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Effect of triazole-based compounds on pre-mRNA splicing in HCT116 cells

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Introduction

Cancer can be described as a disease of unregulated and uncontrolled multiplication of cells, which possess a tremendous ability to proliferate (Chaudhry *et al*., 2022). And despite great efforts, oncological diseases continue to be one of the most urgent public health problems (Lepeltier *et al*., 2020). In 2022 almost 20 million new cancer cases and 9.7 million cancer-associated deaths were recorded. It is approximated that one in five people develop cancer, while one in nine men as well as one in twelve women succumb to cancer. Colorectal cancer was one of the most frequently diagnosed forms of cancer in 2022, making up 9.6% of all diagnosed cancers globally; the number of cancer cases is predicted to rise further (Bray *et al*., 2024).

Reduced oxygen availability, known as hypoxia, is a common feature of the microenvironment of solid tumours (Liao *et al*., 2023; Chen *et al*., 2023). These hypoxic microenvironmental conditions, have been linked with metastasis, malignant progression, as well as increased resistance to treatment strategies such as immunotherapy, chemotherapy, and radiotherapy and poor clinical outcomes after treatment (Kanopka, 2017; Chen *et al*., 2023).

Pre-mRNA splicing is a process under which non-coding regions (introns) are removed, and coding regions (exons) join together to form mature mRNA. Constitutive splicing results in intron removal and exon ligation in the order in which they appear in a gene, alternative splicing differs by skipping certain exons resulting in different mature mRNA. The aforementioned hypoxic microenvironment is one of the most important alternative splicing regulators that influence cancer development (Wang *et al*., 2014; Kanopka, 2017; Farina *et al*., 2020).

Chemotherapy is a widely employed therapeutic strategy in the treatment of various cancers, including colorectal cancers (Florento *et al*., 2012; Anand *et al*., 2022; Fadlallah *et al*., 2024). It also remains as the primary treatment option for advanced-stage malignancies where surgery or radiation therapy are not suitable choices, despite many currently chemotherapy drugs approved for medicinal use exhibiting various adverse effects in patients. In consequence, chemotherapeutic strategies can be greatly bolstered by the design of new chemotherapeutic agents with higher efficacies and increased safety (Anand *et al*., 2022). To contribute to this goal, this work investigates the effect of a group of synthesized triazole-based chemical compounds on the pre-mRNA splicing in HCT116 cells under normal oxygen and hypoxic conditions.

Aim and objectives of this work.

The aim of this work was to investigate the effects of a group of chemically synthesised compounds on the pre-mRNA splicing of tumour-associated genes in HCT116 cancer cells under different oxygen conditions. The following objectives were raised:

1. To evaluate the effects of a set of synthesized triazole-based compounds on the viability of HCT116 cells.
2. To compare the expression of tumour-associated mRNA isoforms in HCT116 cells cultivated under normoxic and hypoxic conditions.
3. To investigate the effects of selected synthesized triazole-based chemical compounds on the formation of tumour-associated mRNA isoforms in normoxic and hypoxic HCT116 cells.

1. Literature review

1.1. Pre-mRNA splicing

1.1.1. Pre-mRNA splicing & mechanism

Splicing of RNA occurs co-transcriptionally or post-transcriptionally inside the cell nucleus. Pre-mRNA is processed by specialised splicing machinery, which removes non-coding sequences (introns). The coding sequences (exons) are retained and joined together to produce mRNA that is then transported to the cytoplasm and translated into proteins. This process greatly influences the ability of cells to adapt to altered cellular conditions (Kanopka, 2017). Typically, human genes contain an average of 8 exons with an average length of 145 nucleotides, while introns are more than 10 times this size and can be far larger. Exon-intron borders contain short pre-mRNA canonical splice site sequences, which are 5' and 3' splicing targets sequences. Introns contain canonical pre-mRNA sequences known as the branch point sequence (BPS) and the polypyrimidine (Poly-Y) tract (Figure 1) (Faustino & Cooper; 2003; Yoshimi & Abdel-Wahab, 2017) The strength of the 3' splice site is determined by the polypyrimidine tract. Stronger splice sites have longer pyrimidine sequences, while weaker sites are segmented by purine/pyrimidine residue interruptions (Liu & Mei, 1996; Coolidge *et al*., 1997; Tse *et al*., 2023).

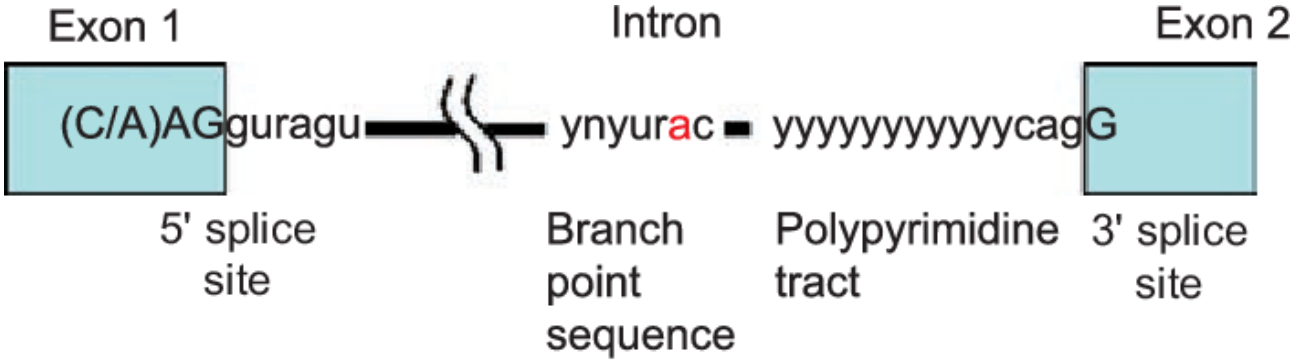

Figure 1. Canonical sequences, including the 5' splice site, the 3' splice site, the branch point sequence and the polypyrimidine tract. C – cytosine, A – adenine, G – guanine, R – purine, Y – pyrimidine (Adapted form Ebbesen *et al*., 2009)

While splicing is an autocatalytic process in prokaryotes, eukaryotes have a more complex and regulated splicing machinery called the spliceosome (Borao *et al*., 2021). The spliceosome consists of five snRNPs, including U1, U2, U4/U6 and U5 snRNPs which are composed of single uridine rich small nuclear RNA (snRNA) and specific proteins, namely the SmB/SmB', SMD1, SmD2, SmD3, SmE, SmF and SmG proteins, which form an Sm ring. (Kastner *et al*., 2019; Nagasawa & Garcia-Blanco, 2023).

