

## SURVEY AND SUMMARY

# A novel view on an old drug, 5-fluorouracil: an unexpected RNA modifier with intriguing impact on cancer cell fate

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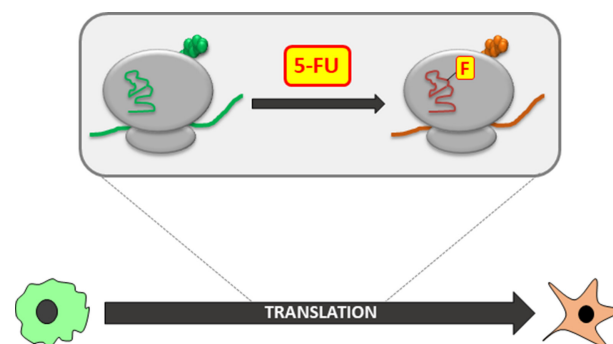
Received March 18, 2021; Revised July 01, 2021; Editorial Decision July 01, 2021; Accepted August 09, 2021

### ABSTRACT

5-Fluorouracil (5-FU) is a chemotherapeutic drug widely used to treat patients with solid tumours, such as colorectal and pancreatic cancers. Colorectal cancer (CRC) is the second leading cause of cancer-related death and half of patients experience tumour recurrence. Used for over 60 years, 5-FU was long thought to exert its cytotoxic effects by altering DNA metabolism. However, 5-FU mode of action is more complex than previously anticipated since 5-FU is an extrinsic source of RNA modifications through its ability to be incorporated into most classes of RNA. In particular, a recent report highlighted that, by its integration into the most abundant RNA, namely ribosomal RNA (rRNA), 5-FU creates fluorinated active ribosomes and induces translational reprogramming. Here, we review the historical knowledge of 5-FU mode of action and discuss progress in the field of 5-FU-induced RNA modifications. The case of rRNA, the essential component of ribosome and translational activity, and the plasticity of which was recently associated with cancer, is highlighted. We

propose that translational reprogramming, induced by 5-FU integration in ribosomes, contributes to 5-FU-driven cell plasticity and ultimately to relapse.

### GRAPHICAL ABSTRACT



### INTRODUCTION

Based on the observation that tumoral tissues used the uracil more rapidly than normal tissues (1,2), 5-Fluorouracil (5-FU) was identified as an antimetabolite chemotherapy as early as 1957 and was approved by the

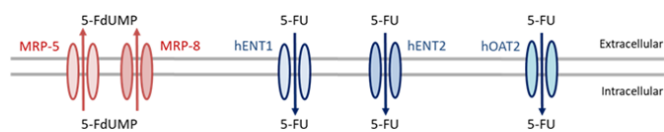
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FDA in 1962 for the treatment of colorectal cancer (CRC) (3). Since its first approval, 5-FU has been widely used either alone or in combination with other drugs to treat many solid cancers of digestive origin (colorectal, anal, pancreatic, oesophageal, gastric and ampullary tumours) and those arising in other organs (i.e. breast, cervix, and head and neck cancers). Today, 5-FU is used to treat several of the deadliest cancers, including CRC and pancreatic ductal adenocarcinoma (PDAC) (Table 1). Even though treatment modalities (dose, timing and administration) vary according to the origin of the tumour, 5-FU remains one of the essential drugs used for cancer management (4).

Inclusion of 5-FU into the regimen used to treat CRC, the second most common cause of cancer-related deaths in western countries, is mandatory. The annual incidence and mortality rates of CRC exceed one and half a million of cases worldwide, respectively (5,6). 5-FU is used as the first-line treatment for CRC patients. However, the rate of response to 5-FU does not exceed 40–60% when 5-FU is used in combination with other molecules such as folinic acid, irinotecan or oxaliplatin, or with targeted therapies such as those based on anti-vascular endothelial growth factor (VEGF) (bevacizumab) or anti-epidermal growth factor (EGFR) (cetuximab) antibodies (7,8). Nevertheless, between 20% and 25% of patients with stage II or stage III cancers do not respond to protocols containing 5-FU, and in these cases, patient overall survival only reaches 50% within 5 years following CRC diagnosis (9) (Table 1). Hence, CRC represents an opportunity for biomedical programs that aim at investigating the mode of action of 5-FU and its secondary effects, as well as resistance mechanisms.

PDAC represents another major type of cancer in which 5-FU treatment has received considerable attention. Indeed, not only 5-FU is administered to patients with resectable pancreatic adenocarcinoma, but it also constitutes the first-line treatment for most PDAC patients. 5-FU is mainly given in association with other systemic agents (e.g. folinic acid, oxaliplatin, irinotecan or gemcitabine), but it can also be combined with radiotherapy or surgery (10). PDAC has fatal outcome in most cases with a 5-year survival rate of only 9%, and an annual estimated death rate of 430 000 worldwide. Moreover, its mortality-to-incidence ratio is close to 1 (Table 1), meaning that the number of patients diagnosed and dying from PDAC each year is almost the same (11,12). Because its incidence has increased by 55% over the last 25 years, PDAC is expected to be the second most frequent cause of cancer-related deaths over the next 10 years (13,14). Consequently, the number of patients suffering from PDAC and exposed to 5-FU will continue to increase in the future. Although several studies have addressed the mechanisms of resistance to 5-FU in PDAC (15,16), further investigation into this resistance is necessary to ensure better patient management.

As described in Table 1, a variety of cancers, aside from CRC and PDAC, are currently treated with 5-FU, generally in combination with systemic and targeted chemotherapy, radiotherapy and/or surgery. However, for such locations, data on resistance mechanisms are even scarcer than for digestive adenocarcinomas (17,18). It is, therefore, crucial to improve the understanding of the mode of action of 5-FU to identify the molecular mechanisms that may be involved



