Research Article

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A promising naphthoquinone [8-hydroxy-2-(2-thienylcarbonyl) naphtho[2,3-b]thiophene-4,9-dione] exerts anti-colorectal cancer activity through ferroptosis and inhibition of MAPK signaling pathway based on RNA sequencing

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Abstract: Naphthoquinones are naturally occurring metabolites with recognized anti-cancer potential but limited clinical application. This study investigated the molecular mechanism of 8-hydroxy-2-(2-thienyl)naphtho[2,3-b]thiophene-4,9-dione (1), a new candidate for colorectal cancer (CRC) treatment, using different experimental settings: MTT, clonogenic, wound healing, and cell cycle assays; as well as RNA sequencing. Naphthoquinone 1 selectively reduced the viability and migration of HT-29 cells by G2/M arrest and changes in their transcriptome signature with significant effect on cellular survival, proliferation, angiogenesis, response to interferon, oxidative stress, and immune response. Impact analysis identified ferroptosis and MAPK pathways as significantly affected. In summary, our results suggest that 1 induces the selective death of CRC cells by inducing oxidative stress, ferroptosis, and MAPK inhibition.

Keywords: naphthoquinones, HT-29, transcriptomics, pharmacological targets, molecular mechanisms

1 Introduction

Colorectal cancer (CRC) is a life-threatening disease documented as the third most commonly diagnosed malignancy and the fourth leading cause of cancer in the world. Historically, CRC has affected regions with a high human development index, such as North American and European countries, where incidence and mortality rates are currently stabilizing or decreasing; whereas trends are rising rapidly in many low-income and middle-income countries, including Central and South America [1,2]. The CRC burden is aggravated by the considerable socioeconomic challenges in these regions; therefore, improvements in treatment options and accessibility to better drugs are critical.

In this scenario, natural products have received great attention for CRC prevention and treatment due to their effectiveness, lack of toxicity, and affordability [3]. Among naturally occurring compounds, naphthoquinones (NQs) appear as attractive metabolites due to their biological and structural properties. Indeed, medicinal plants containing NQs, such as Tabebuia spp. (locally known as lapacho, pau-d’arco, roble, or guayacán), are employed in South America for their analgesic, anti-inflammatory, and anti-neoplastic properties. Moreover, since the seminal studies conducted by the National Cancer Institute (NCI-USA), nearly four decades ago, the clinical importance of NQs has stimulated enormous research interest along with the identification of natural and synthetic quinones with anti-proliferative activity toward a large number of human cancer cell lines, including melanoma, lymphoma, lung, breast, and CRC [4].

In the case of CRC, prominent bioactive NQs have been identified, including 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), 5-hydroxy-1,4-naphthoquinone (juglone), 5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone (shikonin) and β-lapachone. Despite the encouraging experimental evidence, the clinical success of NQ derivatives has been limited
mainly due to their serious and even life-threatening secondary effects [5]. Nevertheless, recent studies have demonstrated that chemical modifications to the NQ nucleus deliver new lead compounds that are both active and safe. In our previous work, it was described that the presence of a furan or thiophene ring on the naphthoquinone scaffold increases the cytotoxicity and selectivity against CRC, leading to the identification of 2-thienyl-8-hydroxynaphtho[2,3-b]thiophene-4,9-dione (1) as the most potent derivative to suppress HT-29 cells with low damaging effects against normal fibroblasts [6]. However, the molecular mechanisms underlying the cytotoxic effect of 1, as well as NQs in general, are still unclear.

In this work, we describe the effect of compound 1 on the viability, proliferation, migration, and cell cycle progression of HT-29 cells. To identify the molecular targets affected by 1, a transcriptome sequencing (RNA-sequencing) analysis was performed.

2 Materials and methods

2.1 Synthesis of test compound

The target compound 8-hydroxy-2-(2-thenoyl)naphtho[2,3-b]thiophene-4,9-dione (1) was prepared as described in ref. [7] to obtain a product with purity >95%. For experiments, stock solutions of 1 were prepared using dimethylsulfoxide (DMSO; Fisher Scientific, USA) and diluted in a complete medium when needed. The final percentage of DMSO was kept to a maximum of 0.1% (v/v).

