNA polymerase II as long primary transcripts, called primary miRNAs (pre-miRNAs) that are transported to the cytoplasm by Ran-GTP/Exportin-5, where DICER1 miRNA biosynthesis gene (19–21) and TRBP2 (TRBP) protein (18), and down-regulation of the DICER1 miRNA biosynthesis gene (19–21). Consistent with these observations, experimental knockdown and genetic defects in miRNA-processing machinery genes, such as DICER1 and TRBP2, cause miRNA global depletion and stimulate tumorigenesis (18, 22–25), suggesting that miRNA impairment actively contributes to cancer development.

Despite the impact of miRNAs on cancer biology, miRNA-based cancer therapy is still in its early stages and mostly limited to target a single miRNA (26, 27). However, because most tumors show a global down-regulation of miRNA expression (5, 6, 9–11), restoration of normal miRNA levels might represent an attractive approach in cancer therapy. Herein, we present an miRNA-based treatment of malignancies in which enoxacin, a small molecule proposed to promote RNA interference and miRNA processing (28), has a powerful cancer-specific growth-inhibitory effect mediated by a TRBP-dependent restoration of the expression of tumor suppressor miRNAs.

Results

Enoxacin Treatment Has a Cancer-Specific Growth-Inhibitory Effect.

Despite the enormous potential that a small molecule that enhances RNA interference might have for cancer therapeutic purposes, the effects of enoxacin in tumor proliferation have not been characterized. Thus, we first analyzed whether enoxacin could predominantly act as a cancer growth inhibitor by examining the effects of the drug in a panel of 12 cancer cell lines from seven common malignancies. The transformed cell lines studied included colorectal (RKO and HCT-116), gastric (SNU-1 and SNU-638), lung (H23, H1299, and A549), breast (MCF-7 and MDA-MB-231), liver (HePG2), leukemia (K562), and lymphoma (RAJI).

Molecular link between miRNA deregulation and cancer development.
Enoxacin Enhances miRNA Production. One critical matter to address is the characterization of the molecular pathways involved in the observed cancer growth-inhibitor phenotype mediated by enoxacin. Enoxacin has been characterized as an enhancer of RNA interference (28), although this mechanism is not naturally used by human cells to silence gene expression posttranscriptionally. At this last level of control, other molecules play a central role in our cells, such as miRNAs (1–3). It was also suggested that enoxacin might promote the processing of miRNAs by comparing the level of three precursor miRNAs (pre-miR-125a, prelet-7, and premiR-30a) (28).

We wondered whether enoxacin could enhance the production of miRNAs with putative tumor-suppressor functions that would explain the antitumoral effects of the drug. We have first addressed this question by measuring the production of miRNAs of cancer cells upon enoxacin treatment. We observed that HCT-116 and RKO enoxacin-treated cells featured an overall increase in the production of 24 mature miRNAs molecules (Fig. S2) and a down-regulation of the corresponding precursor miRNA molecules (Fig. S2). Northern blot analyses of the tumor-suppressor miRNAs let7-a and miR-125a confirmed the enhancement of miRNA production in enoxacin-treated cells (Fig. S2). The case of let-7 is particularly exciting because it targets Cdc34 and leads to Wee1 stabilization and G2/M accumulation (30). Concordantly with these data, we observed that enoxacin use in HCT-116 and RKO cells, in addition to causing G2/M arrest and promoting the processing of let-7, induces Cdc34 down-regulation and the stabilization of the Wee1 protein (Fig. S2).

We also analyzed the global miRNA expression profile of RKO cells upon enoxacin treatment by using a comprehensive expression miRNA microarray platform (9). The expression profile of 731 miRNAs demonstrated that, among the differentially expressed miRNAs (n = 122), enoxacin-treated RKO cancer cells exhibited an overall up-regulation of miRNAs, 81% (100 of 122), whereas only 18% (22 of 122) underwent down-regulation. Strikingly, for the 26 enoxacin-up-regulated miRNAs in which a role in cancer development has been proposed (31, 32), 84.6% had potential tumor-suppressor features (Fig. S2). Most importantly, the restoration of the expression of tumor-suppressor miRNAs in RKO cells upon enoxacin treatment was associated with the down-regulation of their respective target oncoproteins, as we observed for MYC (let-7-a and let7-b) and K-ras (miR-18a*, let7-a, let7-b, miR-143, and miR-205) (Fig. S2). Interestingly, we did not observe down-regulation of tumor suppressor genes regulated by the few oncosuppressive miRNAs induced by enoxacin treatment, such as the miR-21 target proteins Pten and Pdcd4 (Fig. S2).