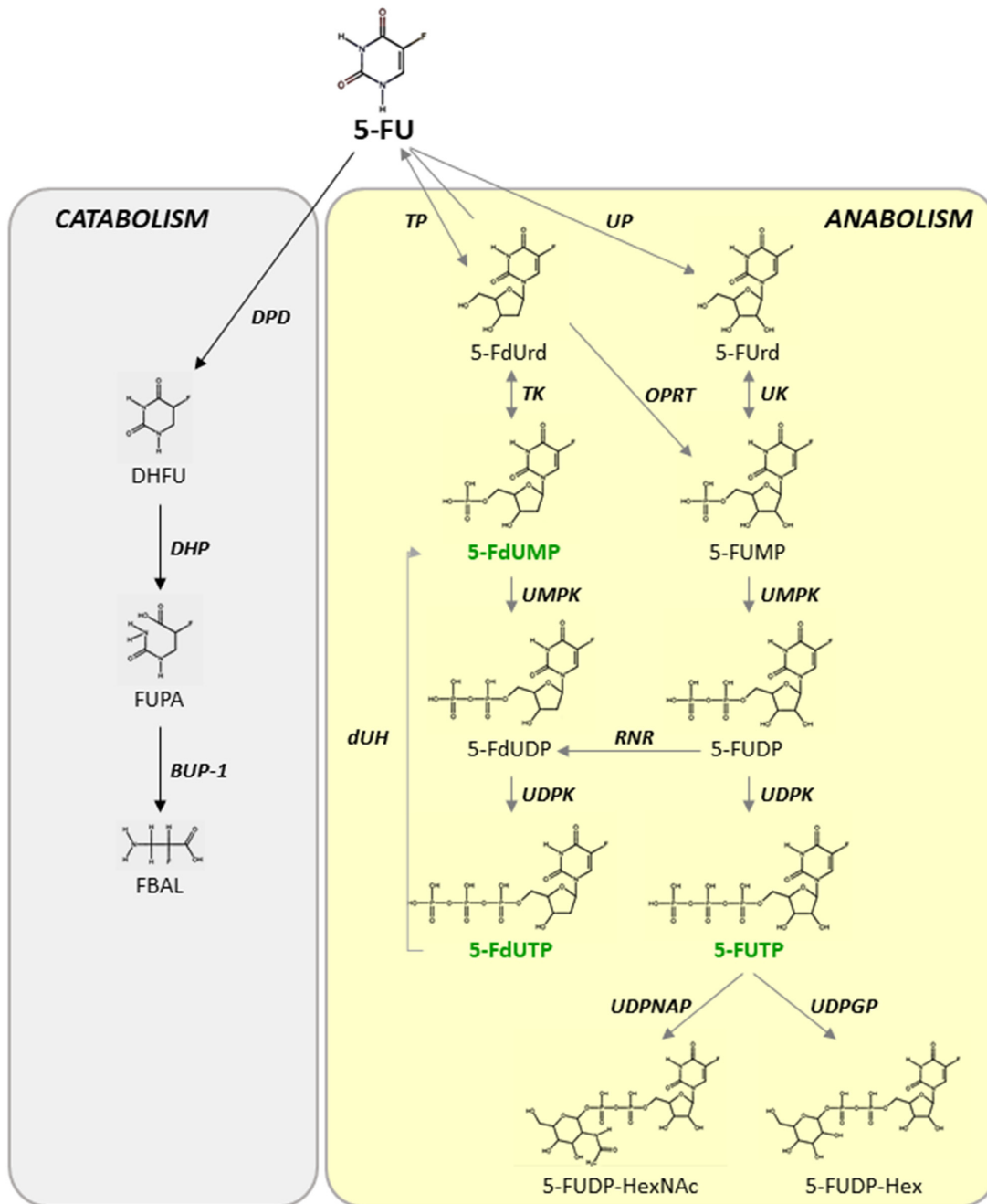
**Figure 1.** Overview of the uptake and efflux of 5-FU in human cells. Uptake (blue) of 5-FU is controlled by the human equilibrative nucleoside transporters hENT1 and hENT2 and by the organic anionic transporter hOAT2. Efflux (red) of 5-fluorodeoxyuridine monophosphate (5-FdUMP), a 5-FU metabolite, is mediated by the multidrug resistance-associated proteins MRP-5 and MRP-8.

in long-term 5-FU based treatment failure (recurrence and metastasis) (19) and to optimize the use of 5-FU for the management of patients with advanced digestive or extra-digestive cancers.

To exert its cytotoxic effect, 5-FU has to reach the tumour site, enter into cells, and be phosphorylated into its three active metabolites that are 5-fluorodeoxyuridine monophosphate (5-FdUMP), 5-fluorodeoxyuridine triphosphate (5-FdUTP) and 5-fluorouridine triphosphate (5-FUTP). Thus, 5-FU follows multistep pathways from transport into the cell to complete metabolism.

5-FU enters into human cells using several types of transmembrane proteins known as human nucleoside transporters (hNT). The hNT include three human concentrative nucleoside transporters (hCNT1, 2 and 3) and four human equilibrative nucleoside transporters proteins (hENT1, 2, 3 and 4) (20). While uridine uptake is performed by all hNTs cited above except hENT4 (20,21), 5-FU transport is achieved by hENT1 and hENT2 only (22–24) (Figure 1). In addition, uptake of 5-FU by organic anionic transporter 2 (hOAT2) has been reported. hOAT2 is highly expressed in the liver and kidney proximal tubules and may be responsible, at least in part, for the hepatic uptake of 5-FU (25–27). Finally, passive transport of 5-FU has also been demonstrated through paracellular and transcellular routes (28). Multidrug resistance-associated protein-5 (MRP-5) and MRP-8, two organic anion transporters ubiquitously expressed in tissues, mediate the efflux of 5-FU through the efflux of 5-FdUMP (29–31).

After administration in patients, approximately 80% of 5-FU is catabolized in the liver into pharmacologically inactive metabolites, 5–20% are excreted in the urine, and only 1–3% contribute to the anabolism pathway responsible for clinical and cytotoxic effects (32). The effects of 5-FU are strongly dependent on the balance between anabolism and catabolism, and therefore, on the various factors involved in these two phases, such as the substrate concentration, the level of enzymes in the various tissues and their enzymatic activities. It is important to emphasize that the substitution of a hydrogen atom with a fluorine atom modifies the conformation of 5-FU only slightly in comparison to uracil and, therefore, the cellular enzymes can metabolize the two substrates indistinctively (33). 5-FU catabolism in the human body is particularly rapid, since the half-life of 5-FU is <20 min (34). 5-FU is mainly degraded by reductive pathways in three steps, leading to pharmacologically inactive metabolites (35,36) (Figure 2). The three enzymes dihydropyrimidine dehydrogenase (DPD), dihydropyrimidinase (DHP) and  $\beta$ -ureidopropionase (BUP-1)