2.2 Cell culture

HT-29 (human colorectal adenocarcinoma cell line; Cat# HTB38™; passages: 10–25), PCS-201-012 (primary dermal fibroblasts; passages: 3–8), MRC-5 (normal human fetal lung fibroblast; Cat# CCL-171; passages: 5–15), and 3T3-L1 (Mus musculus embryo fibroblasts Cat# CL-173; passages: 5–15) as cancerous or normal cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained routinely in McCoy’s 5A (HT-29), Dulbecco’s Modified Eagle’s medium (PCS-201-012 and 3T3-L1), or Eagle’s Minimal Essential Medium (MRC-5) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA or Sao Paulo, Brazil), 1.5 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), and low serum growth supplement (Invitrogen, Waltham, MA, USA) for PCS-201-012; at 37°C and 5% CO₂.

2.3 Antiproliferative assay

The effect of test compound on cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, following the procedures described in [6]. Cells were seeded in 96-well plate (1×10⁵ cells/well) and incubated at 37°C and 5% CO₂ for 24 h. After cells were treated, different concentrations of test compounds (0–40 μM) were dissolved in culture media for 48 h. Subsequently, the media was removed and replaced with MTT solution (0.25 mg/mL). At the end of 4 h, the medium was carefully dried and formazan crystals were dissolved in 100 μL of DMSO, and OD₅₅₀ nm was measured using a plate reader (Multiskan Go, Thermo Scientific, Waltham, MA, USA). The values were plotted as percent relative growth of viable cells against untreated cells considered as control and expressed as the mean ± standard error of mean (SEM) of triplicate samples from three independent experiments.

2.4 Clonogenic assay

Clonogenic assay was used to evaluate the replicative capacity of CRC cells [8]. HT-29 or MRC-5 cells (750 cells/well) were plated into a 6-well plate for 6 h, and treated with 1 (0.86 and 1.73 μM), vehicle (DMSO), or Doxorubicin (1 μM) for 48 h. Then, the medium was replaced and after 7 days of culture, the colonies were fixed (acetic acid:methanol 1:7 vol/vol), stained (0.5% crystal violet), and counted with a stereomicroscope (EZ4 HD, Leica Microsystems, Singapore). A total number of three independent experiments were performed by duplicate.

2.5 Wound healing assay

The influence of 1 upon CRC cell migration was assessed using the wound healing assay. In brief, HT-29 cells (2×10⁵ cells/well) were grown on 24-well plates until
complete confluence. Then, a sterile pipette tip was used to scratch the monolayer, the medium was replaced for FBS-free medium, and cells were treated with 1 (0.5 and 1 μM) or vehicle (DMSO). The culture was monitored with an inverted microscope (Nikon, Melville, NY, USA) equipped with a digital camera, every 24 h for 3 days. Image analysis was performed with TScratch software (http://www.cse-lab.ethz.ch) [9]. A total number of three independent experiments were performed by duplicate.

2.6 Cell cycle analysis

To assess the effect of 1 on cell cycle distribution, HT-29 cells (2 × 10⁵ cells/mL) were exposed to test compound (0.86 and 1.73 μM) or vehicle (DMSO) for 48 h and analyzed using the propidium iodide (PI) flow cytometry kit (ab139418; Abcam, Cambridge, UK) in accordance with the manufacturer’s instruction. Cell-cycle distribution was determined by flow cytometry (Dako, Beckman Coulter Inc., CA, USA). A total of three independent experiments were performed by duplicate.

2.7 Total RNA extraction and analysis

Total RNA of HT-29 cells treated with 1 (1.73 μM, 48 h) was extracted using a commercial kit (GeneJET™; Thermo Fisher Scientific, Vilnius, Lithuania). The concentration, purity, and integrity of the isolated RNA were assessed by Corporación CorpoGen (Bogotá, Colombia) using a NanoDrop 2000c spectrophotometer (Thermo Scientific), a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). The concentration ranged from 49.8 to 97.6 ng/μL; the 260/280 ratios were above 1.9; and the RNA integrity number values were above 7, except for two samples.

2.8 RNA-sequencing analysis

Library preparation and sequencing were conducted at MR DNA (www.mrdnlab.com, Shallowater, TX, USA). Libraries were prepared with total RNA (150–500 ng) using the TruSeq™ RNA LT Sample Preparation kit (Illumina Inc., San Diego, CA, USA) and validated using a Qubit® dsDNA HS Assay Kit (Life Technologies) and the Agilent 2100 Bioanalyzer system, as shown in Table 2. Then, the libraries were pooled at equimolar concentration (2 nM) and 5 pM of the normalized pool was clustered using the cBOT (Illumina Inc.). Sequencing was carried out using a 2 × 150 bp paired-end configuration on the HiSeq 2500 platform for 300 cycles (Illumina Inc.).