Enoxacin Binds to TRBP. It is also important to establish a mechanistic link that could explain how enoxacin promotes miRNA processing. Once we ruled out the possibility that enoxacin treatment increased the expression of the miRNA Machinery proteins (Fig. S3), a clue about the mechanism was provided by the observation that enoxacin increases the binding affinity of the TRBP

Fig. 1. Enoxacin treatment has cancer-specific inhibitory effect. (A) Cell viability assay in 12 cancer cell lines vs. fibroblast cell cultures (Wi-38 and MRC-5) and normal lymphocytes from healthy donors. (B) Colony formation assay in the described cell lines.
TRBP is an integral component of a DICER1-containing complex, in which it plays a critical role in miRNA processing (33, 34). Thus, it is possible that the observed enhancement of miRNA production upon enoxacin treatment is mediated by a direct effect of the drug on the TRBP protein. Indeed, we have identified a physical interaction between enoxacin and the TRBP protein by using two independent methods, surface plasmon resonance (SPR) and isothermal titration calorimetry. In the first approach, we synthesized the wild-type TRBP wild-type protein in bacteria, in addition to a TRBP-mutated protein that has altered amino acids 149–179 and lacks the last 187 amino acids (18) (Fig. 2B). The binding of wild-type and mutant TRBP proteins to enoxacin was first measured by SPR using the BIAcore CM5 sensor chip, which analyzes the ratio of actual to theoretical binding responses (RUactual and RUtheor, respectively). Enoxacin had an RUactual to RUtheor ratio of 0.9, signifying monomeric binding of enoxacin to the immobilized TRBP wild-type protein (Fig. 2B). The mutant form of TRBP was unable to bind to enoxacin (Fig. 2B). We calculated an affinity constant (Kd) of 12.56 μM for the affinity of enoxacin for TRBP wild-type by SPR (Fig. 2B). Time-course experiments confirmed specific enoxacin binding to TRBP, in which a sequential reduction of enoxacin concentration reduced binding response units to the same magnitude (Fig. 2B). The binding kinetics revealed a very stable interaction with a dissociation constant (Kd) of 2.2 × 10⁻⁷ M (Student’s t test P ≤ 0.01) (Fig. 2B). These time-course and kinetics experiments confirmed the absence of binding of enoxacin to the mutant TRBP form (Fig. 2B). Most importantly, isothermal titration calorimetry experiments corroborated the findings obtained with the SRS BIAcore 2000 assays: The binding of enoxacin to the TRBP wild-type protein was entropically driven (ΔS = 138.4 J/K/mol⁻¹), whereas there was no binding between enoxacin and the TRBP-mutant form (Fig. 2C). Thus, there is a direct physical interaction between enoxacin and TRBP that connects the drug and the miRNA-processing pathway.

TRBP Mutant Cancer Cells Are Resistant to the Growth-Inhibitory and miRNA-Processing Effects Mediated by Enoxacin. We decided to investigate an additional link between enoxacin, TRBP protein, miRNA processing, and cellular growth by taking advantage of human cancer cells harboring genetic defects in the TARBP2 gene that encodes the TRBP protein (18). The presence of TARBP2 frameshift mutations in a subset of colorectal, gastric, and endometrial malignancies causes diminished TRBP protein expression and a defect in the processing of miRNAs (18). The reintroduction of TRBP in the mutant cells restores the efficient production of miRNAs and inhibits tumor growth (18). Thus, we assessed the effects of enoxacin in a colorectal cancer cell line harboring an inactivating heterozygous TARBP2 frameshift mutation (Co115), a reconstituted Co115 cell line that it is stably transfected with the TRBP protein (Co115.TRBPWT), and a Co115 cell line stably transfected with the inert TRBP mutant protein (Co115.TRBPmut) (18). In addition, we used another TRBP-defective model by stably transfecting the colorectal cancer cell line RKO with a short hairpin that silences the TRBP protein (RKO.shTRBP). We confirmed that the three TRBP-impaired cell lines (Co115, Co115.TRBPmut, and RKO.shTRBP) had lower levels of wild-type protein expression than the proficient cells (Co115.TRBPWT, RKO, and HCT-116) (Fig. S4). We observed that enoxacin administration led to a small reduction in cell viability in the three cell lines in which TRBP function was defective (Co115, Co115.TRBPmut, and RKO.shTRBP) (Fig. S4). Almost no effect on the clonogenic capacity (Fig. 3A) was observed, relative to the marked reduction of cellular growth for both assays in the TRBP-reconstituted (Co115.TRBPWT) or naturally proficient cells (RKO and HCT-116) (Fig. S4 and Fig. 3A). The EC50 value for enoxacin of the TRBP mutant Co115 cells, EC50 = 238 μM (76.2 μg/mL) (Fig. S4), double the one observed in TRBP wild-type HCT-116 cells (Fig. S1).
We also examined whether the described resistance to growth inhibition upon enoxacin use in TRBP-impaired cells was reflected in the cell cycle. Flow cytometry demonstrated that upon enoxacin treatment, TRBP-co115.xenografted cells exhibited cell-cycle arrest in G2/M phase with an increase from 6 to 38% in this stage (Fig. S4). However, enoxacin treatment of TRBP-impaired cells (co115, co115.TRBPmut, and RKO.shTRBP) did not significantly increase the G2/M cellular fraction (Fig. S4). The cell-death values followed a similar pattern: TRBP-reconstituted cells (co115.TRBPWT) underwent massive apoptosis (97.7%) after enoxacin use that was not observed in mutant TRBP cells (co115, 6.7%) or cells stably transfected with the inert mutant form (co115.TRBPmut, 3.1%) (Fig. S3B). Conversely, depletion of TRBP in RKO cells (RKO.shTRBP) rendered these cells more resistant to enoxacin-mediated cell death than the control cells (RKO) (Fig. S4). Thus, these data imply that the inhibition of cancer-cell growth, the induction of G2/M cell-cycle arrest and cell death by apoptosis upon enoxacin use is mediated by the miRNA-processing protein TRBP.