The spliceosome begins to form with the assembly of the early spliceosome complex (E complex). The U1 snRNP, recruited by serine-arginine (SR) proteins, binds to the 5' splice site, while the splicing factor 1 (SF1) binds to the branch point sequence. The U2 auxiliary factor

(U2AF) subunits U2AF65 and U2AF35 bind to the polypyrimidine tract and the AG-dinucleotide which are located at the 3' splice site, respectively (Black, 2003; Zahler & Roth, 1995; Yan *et al*., 2019). This is followed by the U2 snRNP binding to the branchpoint sequence at the 3' splice site and the removal of the SF1 splicing factor, which results in the formation of the pre-spliceosome (A complex) (Figure 2) (Niño *et al*., 2022). The U4/U6 and U5 snRNPs interact with the pre-spliceosome to form the pre-catalytic spliceosome (B complex) (Faustino & Cooper; 2003; Han *et al*., 2011; Yan *et al*., 2019). The development of the activated spliceosome (B^{act} complex) results from the alteration of the B complex by ATPase/helicase Brr2 which facilitates the dissociation of the U1 snRNP and the U4 snRNP and the recruitment of NineTeen complex (NTC) (which includes numerous proteins such as cdc5, prp19, spf27, etc.) and NTC related (NTR) (which includes proteins such as RBM22 and PPIL1) proteins. Only the B complex, and the preceding pre-B complex contain the five snRNPs (Hang *et al*., 2015; Shi, 2017). The transition of the B^{act} complex into the catalytically activated spliceosome (B* complex) is facilitated by ATPase/helicase Prp2, which allows a transesterification reaction to occur, enabling the generation of an intermediate of 5'-exon and an intron lariat-3'-exon intermediate, resulting in the catalytic step I spliceosome (C complex). The ATPase/helicase Prp16 enables the further transition of the C complex into the step II activated spliceosome (C* complex), which incites the ligation of the 5'-exon to the 3'-exon (Zhang *et al*. 2018). This assembly of the spliceosome occurs recurrently each time an intron is removed from pre-mRNA (Lee & Rio; 2015). The C* complex develops further into the post-splicing complex (P complex), which driven by ATPase/helicase Prp22 releases dissociated ligated exons as mature mRNA, while the intron lariat remains bound to the spliceosome and the P complex transitions into the intron-lariat spliceosome (ILS complex). The ILS complex is later disassembled by ATPase/helicase Prp43, this results in the formation of a free intron lariat (Figure 2) (Wan *et al*., 2017; Yan *et al*., 2019).

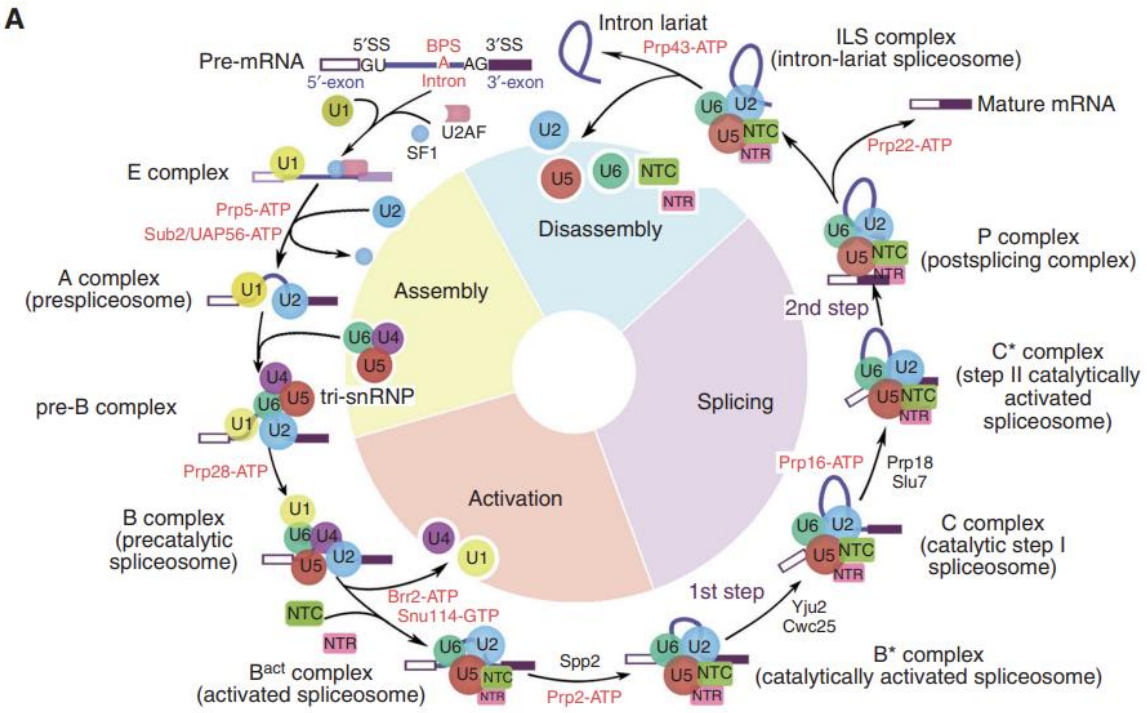

Figure 2. The precursor messenger RNA splicing cycle. Each cycle consists of assembly and activation of the spliceosome, the splicing reaction, and disassembly of the spliceosome. The pre-B complex is the first form of the assembled spliceosome. Following spliceosome developments include: the precatalytic spliceosome, the activated spliceosome, the catalytically activated spliceosome, the catalytic step I spliceosome, the step II catalytically activated spliceosome, the post splicing complex and finally, the intron-lariat spliceosome. The conversion of spliceosome complexes to the next form of spliceosome is executed by the conserved ATPases/helicases (Brr2, Prp2, Prp16, Prp22). The splicing factors Cwc25 and Yju2 encourage the branching reaction, while Prp18 and Slu7 are needed in exon ligation. Disassembly of the ILS complex is executed by the ATPase/helicase Prp43 (Retrieved from Yan *et al*., 2019)

1.1.2. Alternative splicing

As mentioned before, constitutive splicing is the process under which introns are removed, and exons are joined in the sequential order that they appear in the gene (Figure 3A) (Wang *et al*., 2014). Alternative splicing differs from this process by joining different 5' and 3' splice sites which enables a single gene to encode multiple mRNA isoforms (Figure 3B-F). Different mRNA isoforms contain different sequences that determine synthesised protein functions (Faustino & Cooper; 2003; Han *et al*., 2011; Kelemen *et al*., 2013). Alternative splicing appears to occur more extensively in humans compared to model organisms such as mice or fruit flies. This is especially evident when comparing mouse and human genomes, which contain a comparable number of genes, while alternative pre-mRNA splicing occurs in approximately 63% of mouse genes, humans by contrast, experience alternative pre-mRNA splicing in up to 95% of their genes (Kim *et al*., 2004; Kim *et al*., 2007).