2.9 Bioinformatics

Initial bioinformatics analysis was performed at Corporación CorpoGen (Bogotá, Colombia). After the quality was verified with the raw FastQC reads, Trimmomatic [10] was used to remove adapter sequences, trimming low-quality ends, and filtering low-quality reads at phred quality score Q33. Clean high-quality reads were aligned to a human reference genome (GRCh38.p7) with TopHat [11]. The expression level for each transcript was calculated as fragments per kilobase of transcript per million mapped fragments (FPKM) using Cufflinks. Differentially expressed genes (DEGs) were detected with Cuffdiff [12]. Genes were considered differentially expressed using the following threshold: log2(fold change) ≥ 0.5, p-value ≤ 0.001, and false discovery rate (FDR) q-value ≤ 0.05, iPathwayGuide online software (Advaita Corporation, Plymouth, MI, USA) was used to identify significantly impacted pathways, biological processes, molecular function, cellular components, etc.

2.10 Quantitative real-time PCR (RT-qPCR)

The transcription level of 6 DEGs (SPRR1B, HSPA6, SPRR3, IFITM1, IFI44L, and OAS2) was measured to validate the RNA-sequencing analysis. For this, 1.0 μg of RNA was employed to synthesize cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Vilnius, Lithuania). RT-qPCR was performed with the LightCycler® 96 System (Roche, Mannheim, Germany) using FastStart Essential DNA Green Master (Roche) and specific primers (Table 1; Eurofins Genomics, Huntsville, USA). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or hypoxanthine phosphoribosyltransferase 1 (HPRT1) as reference genes. Duplicate cycle threshold (CT) values were analyzed by the comparative CT (∆∆CT) method.

2.11 Statistical analysis

Data were represented as a mean ± SEM. Inhibitory concentration 50 (IC₅₀) was calculated using non-linear
Data were analyzed by one-way analysis of variance (ANOVA) followed by Holm-Sidak’s multiple comparisons test. Statistical significance was considered at $P < 0.05$.

**Ethical approval:** The conducted research is not related to either human or animal use.

### 3 Results

#### 3.1 Compound 1 showed CRC-specific inhibitory activity in vitro

As we recently reported [7], the compound 8-hydroxy-2-(2-thenoyl)naphtho[2,3-b]thiophene-4,9-dione (1), exerted a selective cytotoxic effect against CRC cells (HT-29; IC$_{50}$ of $1.73 \pm 0.08\mu M$) when compared with dermal fibroblasts (PCS-201-012; IC$_{50}$ = 4.59 ± 0.48\mu M). Here, we confirmed the selectivity of compound 1 using fibroblasts from human (MRC-5; IC$_{50}$ = 6.97 ± 0.66\mu M; Figure 1a) and mouse (3T3-L1; IC$_{50}$ = 14.32 ± 2.14\mu M). Moreover, cytotoxic and non-cytotoxic concentrations of 1 (0.86 and 1.73\mu M) inhibited the clonogenic survival of HT-29 cells, while MRC-5 fibroblast remained virtually unaffected (Figure 1b). In addition, Ames test revealed that the test compound did not induce mutagenicity even at high concentrations (4.8 and 9.6\mu M, data not shown). Based on these data, the IC$_{50}$ (1.73\mu M) was selected as the effective concentration of 1, for further experiments.

To test whether compound 1 affected the migratory ability of CRC cells, the wound-healing assay was performed using a monolayer of HT-29 cells grown until