Following our discovery that enoxacin enhances the overall production of miRNAs with putative tumor-suppressor functions in the TRBP-proficient HCT-116 and RKO cells (Fig. 2A and Fig. S2), we wondered whether cells with defects in TRBP would be more refractory to the aforementioned effect. We addressed this matter by measuring the expression levels of the 24 described mature and precursor miRNAs. Co115 TRBP-deficient cells display an impaired expression of mature miRNAs that is improved by TRBP transfection (co115.TRBPWT), but is not enhanced in cells transfected with the TRBP-mutant form (co115.TRBPmut) (18). We observed that enoxacin treatment significantly increased the production of mature miRNAs only in restored Co115 cells, whereas the effect on untransfected-deficient Co115 cells or those transfected with the inert TRBP-mutant form was lower (Fig. 3C and Fig. S5). The same phenomenon was found upon stable depletion of TRBP in the proficient RKO cell line: The enhancement of miRNA production upon enoxacin use was lower in RKO.shTRBP (Fig. 3C and Fig. S5). As expected, a significant down-regulation of the corresponding precursor miRNA molecules upon enoxacin use was observed only in TRBP-proficient cells (co115,TRBPWt) (Fig. S5). Thus, these results reinforce the idea that the small molecule enoxacin exerts its antiproliferative effects in cancer cells by promoting miRNA biogenesis in a TRBP-mediated manner.

Enoxacin Inhibits the Growth of Xenografted Cancer Cells by Promoting TRBP-Mediated Processing of miRNAs. By following the description above of the antiproliferative effect of enoxacin in cultured cancer cells, we translated these results to in vivo animal tumor models. We first used xenografted nude mice model for 4 weeks with daily i.p. injections of 10 mg/kg enoxacin in 5% DMSO, or saline solution in 5% DMSO. We found that the implanted colorectal cancer cell lines RKO and HCT-116 showed potential tumor-growth inhibition upon enoxacin use (Student’s t test: P = 3.12 × 10⁻⁶ and P = 2.05 × 10⁻⁶, respectively) as indicated by tumor weight (Fig. 4A) and growth (Fig. 4B). Interestingly, when the stably TRBP-depleted RKO cell line (RKO.shTRBP) was xenografted, enoxacin treatment was unable to inhibit tumor weight (Fig. 4A) and growth (Fig. 4B). The evidence of TRBP-mediated growth suppression upon enoxacin administration was reinforced by the use of the TRBP mutant cell line Co115. The weight and growth of the Co115 cell xenografts was almost unaffected by enoxacin (Fig. 4B and Fig. S6) and Co115 cells transfected with the mutant TRBP protein (Co115,TRBPmut) were equally insensitive (Fig. 4B and Fig. S6). However, Co115 TRBP-reconstituted (Co115,TRBPWt) xenografts exhibited potent growth inhibition (Student’s t test: P = 6.84 × 10⁻⁵), as reflected by tumor weight and growth upon enoxacin treatment (Fig. 4A and Fig. S6). We next conducted a pathological examination of all xenografted tumors with the different enoxacin and control treatments. We observed that all TRBP–wild-type tumor tissues, including RKO, HCT-116, and Co115,TRBPWt, showed significant necrosis upon enoxacin use (Fig. 4B). However, minimal necrosis upon enoxacin use was observed in the TRBP-deficient (Co115,TRBPmut, and RKO.shTRBP) xenografts (Fig. 4B). Most importantly, at the time of sacrifice, colon, lung, liver, and kidney tissues were resected for pathological analysis and no toxicity was detected in any of the mice used in the assay (Fig. S6). We wondered whether enoxacin also promoted miRNA processing in the described xenografted nude mice model. We found that enoxacin use significantly increased the production of the described 24 mature miRNAs only in TRBP-proficient xenografts (RKO, HCT-116, and Co115,TRBPWt), whereas the effect in impaired TRBP tumors (Co115,TRBPmut, and RKO.shTRBP) was minimal (Fig. 4C and Fig. S7). Conversely, a significant down-regulation of the corresponding precursor miRNA molecules upon enoxacin use was observed only in TRBP-proficient cells (RKO, HCT-116, and Co115,TRBPWt) (Fig. S7). Overall, these experiments confirm a role for enoxacin as a small molecule with in vivo and in vitro antiproliferative effects mediated by a TRBP-associated enhancement of miRNA production.
treatment with enoxacin significantly reduced the number of macro- and micrometastases at mice killing (Fig. 4D and Fig. S8). Thus, our results also suggest a role for enoxacin in the inhibition of tumor dissemination.