**Figure 2.** Overview of the anabolism and catabolism of 5-FU in human cells. The balance between anabolism and catabolism is crucial for the effect of 5-FU on cells. Catabolism is the most rapid process through which 5-FU is degraded in three steps. Anabolism leads to the production of three active metabolites (green): 5-fluorodeoxyuridine monophosphate (5-FdUMP), 5-fluorodeoxyuridine triphosphate (5-FdUTP) and 5-fluorouridine triphosphate (5-FUTP); 5-FdUrd, 5-fluorodeoxyuridine; 5-FdUDP, 5-fluorodeoxyuridine diphosphate; 5-FUrd, 5-fluorouridine; 5-FUMP, 5-fluorouridine monophosphate; 5-FUDP, 5-fluorouridine diphosphate; 5-FUDP-HexNAc, 5-FUDP-N-acetylhexosamine; 5-FUDP-Hex, 5-FUDP-hexose; DHFU, dihydrofluorouracil; FUPA,  $\alpha$ -fluoro- $\beta$ -ureidopropionic acid; FBAL,  $\alpha$ -fluoro- $\beta$ -alanine; TP, thymidine phosphorylase; TK, thymidine kinase; UMPK, UMP kinase; UDPK, UDP kinase; dUH, dUTP hydrolase; UP, uridine phosphorylase; UK, uridine kinase; OPRT, orotate phosphoribosyltransferase; RNR, ribonucleotide reductase; UDPNAP, UDP-N-acetylhexosamine-pyrophosphorylase; UDPGP, UDP-glucose-pyrophosphorylase; DPD, dihydropyrimidine dehydrogenase; DHP, dihydropyrimidinase; BUP-1,  $\beta$ -ureidopropionase. The structural formulas of 5-FU and its metabolites were drawn using the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/edit3/index.html>).

**Table 1.** Most frequent cancers treated with 5-Fluorouracil (5-FU) worldwide in 2018. The top 2 of each column are presented in bold (5,6) – (<https://seer.cancer.gov> - <https://gco.iarc.fr/today/home>)

Most frequent cancer site treated with 5-FU	Cancer subtype treated with chemotherapy including 5-FU	5-year survival (%)	Ranking by Mortality	Mortality/ Incidence (%)	5-Year Prevalence
Colorectal and anal carcinoma	High risk stage II, stage III (locally advanced) and stage IV (metastatic)	64	4	48	<b>4,789,635</b>
Pancreatic adenocarcinoma	Every stage	<b>9</b>	9	<b>94</b>	282,574
Esophageal carcinoma	Every stage except some stage IA	20	6	65	446,522
Gastric adenocarcinoma	Every stage except some stage IA	32	<b>3</b>	51	1,175,507
Hepatocellular carcinoma	Advanced multifocal hepatocellular carcinoma	<b>18</b>	<b>2</b>	<b>70</b>	565,028
Cholangiocarcinoma	Stage IV cholangiocarcinoma	19	17	44	175,086
Cervical carcinoma	Stage IB3 to stage IVA	66	8	55	1,474,265
Breast carcinoma	Stage II to stage IV and some aggressive stage I triple negative carcinoma	91	5	30	<b>6,875,099</b>
Head and Neck carcinoma	Stage II to IV	58 to 65	7	42	1,842,306

are also involved in the degradation of endogenous uracil and thymine (35). Due to its central role in the catabolism of 5-FU and the existence of a genetic polymorphism, DPD has been extensively studied for its role in the efficacy as well as in the toxicity of 5-FU (37). 5-FU anabolism comprises two pathways to produce three active metabolites: 5-FdUMP, 5-FdUTP and 5-FUTP (7,38) (Figure 2). The main pathway converts 5-FU to 5-fluorouridine monophosphate (5-FUMP) either directly by orotate phosphoribosyltransferase (OPRT) or indirectly via 5-fluorouridine (5-FUrd) using uridine phosphorylase (UP) and uridine kinase (UK). An alternative pathway involves the conversion of 5-FU into 5-fluorodeoxyuridine (5-FdUrd) and then into 5-FdUMP.

From all this knowledge about the usage of 5-FU for cancer treatments and of its metabolism, in this review, we point out how integration of 5-FU into RNA and particularly into ribosomal RNA (rRNA) may provide novel mechanistic features that need to be taken into account in order to identify more precisely the limits of this drug that is still widely used. First, after an historical review of data regarding the integration of 5-FU into DNA, we expose the intrinsic ability of 5-FU to incorporate into RNA molecules including rRNA. Second, we uncover how, from recent improvements of analytical technologies, the concept of the F-ribosome emerged. Finally, we discuss how F-ribosomes could constitute a breakthrough for understanding 5-FU resistance and thus, how this innovative concept might help in improving patient management in the future.

## BACKGROUND AND NOVELTIES OF 5-FU INTEGRATION INTO RNA, A REVISITED INTERPLAY

As described above, in cells, 5-FU is converted into active metabolites, namely 5-FdUMP, 5-FdUTP and 5-FUTP. While the first two metabolites have long been associated with some of the cytotoxic effects caused by 5-FU, it is only recently that it has been accepted that 5-FUTP, through its integration into RNA, contributes intensely to 5-FU's anti-proliferative activity. In this section, after a brief overview of the impact of 5-FU on DNA metabolism, we focus on findings linking 5-FU and RNA. We present data demonstrating that 5-FU incorporation into RNA is associated with anti-proliferative effects. Then, we discuss the alteration of

RNA features induced by 5-FU integration in RNA. Finally, we highlight the most abundant RNA species, namely ribosomal RNA (rRNA), which provides promising future avenues.

## Cytotoxic effects of 5-FU due to modifications of DNA metabolism

5-FU was first used in clinic due to the anti-proliferative activity of two of its metabolites, 5-FdUMP and 5-FdUTP, through their deleterious effects on DNA (reviewed in (39)).

One of the impacts of 5-FU on DNA relies on its capacity to induce DNA damage. 5-FdUTP is a substrate for DNA polymerases and is incorporated into DNA. Following 5-FU treatment, the increased concentrations of dUTP and 5-FdUTP overwhelm dUTPase, which hydrolyses dUTP to dUMP. As a consequence, dUTP and 5-FdUTP can be misincorporated into DNA instead of dTTP, causing DNA damage (7). While the incorporation of 5-FU into DNA has largely been demonstrated in human tumour cells (40–43), mouse models such as the murine mammary carcinoma model (44), and CRC patients (45), its contribution to cytotoxicity is not compelling.

Another deleterious effect of 5-FU relies on its capacity to arrest DNA replication, notably via 5-FdUMP, which inhibits thymidylate synthase (TS). TS is an enzyme that acts as the *de novo* source of thymidine by reducing one carbon to a methyl group of dUMP to produce dTMP, which is further phosphorylated to form dTTP. Thymidine is necessary for DNA replication and repair. By interacting with the nucleotide-binding site of TS, 5-FdUMP inhibits binding of the normal substrate dUMP and, consequently, inhibits dTMP synthesis, resulting in abnormally low levels of dTTP and a massive increase in the level of dUTP, which is responsible for DNA replication arrest (7,19).