There are several main types of alternative splicing, including cassette alternative exon (Figure 3C), which is the most widespread type in higher eukaryotes with an approximate frequency of 30%. Another type of alternative splicing includes intron retention (Figure 3F), which in humans is most frequent in untranslated regions and is linked with weak splice sites, as well as lesser intron length and modulation of cis-regulatory elements (Wang *et al*., 2014). Selection of an alternative 5' splice site or 3' splice site, prompts the recognition of a pair or multiple of splice sites at one end of an exon (Figure 3D-E) (Pohl *et al*., 2013). Differing transcripts are achieved by the initiation of transcription at the site of the first exon within a gene or within the upstream 5' end untranslated region. Alternative 5' splice sites facilitate different transcripts that have dissimilar open reading frames , which result in various protein isoforms with divergent N-termini (Sahebi *et al*., 2016). Lastly there are mutually exclusive exons (Figure 3B), which are a subtype of alternative splicing, where two or more splicing events are carried out in coordination and only one of two exons or exon groups is retained in mature mRNA (Pohl *et al*., 2013). Unlike other types of alternative splicing, mutually exclusive splicing can result in isoforms with unchanged sizes if the exchanged sequence is of the same size and does not include a premature stop codon (Birzele *et al*., 2007).

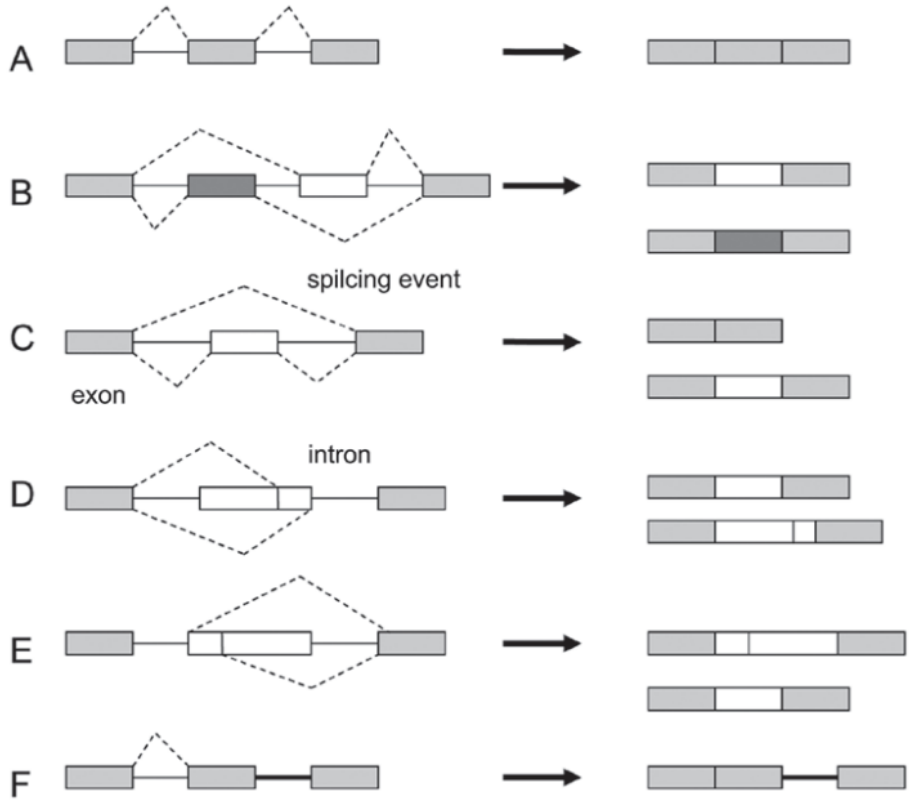

Figure 3. Main types of splicing events, including constitutive splicing (A), mutually exclusive exons (B), cassette alternative exon (C), alternative 3' splice site (D), alternative 5' splice site (E) and intron retention (F) (Retrieved from Wang *et al*., 2014)

1.1.3. Regulation of pre-mRNA alternative splicing

Inclusion of exon sequences in mature mRNA during alternative splicing is determined by the alternative splicing patterns that are established and regulated through the interactions that occur between cis-acting regulatory sequences and trans-acting splicing factors. Cis-acting regulatory sequences are located in either introns or exons and can be positive regulators or negative regulators of alternative splicing. In introns these sequences function as intronic splicing enhancers (ISE) and intronic splicing silencers (ISS), while in exons they function as exonic splicing enhancers (ESE) and exonic splicing silencers (ESS) (Wang & Burge, 2008; Wang *et al*., 2014; Lee & Rio, 2015). Trans-acting splicing factors are RNA-binding proteins that play an additional part in the regulation of splice site selection, by interacting with cis-acting regulatory sequences (Wang & Burge, 2008; Lee & Rio, 2015; Elcheva & Spiegelman 2020). These proteins include heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine-arginine-rich (SR) proteins. Notably, some hnRNP proteins and SR proteins are known to act as splicing repressors or splicing activators (Lee & Rio, 2015).

1.1.3.1. Role of serine/arginine-rich proteins in splicing regulation

The serine-arginine (SR) family of phosphoproteins is a conservative family of splicing factors that are widespread among vertebrates and invertebrates. SR proteins are key regulators in both alternative and constitutive pre-mRNA splicing. These proteins are necessary splicing factors that are essential in early spliceosome assembly (Figure 5) (Kanopka, 2017). In addition to their aforementioned role in the recruitment of the U1 snRNP to the 5' splice site, SR proteins facilitate the stabilisation of U2AF associations with the polypyrimidine tract and the 3' splice site. SR proteins also encourage the recruitment of the U4/U6 and U5 tri-snRNP complex (Sanford *et al*., 2005; Slišković *et al*., 2021). SR proteins are of a comparable two-part structure containing two functional domains, including the N-terminal RNA binding domain, which is made up of several RNA recognition motifs and a C-terminal arginine-serine rich domain (RS) (Figure 4) (Sahebi *et al*. 2016; Kanopka, 2017). The most notable members of the SR family of proteins include the serine and arginine rich splicing factors (SRSFs) (Figure 4) (Zheng *et al*., 2020). These proteins play important roles in the activation, regulation, and repression of constitutive and alternative splicing (Twyffels *et al*., 2011).