**Enoxacin Inhibits the Growth of Human Primary Colorectal Tumors Orthotopically Implanted in Mice by Promoting TRBP-Mediated miRNA Processing.** The establishment of suitable mouse models of cancer showing human-like tumor progression is essential to develop unique therapeutic approaches. In this regard, models of orthotopic implantation of primary human tumors may be more valuable for clinical validation of new drugs than pure s.c. implantation models (35). Thus, we complemented our enoxacin in vivo mouse studies by generating orthotopic models of implanted human primary colorectal tumors. On the basis of the aforementioned results about the TRBP-mediated effect of the drug, we selected a subset of colon tumors that might carry TARBP2 mutations, microsatellite unstable tumors (MSH+) (18). Seven MSI+ tumors were identified from a previously established collection of 84 human primary colorectal tumors. The seven MSI+ colorectal tumors were genetically screened for mutations in TARBP2 and one (14%) had the same TARBP2 mutation as the colorectal cancer cell line Co115: a deletion in a (C)5 repeat in exon 5 that creates a premature stop codon and truncates TRBP (Fig. 5A). The mutation was heterozygous and associated with a decrease in TRBP protein levels (Fig. 5B). When the seven MSI+ tumors were orthotopically reimplanted in the ceacum of three nude mice per patient, only three tumors grew: two TARBP2 wild-type (CRC43 and CRC56) and the TARBP2 mutant (CRC64) (Fig. 5C). When palpable intraabdominal masses for these three tumors were detected, 60 mice were randomized into two groups: a control group (n = 10 for each tumor) treated with saline solution supplied with 5% DMSO and a group (n = 10 for each tumor) treated daily by i.p. injection of a 10 mg/kg enoxacin dose over 15 d. Notably, we observed that enoxacin caused a significant reduction in tumor weight in the human primary colorectal tumors with wild-type TRBP (CRC43 and CRC56) at the time of killing, compared with the DMSO-treated ones (Student’s t test: P = 0.018) (Fig. 5D). Conversely, no significant differences in tumor growth were observed in the TRBP-mutant tumor (CRC64) upon enoxacin use (Student’s t test: P = 0.26). The careful pathological examination of the TRBP wild-type tumor tissues (CRC56 and CRC43) from enoxacin-treated animals showed significant necrosis (65% and 72%, respectively) compared with DMSO-treated mice (5% and 16%) (Student’s t test: P = 0.003 and P = 0.001, respectively) (Fig. 5E).