Finally, to correct 5-FU-induced errors that occur during replication, DNA repair mechanisms take place and contribute to 5-FU cytotoxicity. For example, thymidine DNA glycosylase (TDG) can excise incorporated 5-FU through the base excision repair (BER) mechanism, resulting in DNA strand break accumulation and cell death (46). Another DNA repair mechanism, DNA mismatch repair (MMR), has also been reported to influence 5-FU cytotoxicity in CRC cells (47). A recent study on human colon and

ovarian cancer cells demonstrated that the initiation of the BER mechanism by UNG enzymes is the main mechanism of 5-FU-associated DNA repair that sensitizes cells to 5-FU (48). Overall, the partial efficacy of the repair mechanism, mainly because of 5-FU-induced depletion of the dTTP nucleotide pools, contributes to 5-FU cytotoxicity. In addition, 5-FU excised from DNA might increase the intracellular concentration of the drug and thus favour a cytotoxic effect (47).

Altogether, these studies reveal that 5-FU dysregulates a variety of DNA-based mechanisms, namely the inhibition of TS activity, mis-incorporation into DNA and defective DNA repair. While these types of dysregulation undoubtedly contribute to the cytotoxic effects of 5-FU (49), a growing body of evidence suggests that 5-FU cytotoxicity is also a substantial consequence of defects in RNA metabolism.

### Integration of 5-FU into RNA is implicated in 5-FU driven cytotoxicity

By using approaches that rely on the quantification of radioactively labelled 5-FU metabolites and competition with unlabelled metabolites, a number of studies provided evidence of the incorporation of 5-FU into RNA and unveiled its implication in the 5-FU-driven anti-proliferative effects.

*In vivo* quantification of labelled 5-FU metabolites conducted in mice injected intraperitoneally with solutions of [<sup>3</sup>H]-labelled fluorinated pyrimidines showed that incorporation of 5-FU into RNA accounts for gastrointestinal toxicity in mice (50). Experiments conducted in cell lines using exponentially growing mammary epithelial MCF7 cells treated with [<sup>3</sup>H]5-FU confirmed, through total RNA extraction and nucleoside analysis by high pressure liquid ion exchange chromatography, that incorporation of 5-FU into RNA induces toxicity. Indeed correlation was established between the loss of clonogenic survival of mammary epithelial MCF7 cells and the incorporation of 5-FU into RNA (51).

Further evidence of 5-FU RNA integration driven toxicity has been unveiled using various models, such as mice, yeast and human cell lines. In mice treated with 5-FU, microscopic observations of small intestinal and crypt epithelial cells showed that 5-FU induces apoptosis. Interestingly, administration of 5-FU with uridine to relieve the incorporation of 5-FU into RNA was shown to reduce 5-FU-induced apoptosis in crypt epithelial cells. Moreover, uridine, but not thymidine, was able to inhibit the cytotoxicity of 5-FU observed in the intestinal cells of mice treated with 5-FU regimens (52). In a model of yeast grown in the presence of 5-FU, supplementation of the cultures with UMP reversed the inhibitory effect of 5-FU on cell growth, while the addition of dTMP only showed a slight effect, thus demonstrating the prevalence of RNA-based over a DNA-based toxicity (53). In cell lines, DNA and RNA were isolated from cells treated with 5-FU and hydrolysed to nucleosides. 5-FUrd and 5-FdUrd were quantified by liquid chromatography coupled to mass spectrometry. The incorporation of 5-FU into RNA was up to 15 000-fold higher than that into DNA. Moreover, for a panel of human cell lines, media complementation with uridine, which restored normal RNA metabolism through the reduction of 5-FU in-

corporation into RNA, was shown to compensate for most of the 5-FU-induced cytotoxic effects (54).

Thus, despite the fact that 5-FU was initially described for its deleterious action on the synthesis and integrity of DNA, numerous studies have clearly established that 5-FU cytotoxicity is mainly associated with its incorporation into RNA. However, methodologies used at the time in which these studies were performed assessed the 5-FU integration into the bulk of cellular RNA, without delineating every RNA species.

### Integration of 5-FU into RNA may affect RNA structural and functional features

Because RNA is central to 5-FU toxicity, several studies have explored the consequences of 5-FU integration on the molecular features of different RNA species including messenger RNA (mRNA), transfer RNA (tRNA) and small nuclear RNA (snRNA).

It has been reported that 5-FU impacts mRNA splicing. Indeed, an *in vitro* experiment using  $\beta$ -globin mRNA minigene constructs showed that integration of 5-FU alters pre-mRNA splicing (55). In yeast, transcriptome modifications induced by 5-FU were analysed by high-density DNA microarray technology. Global disruption of pre-mRNA splicing characterized by intron retention was found to affect numerous mRNAs (56). However, these data were challenged by Zhao *et al* (57). These authors revealed that the defect in mRNA splicing does not originate from the incorporation of 5-FU into mRNA since fluorinated mRNA synthesized *in vitro* and injected into *Xenopus* oocytes was not found to be aberrantly spliced. Instead, the incorporation of 5-FU into the spliceosomal U2 snRNA at pseudouridylated sites was shown to inhibit U2 snRNA pseudouridylation and subsequent pre-mRNA splicing (57). Integration of 5-FU not only affects snRNA pseudouridylation but also affects its structure and turnover. Indeed, in sarcoma murine cells, the comparison of electrophoretic migration of snRNA in non-denaturing and denaturing polyacrylamide gels showed that 5-FU alters the structure of U4 and U6 snRNAs, and a reduced turnover of the U1 snRNA was observed in cells treated with 5-FU (58). The impact of 5-FU on mRNA translational efficiency has also been investigated. *In vitro* translation assays using total mRNA or the purified dihydrofolate reductase mRNA isolated from cells grown in presence of 5-FU revealed no major impact of 5-FU (59,60). Moreover *in vitro* translation of the synthetic mRNA encoding TS and containing substitution of 100% of the uracil bases with 5-FU, confirmed that the integration of 5-FU into mRNA does not affect its translation (61).