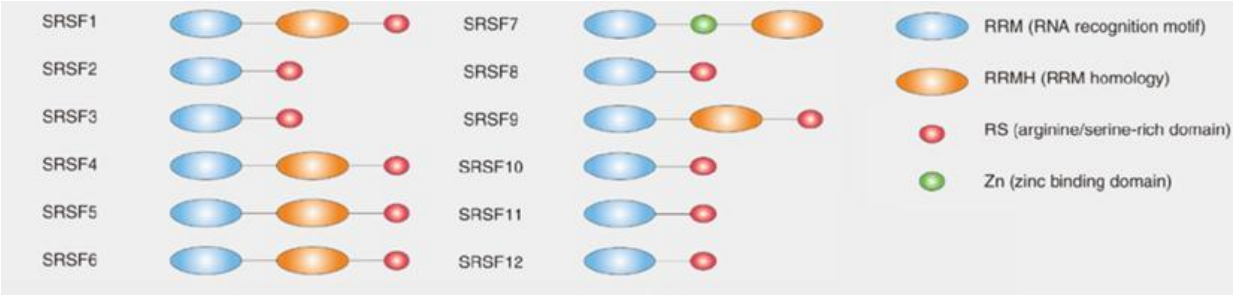

Figure 4. Structure of SR proteins. The serine and arginine rich splicing factors include an RNA recognition motif (RRM), RRM homology (RRMH), an arginine/serine-rich domain (RS) and a zinc binding domain (Zn) (Modified from Zheng *et al*., 2020)

The SRSF1 protein is notable for its additional function in the promotion of mRNA degradation (Twyffels *et al*., 2011). The SRSF1, SRSF3, SRSF5 and SRSF7 splicing factors transport mRNA out of the nucleus, facilitated by a cycle of phosphorylation and dephosphorylation of the arginine/serine-rich domain (RS), which causes SR proteins to move between the nucleus and the cytoplasm (Twyffels *et al*., 2011; Ghosh & Adams, 2011; Wang *et al*., 2014; Zheng *et al*., 2020).

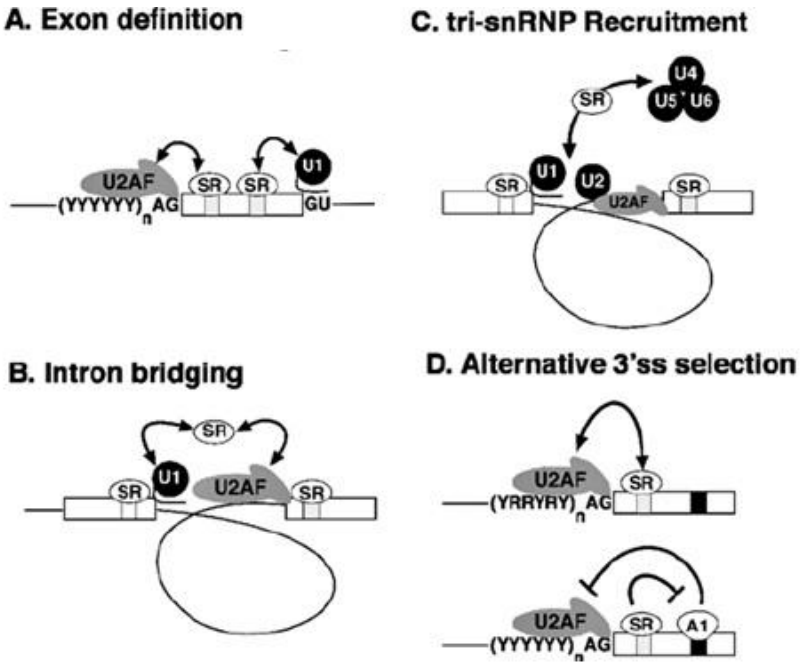

Figure 5. SR proteins in the spliceosome assembly mechanism. Exons are defined by U2AF at a 3' splice site, while a U1 snRNP at a downstream 5' splice site is enabled by SR proteins to associate with exonic splicing enhancer (A); The 5' splice site and the 3' splice site are positioned in apposition early in the splicing process by intron bridging associations between SR proteins and U1 snRNP and U2AF (B); SR proteins recruit the U4, U6 and U5 tri-snRNP to the complex of the pre-spliceosome (C); SR proteins either bind to exonic splicing enhancers and encourage 3' splice site selection by associating with U2AF, or the SR proteins antagonise splicing repressor impacts, thereby encouraging the selection of specific splice sites (D) (Modified from Sanford *et al*., 2005)

1.1.3.2. Role of heterogeneous nuclear ribonucleoproteins in splicing regulation

HnRNP proteins are a class of proteins with varied structures, notable for their role as transcriptional and post-transcriptional regulators. Most hnRNP proteins contain RNA recognition motifs (RRMs) that function as RNA and single strand DNA (ssDNA) binding domains. The hnRNP E and hnRNP K proteins contain a K homology (KH) domain, while hnRNP U protein contains an arginine-glycine-glycine (RGG) domain. Both domain types mediate RNA and ssDNA binding (Figure 6) (Chen *et al*., 2013) Of the hnRNP class of proteins, hnRNPA1, hnRNPA2 and hnRNPA3 have been the most extensively researched, in particular the hnRNPA1 protein, which is known to be active during the splicing process (Black, 2003; Lee & Rio, 2015). The hnRNPA1 protein binds consecutive introns where it facilitates loop formation and the loss of the target exon. (Blanchette & Chabot, 1999; Zhu *et al*., 2001).