### Table 1: Sequences of primers used for real-time qPCR analysis

| Gene symbol | Gene ID | Official name                          | Primer sequences$^a$ | Tm$^b$ | Amplicon Size (pb) |
|-------------|---------|----------------------------------------|----------------------|-------|--------------------|
| GAPDH       | 2597    | Glyceraldehyde-3-phosphate dehydrogenase | F: TCGACAGTCAGCCGCATCTTCTTT 64.6°C 94 |       |                    |
|             |         |                                        | R: AACTCTACTTTGCAGAACCTCAC 64.6°C |       |                    |
| HPRT        | 3251    | Hypoxanthine phosphoribosyltransferase 1 | F: ACCAGTCAACAGGGGACATAA 58.7°C 190 |       |                    |
|             |         |                                        | R: CTTCTGTGGGGTCCTTTTACC 61.2°C |       |                    |
| HSPA6       | 3310    | Heat shock protein family A (Hsp70) member 6 | F: CATCGCCTATGGGCTG6ACGAC 59.8°C 94 |       |                    |
|             |         |                                        | R: GGAGAGAACGCACACATGCAA 58.5°C |       |                    |
| IFI44L      | 10964   | IFN induced protein 44 like             | F: ACAGAGCCAAATGATTCCCTATG 58.6°C 124 |       |                    |
|             |         |                                        | R: TCGATAAACAGCACACACAGTTG 59.9°C |       |                    |
| IFITM1      | 8519    | IFN induced transmembrane protein 1     | F: ACTCAACACTTTCTTCCCCA 59.2°C 231 |       |                    |
|             |         |                                        | R: CTTCTGTCCCTAGACTTACG 59.8°C |       |                    |
| OAS2        | 4939    | 2’-5’-oligoadenylate synthetase 2       | F: AGGGGCTTCTGAGA6CGC 60.8°C 124 |       |                    |
|             |         |                                        | R: TTATCGAGAGTGTACGGTG 58.5°C |       |                    |
| SPRR1B      | 6699    | Small proline-rich protein 1B           | F: TATCTCCTCTCTACACCA 53.8°C 155 |       |                    |
|             |         |                                        | R: TCTCTGGTTTTGGGATG 54.3°C |       |                    |
| SPRR3       | 6707    | Small proline-rich protein 3            | F: CATGAGTTCTACCAGCGAAGCAG 61.9°C 149 |       |                    |
|             |         |                                        | R: TCCAGTTGTTGGAACCTTTTGG 60.9°C |       |                    |

$^a$F: Forward (5’→3’); R: Reverse (5’→3’). $^b$Tm: melting temperature.

| Treatment | Sample | Library size (average) | Raw reads | Clean reads | Mapped reads | Mapping rate (%) | GC% |
|-----------|--------|------------------------|-----------|-------------|--------------|------------------|-----|
| Vehicle (DMSO) | 1      | 588                    | 12,526,531| 11,931,359  | 10,537,310   | 88.3             | 51  |
|            | 2      | 696                    | 14,816,823| 14,044,340  | 12,334,470   | 87.8             | 51  |
|            | 3      | 680                    | 14,596,730| 13,858,108  | 12,218,747   | 88.2             | 51  |
| Compound 1 | 4      | 476                    | 9,689,734 | 9,216,012   | 8,030,070    | 87.1             | 51  |
|            | 5      | 568                    | 12,508,590| 11,895,159  | 10,410,417   | 87.5             | 50  |
|            | 6      | 616                    | 10,433,154| 9,782,330   | 8,609,506    | 88.0             | 50  |

GC%: guanine-cytosine percentage.
confluence and scratched with a pipette tip. As shown in Figure 1c, the recovery rate of the scratched area was significantly decreased by compound 1 (0.5 and 1 \( \mu M \)) in a concentration-dependent manner, 48 and 72 h after treatment.

3.2 Anti-proliferative effect of compound 1 occurs by arresting cell cycle progression

Flow cytometry was employed to determine whether changes in the cell cycle distribution were involved in the reduction of HT-29 viability when exposed to compound 1. Figure 2 shows that treatment with 1 (1.73 \( \mu M \)) resulted in a significantly increased proportion of cells in the G2/M (2N-4N) phase, from 15.15 \( \pm \) 4.62\% (control – 0 \( \mu M \)) to 29.30 \( \pm \) 6.86\%. These results indicate that 1 inhibits CRC cell proliferation through cell cycle arrest at the G2/M phase.

3.3 Transcriptome sequencing revealed that compound 1 reduced the carcinogenic signature of HT-29 cells and promoted cellular death through ferroptosis and inhibition of MAPK signaling

To investigate how compound 1 inhibited the viability of CRC cells, a comparative RNA sequencing (RNA-seq) analysis was conducted using HT-29 cells treated with vehicle (control) or 1 (1.73 \( \mu M \)). As shown in Table 2, the
reads, mapping rates, and GC (guanine-cytosine) content were similar among the samples. After filtering differentially expressed transcripts \( (p < 0.05 \text{ and } \text{FDR}-q \text{ value } < 0.05) \), a total of 491 genes that significantly differed in HT-29 treated cells compared with control counterparts were identified (Figure 3a). Compound 1 significantly reduced the expression of 366 genes while increasing 125 genes. The transcriptomic analysis was validated by the significant correlation between the RNA-seq and RT-qPCR data [least-squares linear regression, \( p < 0.05; R^2 = 0.911 \) (GAPDH) and \( R^2 = 0.908 \) (HPRT)] when studying a selected subset of DEGs.