Regarding tRNA, integration of 5-FU was also shown to affect post-transcriptional RNA modifications, including formation of pseudouridine ( $\psi$ ) and methylation (62,63). *In vitro* binding assays between *in vitro* synthesized 5-FU substituted tRNA (5-FU-tRNA) and yeast purified pseudouridine synthase revealed that 5-FU-tRNA interacts with the enzyme at its catalytic site. Using the release of radioactivity from a tritium labeled pseudouridine synthase RNA substrate as a measure of the enzyme activity, it was further shown that 5-FU-tRNA limited tritium release, indicative

of an inhibition of the enzyme activity (62,63). The interaction between 5-FU-tRNA and the catalytic site of the pseudouridine synthase was later shown to be a covalent complex (64). *In vitro* methylation assay of tRNA by tRNA methyltransferases purified from mice liver and tumours showed that 5-FU inhibits specifically the tRNA uracil-5-methyltransferase (62,63).

More recently, the effect of exposure to 5-FU on the expression of miRNAs and lncRNA was investigated. miRNA microarray technology revealed that 5-FU promotes profound transcriptional reprogramming, leading to altered expression of miRNA in human breast and colorectal cancer cells (65–67). Lately, the role of miRNAs and lncRNAs in 5-FU resistance of CRC was intensively reviewed (68–70). Nevertheless, the depicted underlying mechanism through which 5-FU may impact these non-coding RNAs relies on the capability of 5-FU to alter their expression, leaving the potential 5-FU incorporation into miRNA and lncRNA still undocumented.

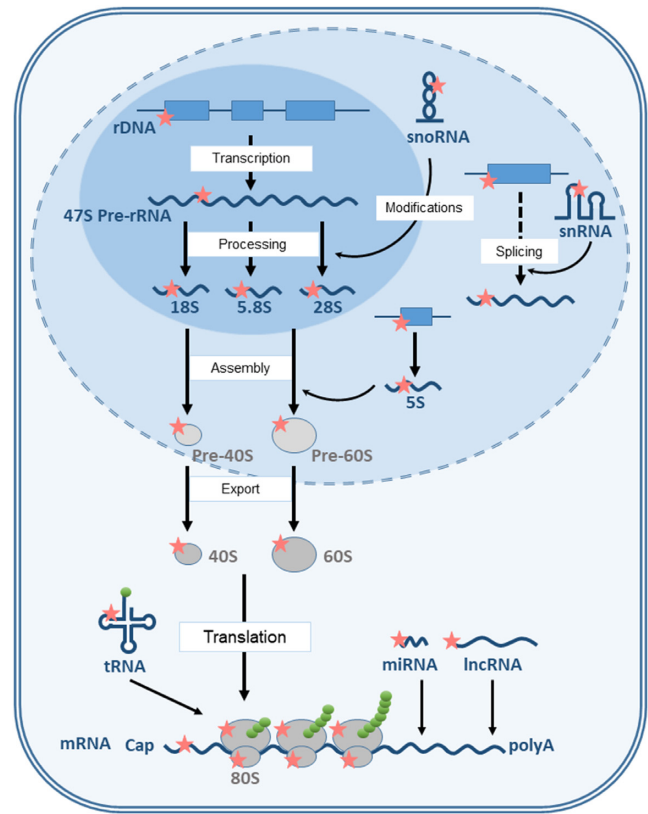
Thus, these results demonstrate that 5-FU is incorporated into several RNA species (Figure 3). Whether this incorporation affects their features remains to be consolidated and extended to all RNA species in the future. Nevertheless, how 5-FU-induced alteration of RNA metabolism impacts cell responses and behaviours or toxicity remains largely unknown.

### 5-FU is incorporated into rRNA and affects ribosome biogenesis (RiBi)

In cells, rRNA is the most abundant RNA species making it the easiest RNA to be technologically apprehended. Thus, for over 50 years, interest in the impact of 5-FU integration into rRNA emerged, although findings remained limited in terms of molecular and phenotypic consequences. Using bacterial models, numerous studies have reported that 5-FU affects ribosome synthesis. Although ribosome biogenesis (RiBi) in bacterial cells differs from that in mammalian cells (71), interest in the impact of 5-FU on mammalian ribosome production has been evaluated given the use of 5-FU as a potent chemotherapy.

RiBi is one of the most energy-consuming processes in the cell that requires hundreds of factors. Synthesis of the different ribosomal components also involves the three RNA Polymerases (RNA Pol) in humans. RNA Pol I is responsible for the synthesis of three of the four rRNAs (28S, 18S and 5.8S). RNA Pol III synthesizes 5S rRNA, and RNA Pol II synthesizes mRNAs encoding for ribosomal proteins (RP). The biogenesis of ribosomes is thus a multi-step process, the limiting step of which is the transcription of rDNA genes by RNA Pol I, which generates a precursor ribosomal RNA (pre-rRNA): the 47S. This pre-rRNA contains the sequences of 18S, 5.8S and 28S rRNAs, separated by internal transcribed spacer sequences (ITS) and surrounded by external transcribed spacer sequences (ETS). This pre-rRNA is cleaved at both ends, generating a subsequent pre-rRNA: 45S. This pre-rRNA is then sequentially processed to produce mature 5.8S, 18S and 28S rRNAs (72).

By analysing the impact of several concentrations of 5-FU on the incorporation of [<sup>3</sup>H]uracil into RNA of rat hepatoma cells, Wilkinson *et al.* showed that 5-FU integra-



**Figure 3.** Overview of all RNA that potentially incorporate 5-FU, with a focus on Ribosome Biogenesis (RiBi). RiBi requires the initial transcription of rDNA genes to a single precursor of ribosomal RNA (pre-rRNA), 47S, in the nucleolus, whereas 5S rRNA is transcribed in the nucleoplasm. 47S is subsequently cleaved in several pre-rRNA to ultimately provide 18S, 5.8S and 28S rRNAs. During this processing step, rRNAs are post-transcriptionally modified by protein-processing complexes including fibrillarin (FBL) complexed with C/D box snoRNAs and DKC1 complexed with H/AHA box snoRNAs, which are responsible for 2'-O-methylation and pseudouridylation modifications, respectively. RiBi also requires the transcription of mRNAs encoding ribosomal proteins (RP, not shown here), which, after being translated in the cytoplasm and imported into the nucleus, are assembled into small pre-40S (18S and 32 RPS) and large pre-60S (5S+5.8S+28S and 47 RPL) ribosomal subunits. The two mature subunits are then exported into the cytoplasm and assembled into mature ribosomes (80S), ready to achieve translation with mRNA and tRNA. The extrinsic RNA epitranscriptome created by 5-FU (red star) enforces the expansion of the DNA-based initial view of the deleterious effect of 5-FU on phenotype.