Tissue samples from the tumors were also collected for analysis of miRNA expression profiles. Upon enoxacin treatment, we observed increased expression of the 24 studied mature miRNAs in the TRBP+ implanted colorectal cancer orthotopic primary tumors (CRC56 and CRC43) relative to DMSO-treated mice (Student’s t test: CRC56, P = 0.012; CRC43, P = 0.023) (Fig. 5F and Fig. S9). Importantly, no significant changes in miRNA production were detected when the implanted orthotopic TRBPMut tumor (CRC46) was treated with enoxacin (Student’s t test: P = 0.78) (Fig. 5F and Fig. S9). As expected, a significant down-regulation of the corresponding precursor miRNA molecules upon enoxacin use was only observed in CRC56 and CRC43 wild-type TRBP tumors (Fig. S9). Finally, we used the expression miRNA microarray platform (9) to analyze two primary human orthotopically transplanted tumors (TRBP wild-type CRC43 and mutant CRC64). The expression profile of 731 miRNAs demonstrated that, upon enoxacin use, there was an overall up-regulation of miRNA expression levels in the TRBP wild-type tumor (CRC43) shifting the microRNAome to a more “normal colon expression profile” that clustered its miRNA transcriptome within the primary normal colon mucosa branch (four samples were used) (Fig. 5G). Among the enoxacin-up-regulated miRNAs in the CRC43 tumor with a proposed role in cancer, 74% (49 of 66) had potential tumor suppressor features (Fig. S9). The restoration of the expression of tumor-suppressor miRNAs in the tumors upon enoxacin treatment was associated with the down-regulation of their respective target oncoproteins, such as we observed for MYC (let-7a, and let-7b) and K-ras (miR-18a*, let-7a, let-7b, miR-143, and miR-205) (Fig. S9).

Conversely, we observed a minimal effect on the miRNA expression profile of the TRBP mutant CRC64 tumor upon enoxacin use: It was unable to “shift” its microRNAome to the normal colon expression signature branch (Fig. 5G). Only 10 miRNAs (1.3%) were up-regulated (Fig. S9) and the downstream target oncoproteins of the miRNAs remained unchanged (Fig. S9). These findings emphasize the central role of TRBP-mediated miRNA processing in mediating the cancer-specific growth-inhibitor effect of enoxacin reported here.

**Discussion**

Human tumors have aberrant miRNA expression profiles (microRNAomes) that occur in the context of genetic (18, 24, 36) and epigenetic (13–16) lesions in miRNA loci and the miRNA processing machinery, or associated with upstream events in transforming and growth-inhibitor genes, such as MYC (17). From a functional standpoint, some of the cancer-related miRNAs can act as oncogenes or tumor suppressors (7, 8, 31, 32), opening up the possibility of searching for drugs that might regulate miRNA expression or use artificial miRNAs as potential antitumoral agents. Most of the achievements in this area concern miRNAs with known oncogenic roles. miRNAs can be inhibited in several ways,
such as by complementary nucleic acid analogs that block the unique signature of miRNAs (antagomirs) by “base-pairing” (37). Covalent modifications of the analog inhibitor include locked nucleic acids (LNAs) 2′-O-methyl and 2′-O-methoxymethyl (26). Alternatively, it is possible to use a sponge vector expressing miRNA target sites to saturate the endogenous miRNA (38). For miRNAs with tumor-suppressor roles, fewer examples exist, a prime example being the systemic adeno-associated virus-mediated delivery of miR-26a in a hepatocellular carcinoma mouse model, which suppresses tumorigenesis (39). Similar results have recently been obtained in a mouse model of lung cancer and xenografted prostate tumors for the exogenous delivery of let-7 and miR-16, respectively (40, 41). However, if most human tumors are characterized by a defect in miRNA production and global miRNA down-regulation (5, 6, 9–11), it is tempting to propose that restoring the global microRNAome can also have a therapeutic effect. This is the same line of reasoning as for DNA demethylating agents and histone deacetylase inhibitors that, even without the existence of any target specificity, have received clinical approval for the treatment of certain hematological malignancies (42, 43). Enoxacin belongs to the family of synthetic antibacterial compounds based on a fluoroquinolone skeleton (44). Fluoroquinolones are commonly used broad-spectrum antibiotics (44) that are relatively nontoxic and inhibit type II DNA topoisomerase in mammalian cells and bacterial DNA gyrase. Enoxacin has been used to treat bacterial infections such as urinary tract infections (45). Most importantly, of 10 fluoroquinolones analyzed, enoxacin was the only one capable of enhancing RNAi activity of exogenous delivery of let-7 and miR-16, respectively (40, 41). This work was supported by Grants SAF2007-00027-9141 and SAF2008-128:683. Materials and Methods

Human cancer cell lines were obtained from the American Type Culture Collection. Total RNA was isolated by TRizol (Invitrogen). TaqMan MiRNA assays were used to quantify the levels of mature miRNAs (18). SuperScript III Platinum One-Step RT-qPCR kit (Invitrogen) was used to quantify precursor miRNAs (18). miRNA expression study by microarray analysis, protein blotting, and of focal microscopy were developed as described (18). For the in vivo nude mice xenografts, orthotopics, and lungfiver metastases experiments, 5-wk-old male nu/nu Swiss mice (Harlam) were used. Additional experimental details are provided in SI Materials and Methods.

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