Gene ontology (GO) analysis was performed using iPathwayGuide online software on all DEGs. Figure 3b shows the top-10 list of GO terms related to biological process, molecular function, and cellular components, with a bar chart presenting the \( p \) values obtained from the analysis. According to the results, a significant enrichment of terms related to cancer progression (i.e., cell proliferation, cell migration, angiogenesis, cell–cell junction, cell–cell signaling, response to oxidative stress, and immune response) terms were induced by 1. The impact analysis revealed the most influenced pathways (Figure 3c), highlighting the significant enrichment of cell adhesion molecules (KEGG: 04514) and ferroptosis (KEGG: 04216) which are linked to cellular interactions (immune response, antigen recognition, and cellular adhesion) and regulated cell death, respectively.

In particular, treatment with compound 1 resulted in significant association with terms (i.e., defense response to virus) and pathways (i.e., influenza A, herpes simplex infection, human papillomavirus infection) associated with anti-viral immune response, which is a consequence of the marked reduction of interferon regulated
Simultaneously, the analysis of the 10 up-regulated genes associated with resistance to chemotherapy, tissues from CRC patients and their up-regulated genes involved in glutamate/cysteine transport (SLC3A2 and SLC7A11) and iron storage (FTH1-ferritin), which are down-regulated in CRC or dysplastic adenoma [14–17]. Alternatively, compound 1 reduced the expression of genes involved in iron efflux (SLC40A1-Ferroportin-1) and degradation of ferritin (LC3), which are interestingly overexpressed in CRC [17,18].

4 Discussion

Novel compounds that specifically and effectively target cancer cells are crucial for developing promising treatments. Molecules inspired in natural products, such as substituted naphtho[2,3-b]thiophene-4,9-diones, are proposed as leading structures with several studies and granted patents showing their strong anti-cancer effect [19,20]. In this work, we focused on the study of the 8-hydroxy-2-(2-thienyl)naphtho[2,3-b]thiophene-4,9-dione (1), previously identified as a potent anti-proliferative compound, with IC_{50} values ranging from 0.29 μM (HaCaT) to 1.78 μM (HT-29) [6,7]. Consistently, we found that treatment with 1 induced selective cytotoxicity against HT-29 cells while human and mice fibroblasts from different origins (skin, lung, and embryo) were
| Genesymbol | Description | Function | Association with CRC | LogFC | q value | Ref. |
|------------|-------------|----------|----------------------|-------|---------|------|
| IFITM1     | IFN-induced transmembrane protein 1 | IFN-induced antiviral protein. Plays a key role in the antiproliferative action of IFN-γ (inhibition of ERK activation or arresting cell growth in G1-phase trough p53) | ↑ Expression in CRC tissue that is associated with an aggressive phenotype and poor prognosis | -4.769 | 0.004 | [36,37] |
| IRI44L     | IFN-induced protein 44-like | Exhibits a low antiviral activity against hepatitis C virus | ↑ Expression in HT-29 cells resistant to oxaliplatin | -4.196 | 0.004 | [38] |
| OAS2       | 2′-5′-oligoadenylate synthase 2 | IFN-induced, dsRNA-activated antiviral enzyme | ↓ mRNA expression in high pro-migratory profile cancer-associated fibroblasts from CRC patients | -3.127 | 0.004 | [39] |
| XAF1       | XIAP-associated factor 1 | Seems to function as a negative regulator of members of the IAP (inhibitor of apoptosis protein) family | ↓ Expression in several cell lines and CRC tissue, which is associated with advance stage and high grade of tumor The restoration of XAF1 expression in CRC cells induces apoptosis and enhanced chemotherapy sensitivity | -3.059 | 0.004 | [40,41] |
| EPSTI1     | Epithelial–stromal interaction protein 1 | — | ↑ Expression in sporadic and IBD-associated CRC cell lines with knockdown expression of Nkx2-3, a transcription factor down-regulated in CRC ↓ Expression in CRC cells with strong EMT phenotype | -3.046 | 0.004 | [42,43] |
| MX2        | IFN-induced GTP-binding protein Mx2 | IFN-induced dynamin-like GTPase with potent antiviral activity | — | -2.979 | 0.004 | — |
| RSAD2      | IFN-inducible iron-sulfur (4Fe-4S) cluster-binding antiviral protein | IFN-inducible iron-sulfur (4Fe-4S) cluster-binding antiviral protein | ↑ Expression in CRC samples when compared with colorectal adenomas | -2.934 | 0.004 | [44] |
| PAX4       | Paired box protein Pax-4 | Plays an important role in the differentiation and development of pancreatic islet beta cells | ↑ mRNA levels in HCT116 cells with induced invasiveness and migration | -2.927 | 0.045 | [45] |
| FFAR2      | Free fatty acid receptor 2 | G protein-coupled receptor that is activated by the short-chain fatty acids (SCFAs) | ↑ mRNA and protein expression in CRC specimens ↓ mRNA and protein levels in human CRC tissue Flar2−/− mice are more susceptible to AOM-DSS ↑ mRNA expression of FFAR2 is detected only in HT-29 cells out of 8 CRC cell lines | -2.824 | 0.004 | [46,47] |
| CMPK2      | UMP-CMP kinase 2, mitochondrial | May participate in dUTP and dCTP synthesis in mitochondria | ↓ mRNA expression is associated with a lower CRC risk score | -2.792 | 0.004 | [48] |