tion does not inhibit the synthesis of 45S pre-rRNA, but rather affected its maturation (73,74). Indeed, after the electrophoresis of RNA on polyacrylamide-agarose gels, gels were scanned at 260 nm and sliced for [<sup>3</sup>H] radioactivity counting. The amount of [<sup>3</sup>H] labelled 45S pre-rRNA increased compared with the amount of downstream [<sup>3</sup>H] labelled 38S intermediate. In addition, 18S and 28S rRNAs, constitutive of the mature ribosome, were produced in lower amounts, with 18S rRNA being more sensitive to 5-FU than 28S rRNA (73,74). In human CRC cells treated with [<sup>3</sup>H]5-FU, the incorporation of labelled 5-FU into 45S pre-rRNA and the production of labelled mature 18S and 28S rRNAs were analysed by RNA fractionation by polyacrylamide-agarose electrophoresis followed by [<sup>3</sup>H] fluorography to

demonstrate that 5-FU is incorporated into rRNA. In human CRC cells treated with 5-FU, northern blotting, using a set of [<sup>32</sup>P] labelled probes, confirmed that 5-FU affects 45S pre-rRNA maturation (75). In a large-scale study aiming at describing the impact of a panel of 36 chemotherapeutic drugs on rDNA transcription and rRNA processing, human fibrosarcoma cells were cultured with drugs, phosphate depleted and labelled with [<sup>32</sup>P] orthophosphate. The isolated RNAs were separated by agarose gel electrophoresis for quantification by phosphorimager analysis of all rRNA species. This approach confirmed that 5-FU does not impact rDNA transcription, but it does affect pre-rRNA processing (76).

Since 5-FU affects RiBi, it cannot be excluded that 5-FU may cause what is recognized in the literature as ribosomal stress (77). Ribosomal stress occurs when inhibition of rRNA processing triggers a disequilibrium between the quantity of rRNA and that of RPs. In this process, two key RPs, namely RPL5 and RPL11, bind to MDM2, thus prevent the interaction between MDM2 and P53, and consequently stabilize and activate P53, leading to cell cycle arrest and apoptosis (78). Nevertheless, providing that P53 needs to be free of any mutations to be functional, this potential 5-FU induced cytotoxic effect, if occurring in some circumstances, cannot account for a generalized mode of action of 5-FU since many cancers are characterized by *p53* mutations.

Hence, these studies demonstrated, using approaches available at their time of publication, that 5-FU is incorporated into rRNA precursors and affects RiBi (Figure 3). However, no demonstration of the presence of 5-FU into ribosomes could be done.

### 5-FU INTEGRATION IN rRNA GENERATES THE FLUORINATED RIBOSOME (F-RIBOSOME)

To improve our understanding of the consequences of 5-FU incorporation into rRNA, the development of technologies aimed at detecting and quantifying the 5-FU molecules incorporated into RNA turned out to be crucial. Here, we discuss how progress in the detection of 5-FU anabolites in RNA allowed to identify and quantify the 5-FU molecules integrated in the ribosomes, and propose that, by creating an epitranscriptomic-like source of rRNA modification, 5-FU creates a novel biological object called F-ribosome.

#### Analytical detection of 5-FU anabolites in RNA from old to new technologies

The study of the distribution of 5-FU metabolites in different biological samples (cells, cellular compartments, DNA, RNA, etc.) requires advanced analytical tools to reach greater sensitivity and selectivity. Several technologies enabling such analyses have been described: radiolabelled assay, nuclear magnetic resonance (NMR), capillary electrophoresis, gas chromatography coupled with mass spectrometry (GC-MS), and, more recently, liquid chromatography coupled with mass spectrometry (LC-MS).

Radiolabelled assays were mostly used in the 1980s, particularly to study the incorporation of 5-FU into DNA

and RNA (79). For these assays, cells were incubated with [<sup>3</sup>H]FU, RNA and DNA were separated by differential extraction and then radioactivity was measured in each extract. This approach is highly sensitive; however, it is limited to laboratories authorised to conduct radiolabelling. In addition, since the signal measured is identical, irrespective of the metabolite, this technology relies on pure DNA and RNA extracts. Another drawback of this approach is that the treatment of samples with radiolabelled [<sup>3</sup>H]5-FU is necessary, preventing its use on patient samples. It thus became obsolete and has been replaced by methods using chromatographic separation.

<sup>19</sup>F nuclear magnetic resonance (<sup>19</sup>F NMR) is a non-invasive spectroscopic method that is used to identify and quantify all 5-FU anabolites in a single run (80,81). It was applied to several biological samples such as intracellular extracts from cultured cells (80), tumour tissues (82,83) and RNA (82). To the best of our knowledge, it has not been used to analyse DNA. <sup>19</sup>F NMR is specific to fluorinated compounds and has to be coupled with <sup>31</sup>P NMR to study endogenous nucleotides and nucleosides (81).

Two capillary electrophoresis methods coupled with UV detection have been reported for the quantification of 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP and 5-FUTP (84,85). The most recently applied method to quantify 5-FU and 5-FdUMP in cells and tissues failed to detect 5-FdUMP (85), emphasizing the lack of sensitivity of the method.

GC-MS methods reported in the literature were used to study the incorporation of the 5-FU metabolite into RNA and DNA (45,83). While 5-FU can be analysed by GC, 5-FUMP and 5-FdUMP cannot be determined as such for physicochemical reasons. In these studies, both metabolites had to undergo two enzymatic steps to be transformed into 5-FU. The procedure was time-consuming and presented the following major drawback: since 5-FU was quantified as a surrogate for 5-FUMP and 5-FdUMP, RNA had to be free of any DNA contamination and vice versa during sample preparation, due to the risk of overestimating the concentrations of metabolites.

LC-MS represents the method of choice well described in the literature for the analysis of anabolic metabolites of 5-FU. Detection by MS provides both high sensitivity and selectivity. LC-MS technology is able to quantify nucleosides as well as nucleotide anabolites, although the quantification of nucleotides, particularly tri-phosphates, remains challenging. Thus, few assays have described the analysis of both types of compounds (86–89). Methods have been applied on cultured cells (86,87,89), peripheral blood mononuclear cells (88), and DNA and RNA (54,86).

Recently, our laboratory set up a highly sensitive technology based on LC coupled with high resolution mass spectrometry (LC-HRMS) using Orbitrap technology for the simultaneous determination of the ten anabolic metabolites (nucleosides, nucleotides and sugar nucleosides) of 5-FU (86). The method was applied to measure the proportion of each anabolic 5-FU metabolite in cellular contents and to study their incorporation into RNA and DNA. After extraction and digestion, a dephosphorylation step was added in order to quantify 5-FUrd in RNA and 5-FdUrd in DNA rather than 5-FUMP and 5-FdUMP since a better sensitivity was achieved for nucleosides rather than nucleotides

(86). This method also provided the measurement of endogenous nucleosides and nucleotides, thus enabling measuring the consequences of inhibition of enzymes, such as TS or pseudouridine synthase, in the same analytical run. More importantly, this method allowed us to report for the first time the incorporation rate of 5-FUrd into rRNA, namely 28S and 18S (86).