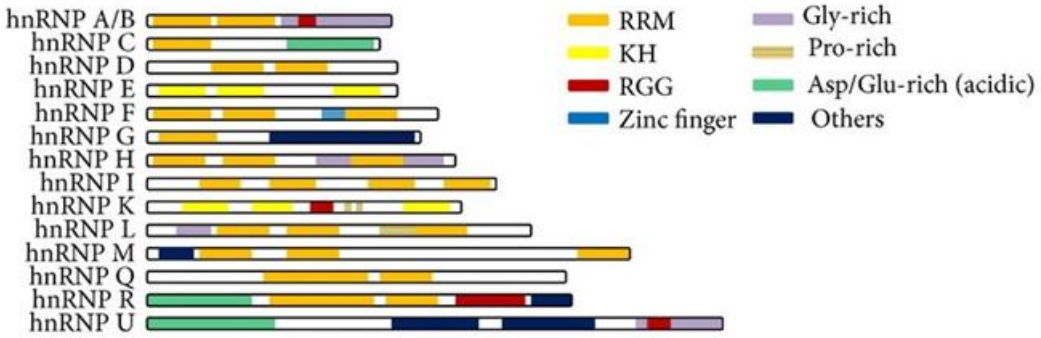

Figure 6. hnRNP structure. Most hnRNPs contain RRM domains, apart from hnRNP E, hnRNP K and hnRNP U. hnRNP E and hnRNP K contain KH domains, while hnRNP U contains a RGG domain. (Modified from Chen *et al*., 2013)

hnRNP A2/B1 proteins in addition to pre-mRNA splicing, participate in mRNA trafficking and decay while hnRNP C also promotes translation (Widagdo & Anggono, 2018). hnRNPL proteins in conjunction with hnRNPA1 proteins regulate spliceosome assembly by prolonging U1 snRNA and pre-mRNA interactions (Chiou *et al*., 2013).

1.2. Hypoxic microenvironment and cancer development

1.2.1. Cellular hypoxia, response to hypoxia and association with cancer

Aerobic organisms, depend on the presence of oxygen to ensure survival. Their cells utilise oxygen in the generation of cellular energy in the form of adenosine triphosphate (ATP), via oxidative phosphorylation. Under normal oxygen conditions (according to Simon & Keith (2008) ranging between 2% and 9% in most cells) a cell maintains a constant ratio of adenosine triphosphate and adenosine diphosphate. A deficit of oxygen, known as hypoxia, will have consequences on cell viability due to it being the final acceptor of electrons in the mitochondrial

respiratory chain (Gilany & Vafakhah, 2010). Although hypoxia is encountered in physiological processes such as embryonic development, it can also result from a variety of pathological and physiological conditions. These conditions include altitude sickness, obstructive sleep apnea or pulmonary diseases, hampered ability of blood to carry oxygen due to anaemia or carbon monoxide poisoning, obstruction of cell ability to process oxygen due to intoxication with substances such as cyanide, and most notably cancer: the formation of aberrant blood vessels with poor blood flow in tumours (Höckel & Vaupel, 2001; Harris, 2002; Gilany & Vafakhah, 2010).

Response to hypoxic conditions in cells according to the length of occurrence, include an acute, an intermediate and a chronic response (Gilany & Vafakhah, 2010; Muz *et al*., 2015). Acute hypoxia results from a brief exposure to hypoxic conditions, often occurring when blood flow is obstructed for at least several minutes. However, enduring changes in blood flow and insufficient supply of oxygen results in chronic hypoxia, which contributes to long-term changes of cellular processes. Chronic hypoxia induced changes include higher frequency of DNA breaks, increased frequency of DNA replication errors as DNA repair systems are impaired, including homologous recombination and mismatch repair, which could lead to genetic instability and mutagenesis (Muz *et al*., 2015).

As tumours rapidly develop, the distance between cancer cells and blood vessels increases and the diffusion of oxygen decreases, this is further compounded by the formation of abnormal blood vessels that do not facilitate blood-flow to the tumour, resulting in chronic hypoxia becoming prevalent in the microenvironment of solid tumours (Nejad *et al*., 2021; Chen *et al*. 2023). Response to hypoxic conditions in tumour cells include changes in gene expression, promotion of autophagy, suppression of apoptosis, stimulation of the epithelial-mesenchymal transition, increased angiogenesis, increased local invasive growth, regional and distant tumour cell spreading, and enhanced aggressiveness of tumours by clonal selection (a process by which clones within the tumour, with the fittest traits, experience “positive” selection) (Höckel & Vaupel, 2001; Nejad *et al*., 2021). Hypoxic microenvironmental conditions in cancer cells mediate the effects of conventional cancer therapy, including radiotherapy, chemotherapy, phototherapy and immunotherapy, and are associated with poor prognosis in cancer patients as well as malignant progression and tumour metastasis (Nejad *et al*., 2021; Chen *et al*. 2023).

1.2.2. Hypoxia-inducible transcription factors (HIF)

Maintenance of oxygen homeostasis relies on hypoxia-inducible factors (HIF), which mediate transcription. HIFs activate the transcription of genes associated with erythropoiesis and angiogenesis to increase oxygen supply, and modulate the balance between oxidative and glycolytic

metabolism, thereby matching oxygen demand with the available supply. Moreover, HIFs regulate the transcription of genes associated with cell proliferation and apoptosis (Semenza, 2007; Rankin & Giaccia, 2008; Jun *et al*., 2017; Chee *et al*., 2019; Wicks & Semenza, 2022).

The HIF transcription factor is structured as a heterodimer that includes a HIF-α subunit stabilised by hypoxia and a HIF-β (aryl hydrocarbon nuclear receptor translocator (ARNT)). While the HIF- α subunit under normal oxygen conditions is continuously degraded, under hypoxic conditions it stabilises and thus serves as an oxygen sensor. The β subunit is stably expressed in cells and not affected by oxygen conditions. Three HIF-α subunits in eukaryotic cells are known, including HIF-1α, HIF-2α, and HIF-3α (Berra et al., 2001; Graham & Presnell, 2017; Jun *et al*., 2017; Serocki *et al*., 2018; Iglesias *et al*., 2019; Wicks & Semenza, 2022). The HIF-1α protein contains: an oxygen-dependent degradation (ODD) domain, two Per-Arnt-Sim (PAS) domains (PAS-A and PAS-B), a basic helix–loop–helix (bHLH) domain, N- and C-terminal transactivation domains (TAD) bridged by an inhibitory domain. The ODD domain is essential in the ubiquitin-proteasome pathway (an essential pathway in protein degradation, by tagging proteins with ubiquitin). The PAS domains are associated with dimerization and specificity of target genes. The bHLH domain is involved in DNA binding and dimerization (Lee *et al*., 2004; Rankin & Giaccia, 2008; Li *et al*., 2022). The C-terminal transactivation domain of HIF-α contains a conserved asparagine residue that is hydroxylated under normal oxygen conditions, which blocks HIF-α engagement with transcriptional coactivators such as p300/CBP. Th N-terminal transactivation domain (Lando *et al*., 2002; Lee *et al*., 2004; Wicks & Semenza, 2022). The N-terminal transactivation domain also associates with transcriptional coactivators p300/CBP; however, its interaction is comparatively weaker (Yfantis *et al*., 2023). The structure of HIF-2α is similar to that of HIF-1α, as it contains the same domains as HIF-1α. HIF-3α contains bHLH, PAS, and ODD domains, as well as a N-terminal transactivation domain; however, it does not contain a C-terminal transactivation domain (Figure 7) (Rankin & Giaccia, 2008; Rani *et al*., 2022).