LogFC: Log2 fold change.
Table 4: Top ten differentially expressed genes (up-regulated)

| Gene Symbol | Description | Function | Association with CRC | LogFC | q value | Ref. |
|-------------|-------------|----------|----------------------|-------|---------|------|
| SPRR1B      | Cornifin-B  | Cross-linked envelope protein of keratinocytes | —       | 3.541 | 0.004   |      |
|             |             | Molecular chaperone implicated in a wide variety of cellular processes | ↑ Expression when cell death was induced by electrohyperthermia using HT-29 CRC xenografts | 2.463 | 0.004   | [30] |
| HSPA6       | Heat shock 70 kDa protein 6 | Cross-linked envelope protein of keratinocytes | ↑ Expression is involved in colorectal tumorigenesis, CRC proliferation, and lymphovascular invasion | 2.438 | 0.012   | [49,50] |
| SPRR3       | Small proline-rich protein 3 | Cross-linked envelope protein of keratinocytes | ↑ Expression in CRC tissue and cell lines (i.e., HT-29) | 2.335 | 0.004   | [51,52] |
| AKAP12      | A-kinase anchor protein 12 | Anchoring protein that mediates the subcellular compartmentation of protein kinase A and C. (Tumor suppressor gene) | ↓ mRNA expression or methylation of gene promoter in CRC tissue | 2.248 | 0.009   | [53] |
|             |             |                                                   | ► Represents a potential molecular biomarker for predicting CRC malignancy |       |         |      |
|             |             |                                                   | ► The re-expression of AKAP12 induced apoptosis, reduced colony formation and migration of LoVo cells in vitro and in vivo |       |         |      |
| SPRR1A      | Cornifin-A  | Cross-linked envelope protein of keratinocytes | ↓ mRNA expression in a mouse model of CRC | 2.271 | 0.004   | [54] |
|             |             |                                                   | ↓ Expression and ↓ methylation in CRC tissue |       |         |      |
| SCEL        | Sciellin    | May function in the assembly or regulation of proteins in the cornified envelope | ↓ Expression in metastatic cell lines promoted CRC cell migration and invasion (↑Vimentin; ↓E-cadherin), while overexpression had the opposite effect (MET inducer?) | 2.171 | 0.004   | [54] |
|             |             |                                                   | ↓ Expression in CRC specimens with higher clinical stage and hepatic metastasis |       |         |      |
| DHR59       | Dehydrogenase/reductase SDR family member 9 | 3-Alpha-hydroxysteroid dehydrogenase that participates in dihydroxyprogesterone and retinoic acid biosynthesis | ↓ mRNA expression in CRC clinical samples. It might represent a useful biomarker of prognosis | 2.163 | 0.004   | [55] |
| LRRN4       | Leucine-rich repeat neuronal protein 4 | May play an important role in hippocampus-dependent long-lasting memory | Identified as a low abundance protein in CRC tissues | 2.009 | 0.004   | [56] |
| AKR1C1      | Aldo-keto reductase family 1 member C1 | Converts progesterone to its inactive form. In the liver and intestine may have a role in the transport of bile | ↓ mRNA expression at the tumor center when compared to normal mucosa | 1.659 | 0.004   | [57] |
| CST1        | Cystatin-SN | Cysteine proteinase inhibitors | ↓ mRNA levels in CRC cell lines (CaCo-2 and SW480) and affected tissue from patients | 1.611 | 0.004   | [58] |
|             |             |                                                   | ↓ Levels in urine from CRC patients |       |         |      |

LogFC: Log2 fold change.
significantly less affected. Here, we also showed that 1 had a major impact on HT-29 cells proliferation and migration, as demonstrated by the absence of colony formation and the reduction of wounded area, at toxic and non-toxic concentrations. Furthermore, this derivate triggers the arrest of cell cycle progression at G2/M phase, as supported by flow cytometry analysis.