### The F-ribosome, a novel ribosome species, created by 5-FU

While incorporation of 5-FU into RNA has been studied since the early seventies using methodologies that roughly assess the quantity of 5-FU integrated into RNA, these methodologies were rather inadequate for distinguishing rRNA from all species of RNA when measuring the incorporation of labelled 5-FU metabolites.

The recent technological developments from our laboratory not only pushed the LC-HRMS technology up to measuring the incorporation rate of 5-FUrd in 28S and 18S rRNAs as detailed above, but further allowed the detection and quantification of 5-FUrd present in the whole ribosome (86). From cells treated with 5-FU, cytoplasmic ribosomes were isolated and their rRNA subsequently purified as a pool of 28S, 18S, 5.8S and 5S rRNA. Since the nucleotide sequence, and thus the number of each endogenous nucleoside is known for human 28S, 18S, 5.8S and 5S rRNAs, the quantification of the amount of 5-FUrd relative to that of endogenous nucleosides provides the number of 5-FUrd molecules per rRNA, and per ribosome accordingly.

Quantification of the amount of 5-FUrd from ribosomes in several CRC cell lines treated for 24 h with 10  $\mu$ M 5-FU showed that 5-FU treatment results in the production of fluorinated ribosomes that we called F-ribosomes (i.e., ribosomes with 5-FU incorporated in their rRNA) (90). We thus demonstrated that 5-FU-treated cells produce F-ribosomes bearing extrinsic chemical rRNA modifications (Figure 3). To support the notion that 5-FU creates novel ribosomes, we recently investigated the activity of F-ribosomes using an *in vitro* hybrid translational assay (91). We found that 5-FU incorporation in the ribosome modifies its intrinsic translational activity (90).

Quite interestingly, recent findings showed that 5-FU treatment is associated with a full translational reprogramming. These data support the intriguing possibility that the F-ribosome intrinsic translational activity could contribute to such translational reprogramming. Two early large-scale studies proposed that 5-FU modulates the translation of a subset of mRNAs. However, the alteration of translational efficiency was not directly investigated, and 5-FU treated CRC cells underwent additional modifications, i.e. stable modifications of TS expression (92) or Hsp70 immunoprecipitation (93). Recently, by coupling polysome profiling with mRNA expression microarray, we showed that 5-FU induces a global translational reprogramming in three CRC cell lines (94). We identified 313 genes coding for mRNA, the translational efficiency of which is selectively modulated following 5-FU treatment. Enrichment analysis revealed that translationally down-regulated mRNAs are mainly implicated in DNA replication, as usually observed in response to 5-FU reinforcing the notion that translational control also contributes to this 5-FU induced effect. Inter-

estingly, while 5-FU decreases global translation slightly, it promotes the translational efficiency of over 280 genes (the full list is available in (94) Supplementary Table S2). These translationally up-regulated genes are implicated in transcription regulation and have roles in the translational machinery including translation initiation and elongation factors, tRNA maturation factors and ribosomal proteins (the genes are indicated in (94) Supplementary Table S3). Among the mRNAs whose translation is up-regulated in response to 5-FU, some are involved in pathways controlling cell proliferation and tumorigenesis such as Wnt and integrin signalling pathways also known as pathways involved in 5-FU resistance (49,95) (Table 2). Again, this suggests that translational control could be part of the cell response mechanisms that finally lead to reshape gene expression regulation favouring emergence of resistant cells. In parallel, we unveiled that a miRNA-based mechanism was involved in the 5-FU-dependent translation, reduced miR-155 expression increasing the translation of HIVEP2 mRNA translation in response to 5-FU. Whether this miRNA-based mechanism works in concert with the rRNA-based mechanism remains to be determined. Nevertheless, whatever the molecular mechanisms engaged in this translational reprogramming, gaining access to the translome deeply remodelled by 5-FU provides a reservoir of potentially actionable protein targets, the modulation of which could increase the sensitivity of cancer cells to 5-FU (Figure 4).

Still, whether translational reprogramming is a direct consequence of 5-FU incorporation into ribosomes and into the other RNA being part of the translational apparatus or involved in its fine tuning remains to be fully demonstrated.

## CONCLUSION AND PERSPECTIVES

Biological epitranscriptomic rRNA modifications (2'-O-ribose-methylation, pseudouridylation, base modification) are implicated in the control of ribosome translation and cancer cell fate (96–98).

2'-O-ribose-methylation (2'-O-Me) is the most abundant modification, with 106 sites mapped in human rRNA (99). While ribosomes were usually considered to be constitutively methylated at each 2'-O-Me site in healthy proliferating cells (100–102), our laboratory reported that rRNA 2'-O-Me can be altered at some specific sites during mammary tumorigenesis (103–105), and that alteration of 2'-O-Me rRNA directly affects the intrinsic translational activity of ribosomes (106). These data support the importance of ribosome epitranscriptomics in regulating translation and contributing to the phenotypes of breast tumours.