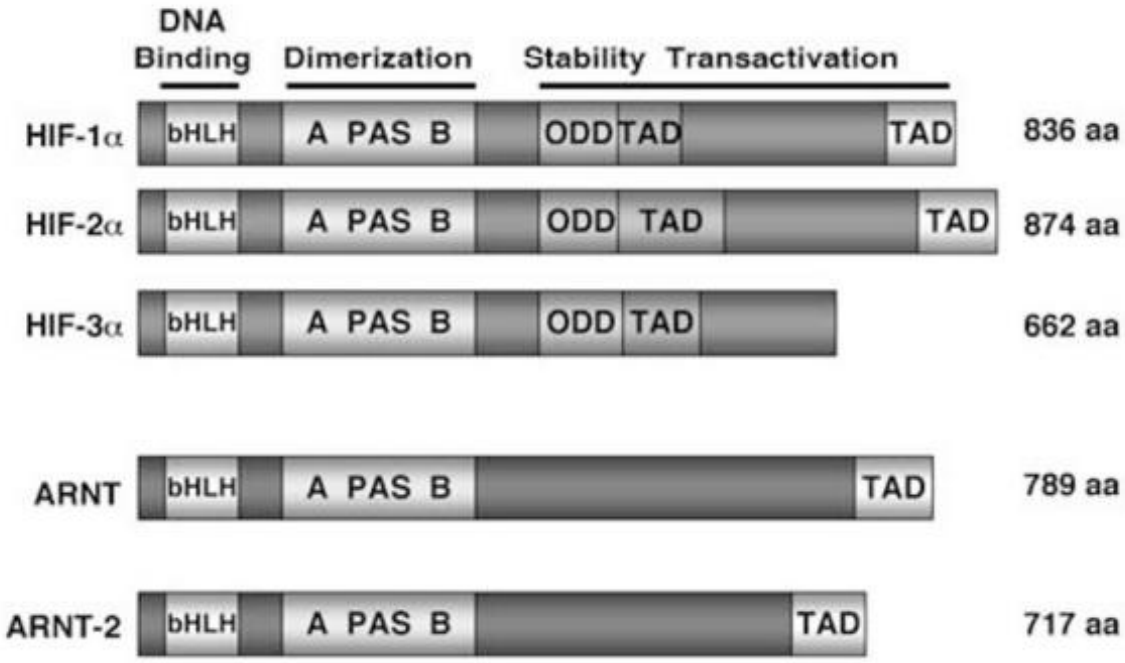

Figure 7. Visual representation of HIF protein structure and domains. bHLH - basic helix–loop–helix domain, PAS - Per-Arnt-Sim domain, ODD - oxygen-dependent degradation domain, TAD - transactivation domain. (Adapted from Rankin & Giaccia, 2008)

Under normal oxygen conditions, HIF-α is continuously degraded, this oxygen dependent degradation is activated by prolyl hydroxylase (PHD) proteins hydroxylating proline residues on HIF-α proteins. Following this, HIF-α binds to E3 ubiquitin ligase von Hippel-Lindau tumour-suppressor protein (pVHL). This binding facilitates interaction with the Elongin C protein, leading to the recruitment of the E3 ubiquitin-protein ligase complex, which ubiquitinates HIF-α marking it for proteasomal degradation by the 26S proteasome complex as shown in Figure 8 (Groulx & Lee, 2002; Semenza, 2007; Rankin & Giaccia, 2008; Jun *et al*., 2017).

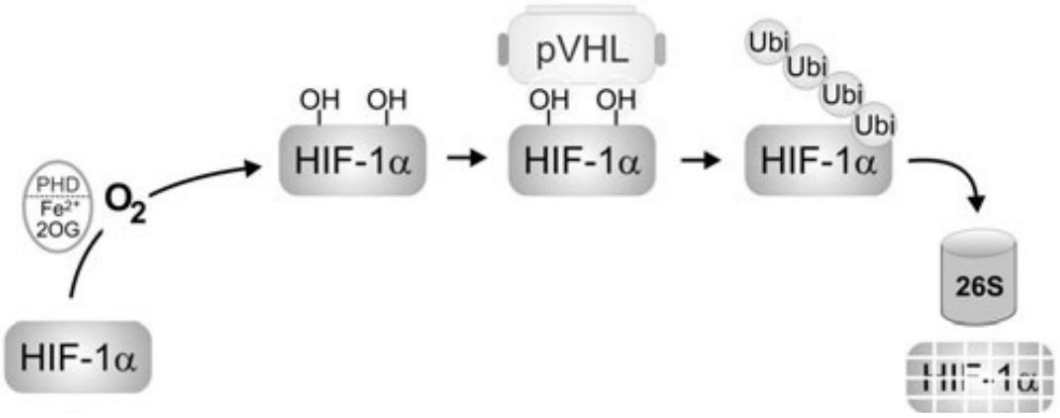

Figure 8. Degradation of HIF-1α protein under normal oxygen conditions. 26S – 26S proteasome; HIF-α – hypoxia inducible factor; PHD – prolyl hydroxylase; pVHL – von Hippel Lindau protein; Ubi – ubiquitin (Modified from Dehne & Brüne, 2014)

The transcriptional activity of HIF is further regulated by factor inhibiting HIF-1 (FIH-1), which under normal oxygen conditions directs the α protein to degradation. (Semenza, 2007; Jun *et al*., 2017; Wicks & Semenza, 2022). However, during hypoxia PHD and FIH functioning is supressed, which enables the stabilisation of HIF-α proteins and their translocation to the nucleus, where they dimerise with HIF-1β. The resulting HIF-α and HIF-β heterodimer binds to hypoxia response elements (elements that contain the sequence 5'-(A/G)CGTG-3' that serves as a HIF binding site) in target genes, recruiting coactivator proteins, leading to increased transcription of target genes (Figure 9) (Semenza, 2007; Jun *et al*., 2017).