To date, it is accepted that naphthoquinone-based compounds exert their cytotoxicity inducing oxidative stress by redox cycling of the quinone through one-electron reduction by NADPH-cytochrome P-450 oxidoreductase (CPR) to the semiquinone radical, followed by a two-electron reduction by NAD(P)H:quinone oxidoreductase 1 (NQO-1) to hydroquinone [19]. Therefore, most pharmacological studies of NQs focus on the determination of the levels of superoxide and reactive oxygen species (ROS); hence, their exact molecular mechanisms still remain largely unknown. In the case of compound 1, Bannwitz et al. reported that its anti-proliferative effect was combined with low membrane-damaging effects [7]. Furthermore, they showed that several naphtho[2,3-b]thiophene-4,9-diones are activated by CPR or NQO-1; however, their ability to induce cell death was poorly correlated with the amount of superoxide, indicating that other mechanisms are involved. In our study, we profiled the transcriptomic alterations induced by compound 1 in the CRC cell line HT-29 using RNA-seq validated with RT-qPCR. The transcriptomic analysis revealed that derivative 1 promoted the differential expression of targets crucial for cellular survival, migration, signaling, and junction; immune response; oxidative stress response, among others. The detailed analysis of the top 10 DEGs, either up- or down-regulated (Tables 3 and 4), allowed us to verify that compound 1 reduced the carcinogenic nature of CRC cells since at least 50% of the DEGs were identified as altered in the opposite direction in CRC. This observation was particularly important in the case of IRGs, which were significantly down-regulated by test compound. Although interferon (IFN) signaling has traditionally been connected with pro-apoptotic tumor-suppressor functions, emerging data have revealed that in, certain contexts, IFN-activated pathways might mediate cellular growth, metastasis, and resistance to certain therapies [21]. In the case of CRC, it is described that a feature of an irinotecan-resistant cell line is the up-regulated expression of IRGs (i.e., IFIT1, GIP3, IFI35, IFITM1, OAS1, and IFIT3) [22].

Moreover, the impact analysis of the altered pathways added important information regarding the molecular mechanism underlying the cytotoxic effect of 1. This analysis evidenced that compound 1 influenced several signaling pathways that promoted cellular death (ferroptosis) or cellular proliferation and survival (MAPK and PI3K-AKT signaling pathways). Ferroptosis is a type of regulated cell death caused by the accumulation of iron-dependent lipid peroxides that are generated during excessive ROS production [23], which is triggered by (a) inhibitors of the cystine/glutamate antiporter (system Xc\(^-\) SLC3A2/SLC7A11) that reduce the synthesis of glutathione content causing oxidative damage; or (b) inhibitors of glutathione peroxidase 4 (GPx4) leading to uncontrolled lipid peroxidation [23]. From our transcriptomic analysis, several events might be causing the oxidative death induced by 1: (1) accumulation of intracellular iron content via increasing ferritin (FTH1) and decreasing ferroportin-1 (SLC40A1) and LC3 (MAP1LC3A) expression; (2) elevation of ROS (redox cycling) along with depletion of intracellular glutathione, which in turn leads to the observed up-regulation of glutamate cysteine ligase (GLC); or (3) decrease in Xc\(^-\) transport function, either by direct or indirect blockage, which is expected to decrease the levels of glutathione, which in turn will induce the expression of SLC7A11 and SLC3A2 (Figure 4). Furthermore, compound 1 also regulated the expression of several heat shock proteins (HSPs), which are involved in signaling pathways that affect iron metabolism and ferroptosis. Specifically, treatment with derivative 1 significantly reduces the expression of HSPB1 (also known as HSP-27), whose levels are often elevated and associated with chemotherapy resistance and worse clinical outcome in CRC [24]. Interestingly, a protective effect against oxidative stress by reducing iron uptake has been described for HSPB1 [23]; together with the observation that its inhibition induced the accumulation of intracellular iron leading to lipid peroxidation and injury of cancer cells when ferroptosis was induced by erastin, whereas the overexpression of HSPB1 inhibited this effect [25]. Alternatively, compound 1 up-regulated the expression of HSPA6 (HSP70B) and HSPH1 (HSP110 or HSP105), molecular chaperones which are known for improving cell survival during extreme stress. Although higher expression of HSP is not considered favorable for CRC patients [26]; it has been demonstrated that the expression of HSPH1 and HSPA6, might favor immune recognition of tumors [27,28] or inhibit the proliferation, migration, and invasion of cancer cells by cytotoxic treatments [29,30], respectively.