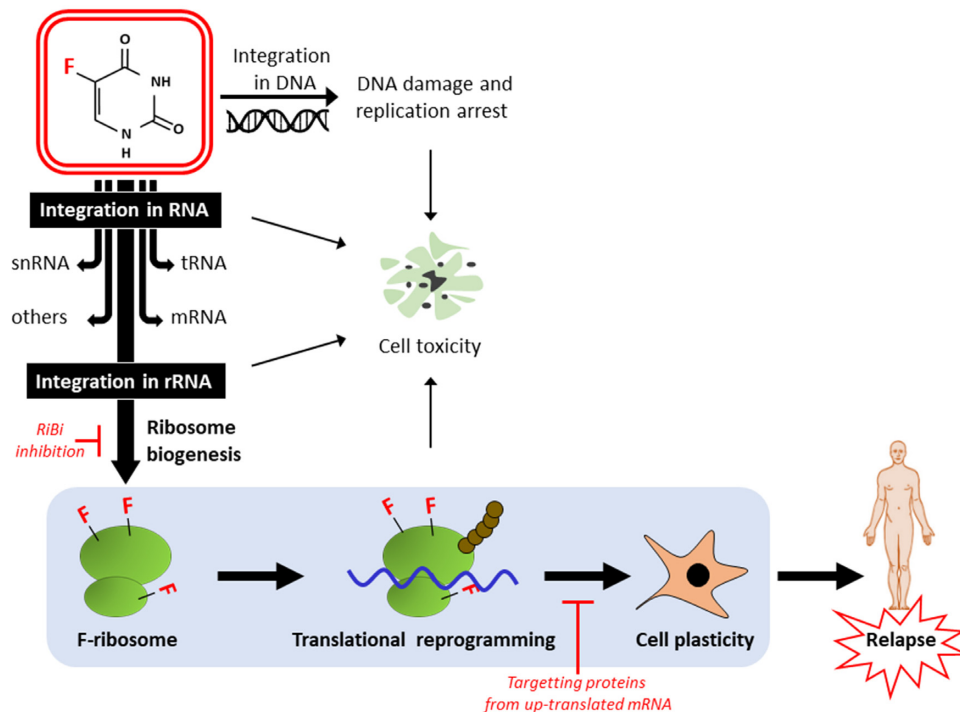
Pseudouridylation, which involves the isomerisation of uridine into  $\psi$ , accounts for 95 modified sites in human RNA (107). Defects in  $\psi$  have been linked with X-linked dyskeratosis congenita (X-DC) associated with an increased risk of developing tumours (108), and rRNA  $\psi$  defects are responsible for alterations of ribosomal translational activity (109,110) and cell fate (111–114).

Methylation and acetylation of bases that are responsible for >12 additional epitranscriptomic rRNA modifications in humans (96,115) also contribute to the modulation of ribosome activity and human cell fate. For ex-



**Table 2.** Signalling pathways affected by translational reprogramming in response to 5-FU. Bioinformatic non-statistical analysis using the list of translationally up-regulated genes identified in (94) using PANTHER (<http://pantherdb.org>, Functional classification viewed in graphic charts' using 'pathways' parameters) (124)

Pathway name (ID)	Number of genes involved in the pathway	Gene hit against total number of gene in pathway (%)	Genes
Wnt signalling pathway (P00057)	6	5.0	FZD1, FZD5, DACT1, MYH6, GNG7, GNG10
Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	6	5.0	MYH6, SOCS6, GNG7, GNG10, RHOQ, SOCS7
Gonadotropin-releasing hormone receptor pathway (P06664)	4	3.3	ACVR2B, NR3C1, MAP3K13, MAP4K4
Integrin signalling pathway (P00034)	4	3.3	RAP2B, FRK, MEGF9, RHOQ
Angiogenesis (P00005)	4	3.3	FZD1, FZD5, PDGFC, PDGFA



**Figure 4.** Model showing that translational reprogramming, induced by 5-FU integration in ribosomes, contributes to 5-FU-driven cell plasticity and relapse. 5-FU is generally recognized as a disruptor of DNA metabolism contributing to its cytotoxicity. However, 5-FU is mainly incorporated into RNA. Through its integration in rRNA, the most abundant RNA, 5-FU produces active F-ribosomes which induce translational reprogramming accompanied by an incomplete cytotoxicity. Instead, cell plasticity takes place and will ultimately contribute to relapse.

ample, deficiency of NSUN5 (28S rRNA (cytosine-C(5))-methyltransferase) or ZCCHC4 (rRNA N6-adenosine-methyltransferase), which introduces the m<sup>5</sup>C3782 or m<sup>6</sup>A4220 modification on 28S rRNA respectively, induces translational reprogramming and affects cell proliferation (116,117).

Taken together, these studies highlight the ribosome, and more particularly, epitranscriptomic rRNA modifications, as key players in translational regulation and tumorigenesis. Any alteration in biological rRNA epitranscriptomics leads to profound changes in the translational activity of the ribosome and is associated with new phenotypes (103–105,118,119). Through its stable integration into rRNA, 5-FU induces extrinsic post-transcriptomic modifications of the ribosomes, thus creating a novel biological object, which is an artificially modified ribosome, the F-ribosome.

Following this notion, it can be proposed that rRNA modification by 5-FU creates a source of epitranscriptomic-like rRNA modification which may affect cell fate and notably contribute to the emergence of 5-FU resistant cells (Figure 4).

The ribosome is a complex macromolecular machinery that has a pivotal role in the growth and proliferation of mammalian cells. However, the ribosome is rarely envisaged when studying the mechanisms of resistance to anti-cancer therapies, such as 5-FU-based treatments. Here, we placed the ribosome as well as RNA-mediated translational regulation at the heart of the action of 5-FU and propose that a novel view of this old drug can contribute to managing the impact of 5-FU in therapy by the medical community. In this biological model, combination of 5-FU with RiBi inhibitors and/or drugs targeting proteins encoded by trans-

lationally deregulated mRNAs could be evaluated to counteract cell plasticity and relapse as a consequence (Figure 4).

At present one of the most extensively studied issues in oncology is the drug resistance, as this accounts for the vast majority of cancer-related deaths. Significant changes in tumour cells occur upon treatment and the resulting resistance process is currently modelled as a succession of three phases, named sensitivity, tolerance and recurrence (120,121). A wide majority of studies explore the molecular mechanisms underlying the drug tolerance through analysis of genetic as well as non-genetic mechanisms such as epigenetic and transcriptomic reprogramming (121–123). However, it appears obvious that being the last step of gene expression, translation arises as an inescapable layer of gene expression regulation that, although underestimated for a long time, should now be fully considered to elucidate the molecular mechanisms underlying drug-tolerance, drug-resistance and recurrence following anti-cancer treatments containing 5-FU.

## ACKNOWLEDGEMENTS

We thank Brigitte Manship for editing the manuscript.

## FUNDING

Institut National de la Santé et de la Recherche Médicale (Inserm); Centre National de la Recherche Scientifique (CNRS); Université Claude Bernard Lyon 1 (UCBL1); Agence Nationale de la Recherche (ANR) [ANR-13-BSV8-0012-01 RIBOMETH, ANR-19-CE12-0004 ACTIMETH]; Institut National du Cancer (INCa) [PLBIO 2018-131 FluoRib]; PAIR Sein program [ARC INCa LNCC 7625 RiboTEM]; SIRIC program [INCa-DGOS-Inserm 12563 LYriCAN]; Laboratoire d'Excellence (Labex) [DEV-CAN2UMAN]; Fondation ARC pour la Recherche sur le Cancer [20161204686 MARACAS]; Ligue contre le Cancer (Drôme, Rhône, Isère, Puy-de-Dôme, Allier).  
*Conflict of interest statement.* None declared.

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