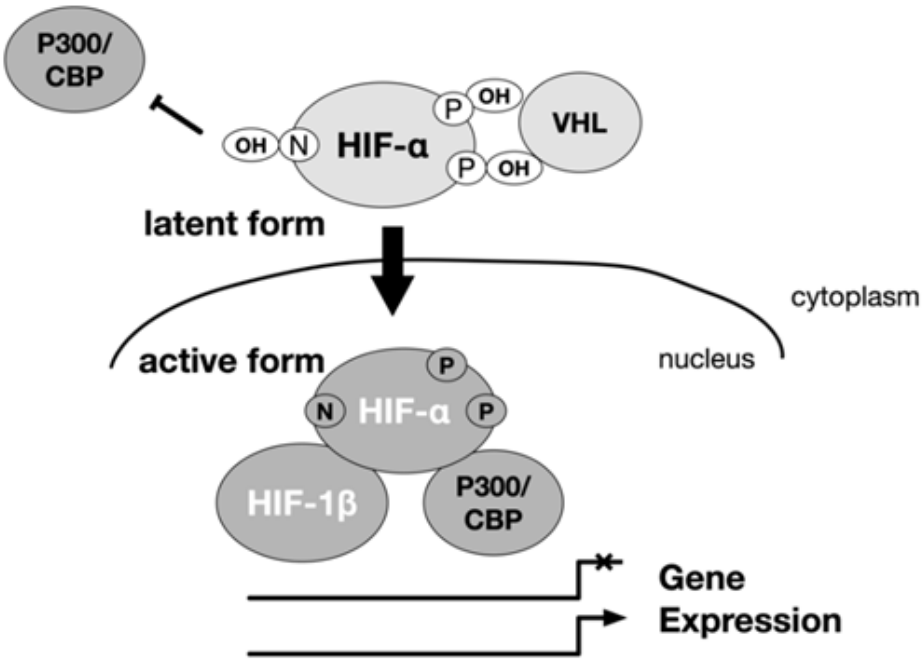

Figure 9. HIF- α activation as a transcription factor. HIF – hypoxia inducible factor; p300 – histone acetyltransferase p300; CBP – CREB-binding protein (Modified from Hirota, 2021)

The elevated expression, specifically of HIF-1α and HIF-2α proteins, is associated with an increase in patient mortality rates across numerous solid cancers. In cancer cells, the expression of HIF regulated genes plays a multifaceted role in the progression of cancer, contributing to tumour vascularisation, metabolic reprograming, enhanced cell mortality, invasion, resistance to chemotherapy and radiotherapy, as well as cancer stem cell specification and immune evasion (Wicks & Semenza, 2022).

1.2.3. Hypoxic microenvironment induced pre-mRNA splicing changes

Under pathological conditions, notably human cancers, changes in alternative splicing patterns of genes and splicing factor expression have been shown. Numerous pre-mRNA transcripts have been identified whose splicing regulation is altered in response to hypoxic conditions

(Kanopka, 2017). Alternative splicing changes induced by hypoxic conditions, are known to occur in tumours. Such changes have been observed in lung, colon, breast, head and neck cancers (Farina *et al*., 2020). Hypoxia is also known to cause SR protein hyperphosphorylation, which leads to SR proteins interacting with RNA sequences with which they do not associate in normoxic cells. Under hypoxia, these SR proteins are recruited to pre-mRNA, where they recruit splicing factors to sites that are unused under normal oxygen conditions. By these means, SR proteins encourage expression of mRNAs that code for proteins that encourage adaptation to hypoxic conditions (Kanopka, 2017). Hypoxia also plays a part in splicing changes by indirectly encouraging the formation of cytosolic stress granules, which contain stalled translation pre-initiation complexes comprised of mRNAs, translation initiating factors, ribosomal subunits and RNA binding proteins. These stress cytosolic granules modulate hypoxia-induced alternative splicing by amassing SRSF splicing factors and CUGBP Elav-like family (CELF) proteins that encourage decay of mRNA that contain premature stop codons (Farina *et al*., 2020). Many mRNA isoforms that are generated by alternative splicing of pre-mRNA are regulated and preferentially re-expressed in tumour cells, which encourage tumour growth and survival (David & Manley, 2010).

1.3. Specific mRNA isoform formation in disease

1.3.1. *Caspase-9*: crucial mediators in the apoptotic pathway

The caspase-9 (CASP9) protein serves a crucial role in the cellular apoptosis pathway that is triggered by various factors, such as chemotherapy, radiation or stress agents. The caspase-9 protein is known to function as a tumour suppressor (Goehe *et al*., 2010). It belongs to the caspase family of protease enzymes which in human cells contains 12 members (Hong *et al*., 2020; Sahoo *et al*., 2023). The caspase-9 protein becomes active at the establishment of a trimeric complex known as the apoptosome. Splicing of the caspase-9 pre-mRNA is known to produce pro-apoptotic and anti-apoptotic isoforms with differing roles and functions. One is the apoptosis inducing caspase-9a isoform and the other is the survival encouraging caspase-9b isoform (Seol & Billiar, 1999; Hajra & Liu, 2004; Shultz *et al*., 2010). The two isoforms differ in the inclusion or exclusion of a 4-exon cassette, which encompasses exons 3, 4, 5 and 6, and codes a catalytic domain. The retainment of this cassette of exons in the mature mRNA product results in the pro-apoptotic caspase-9a variant, while their exclusion results in the anti-apoptotic caspase-9b protein. The caspase-9b isoform supresses caspase-9a activity by excluding caspase-9a from binding to the apoptosome (Seol & Billiar, 1999; Goehe *et al*., 2010). Caspase-9 dysregulation, namely the alteration of caspase-9a/caspase-9b isoform ratio in favour of caspase-9b, is associated with various cancers (Shultz *et al*., 2010; Avrutsky & Troy, 2021).