Another significantly impacted pathway by 1 was the mitogen-activated protein kinase (MAPK) pathway, including ERK, p38, and c-Jun NH\(_2\)-terminal kinase
JNK, which is recognized for controlling cellular responses to the environment and regulate cell cycle, differentiation, growth, and cell senescence, all of which are critical for cancer initiation and progression [31]. Accumulating evidence has demonstrated that naphthoquinone derivatives modulate the MAPK pathway; however, studies are conflicting since some show that cell death is induced by activation of ERK, p38, and/or JNK [32,33], while others demonstrate the opposite [34]; suggesting that MAPK regulation is dependent on the compound structure or the cellular context. In HT-29 cells, we found that compound 1 significantly reduced the expression of ERK, which might explain the inhibition of cell proliferation and migration. In addition, perturbation analysis indicated that activation of JNK and p38 might also be affected by 1, which could enhance the cytotoxic effect of compound 1. Similarly, compound 1 showed a significant impact on PI3K-AKT signaling pathway that could be explained since some molecular targets are shared with the Ras/Raf/MEK/ERK pathway, highlighted as significantly impacted in the perturbation analysis. Interestingly, a recent study demonstrated a common inhibition of ERK, AKT, and STAT3 signaling pathways when cancer cells were treated with thiolated naphthoquinone derivatives [35].

Our experimental results demonstrated the value of transcriptomic sequencing as a tool to discover the molecular targets underlying the anti-proliferative effect of naphtho[2,3-b]thiophene-4,9-diones, showing that the induction ferroptosis and the inhibition of MAPK and PI3K-AKT pathways are certainly involved in the cytotoxicity promoted by 1. However, our study is limited by the lack of supporting evidence employing other molecular assays (i.e., MTT assay in the presence of ferroptosis inhibitors or western blot for ERK phosphorylation); therefore, additional studies should be performed to find the exact mechanism through which compound 1 induces cellular death and the interconnection between oxidative stress, ferroptosis, and MAPK (ERK)/PI3K-AKT activation.

In conclusion, this study demonstrated that treatment with 8-hydroxy-2-(2-thenoyl)naphtho[2,3-b]thiophene-4,9-dione (1) inhibited selectively the viability, proliferation, and migration of HT-29 CRC cell line by inducing cell cycle arrest, ferroptosis, and inhibiting the MAPK pathway, affecting specifically the activation of ERK with the consequent perturbation of the common downstream targets in the PI3K-AKT pathway. The identification of the affected molecular targets was performed through RNA-seq, confirming the utility of

**Figure 4:** Compound 1 significantly impacts the expression of targets within ferroptosis pathway. iPathwayGuide online software was used to perform the impact analysis. (a) The difference in mRNA expression between HT-29 cells treated with compound 1 (1.73 μM) or vehicle (control) are shown with a heat map at the top left displayed as Log2 fold change (LogFC). Blue indicates down-regulated genes and red indicates up-regulated genes (log2 |fold change| ≥ 0.5, p-value ≤ 0.001, and q-value ≤ 0.05).
transcriptomic analysis to study naphthoquinone-based compounds to identify novel therapeutic targets. Indeed, this is the first report of the induction of ferroptosis, an oxidative type of cell death, by naphthoquinone-based compounds, suggesting that their targeting on oxidative stress might be linked to reprogramming of iron metabolism as an interesting mechanism for CRC therapy. Further study is necessary to elucidate the specific link between oxidative stress–ferroptosis–MAPK signaling when cell death is induced by 1, as well as the efficacy of this leading compound using in vivo models.

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Author contributions: LF conceived the study; LF and YO supervised the study and designed experiments; KM synthesized compound 1; DC, DR, and YO performed the experiments; YO and LF wrote the manuscript. All authors read and approved the final version of the manuscript.

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