1.3.2. *FAS*: a regulator in the apoptotic pathway

The *FAS* (Fas cell surface death receptor) receptor gene is another key regulator in the apoptotic pathway, that is subject to alternative splicing. It is expressed on the surface of numerous cell types, where the FAS receptor is triggered by the cytotoxic T cell-generated FAS ligand, which induces a chain of events that lead to programmed cell death (Bouillet & O'Reilly, 2009). FAS pre-mRNA is alternatively spliced to produce multiple isoforms, including the soluble FAS isoform that omits exon 6 (Ruberti *et al*., 1996). The resulting soluble FAS protein is missing the transmembrane domain that is coded by exon 6, and the protein isoforms that are expressed by cancer cells function as decoy receptors for the FAS ligand; the FAS ligand interacts with the decoy receptor and apoptosis signals are inhibited, thereby enabling cancer cells to avoid apoptosis (Cheng *et al*., 1994). Increased expression of the soluble FAS protein is known to occur in numerous cancer types. It has been shown that a hypoxic microenvironment alters alternative splicing of FAS pre-mRNA and encourages the expression of soluble FAS mRNA. In addition, it was shown that due to increased modification level of U2AF35 and U2AF65 proteins in hypoxic cells, there is a diminished interaction efficiency of these proteins with RNA. This leads to a decrease in the full-length FAS isoform and an increase in the soluble FAS mRNA isoform (Vilys *et al*., 2020).

1.3.3. *PUF60*: splicing regulator

Poly-U-binding factor 60 kDa (PUF60) is an important splicing factor that plays a crucial function in the regulation of RNA splicing and transcription. PUF60 proteins synergistically bind along with the U2AF65 splicing factor to RNA by engaging with the pyrimidine region, thus encouraging splicing of introns that have weak 3’ splice sites (Page-McCaw *et al*., 1999; Chen *et al*., 2025). The PUF60 protein contains two central RNA recognition motifs and a U2AF homology motif (UHM), an RNA recognition motif that binds to UHM ligand motifs (ULM), at its C-terminal. ULMs are a type of linear peptide motifs that contain tryptophan. The UHMs of PUF60 and U2AF65 proteins simultaneously and non-competitively bind to specific ULMs at the N-terminal region of splicing factor SF3b155, which is a critical U2 snRNP component that functions as foundation for spliceosome assembly, and both proteins recruit SF3b155 to the 3’ splice site (Corsini *et al*., 2009). U2AF and PUF60 proteins promote the association of U2 snRNP with pre-mRNA (Page-McCaw *et al*., 1999). Fluctuations of PUF60 protein levels result in changes in selection of alternative splice sites, for example alternative splice site selection in Amyloid Beta Protein (APP) (cell surface receptor coding gene, associated with Alzheimer’s disease) pre-mRNA is regulated by PUF60 protein levels (Hastings *et al*., 2007; Baumkotter *et al* 2014; Pfundstein *et al*., 2022). The majority of cancer cells exhibit substantial overexpression of PUF60, suggesting that

it may be a suitable target for therapeutic agents in the treatment of oncological diseases (Chen *et al*., 2025).

1.3.4. *FGFR1OP*: a ciliogenesis, cell growth and centrosome mediator

The Fibroblast Growth Factor Receptor 1(*FGFR1*) oncogene partner (FGFR1OP) protein is associated with ciliogenesis, cell growth, cellular motility, as well as centrosome mediation (Lee & Stearns, 2013; Tollenaere *et al*., 2015). Additionally, the protein seems to be necessary in cell cycle progression (Acquaviva *et al*., 2009). *FGFROP1* serves as a fusion partner for *FGFR1*, which is triggered by chromosomal translocations that occur in haematological malignancies. These translocations usually arise in patients with myeloproliferative neoplasms, in whom it initially manifests as eosinophilia and lymphadenopathy, which eventually leads to acute myeloid or mixed lineage leukaemia. The fusion protein that results from fused *FGFR1OP* and *FGFR1* genes, functions as a non-transmembrane-type FGFR kinase in myeloproliferative syndrome and peripheral T-cell lymphoma (Figure 10). This fusion protein has an oncogenic potential due to the lack of extracellular and transmembrane domains, which results in alterations in subcellular localisation (Katoh, 2016). Different transcripts of *FGFR1OP* result from either exon 7 or exon 11 being excluded from the forming mRNA during the splicing process (Popovici *et al*., 1999). Moreover, FGFR1OP insufficiency associated with the development of Crohn's Disease (Trsan *et al*., 2024).

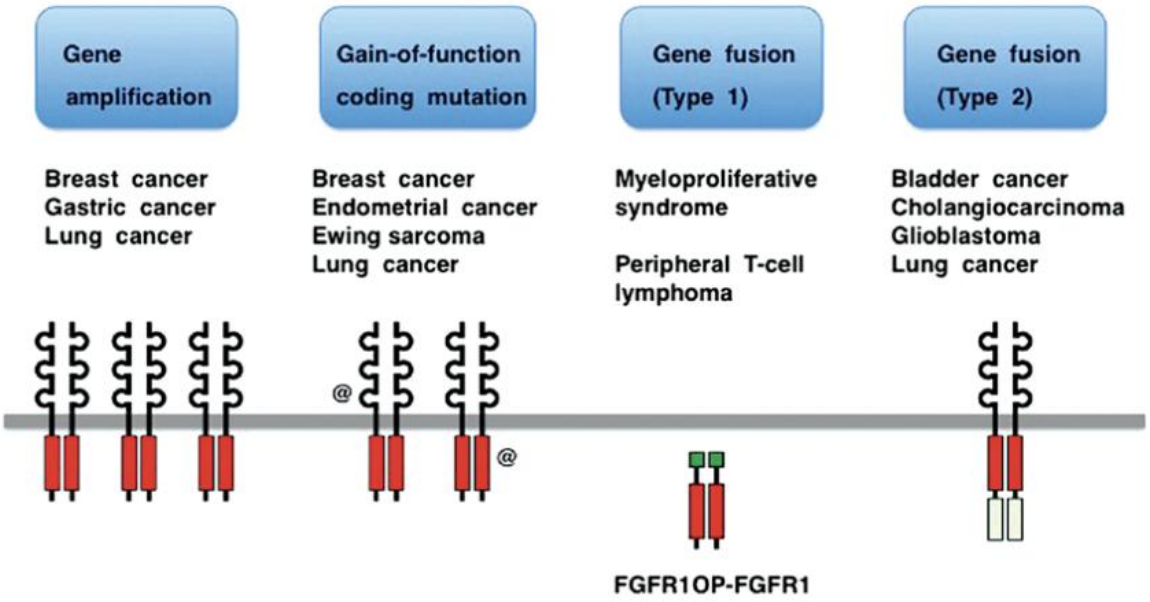

Figure 10. Fibroblast growth factor receptor activation in cancer due to gene amplification, coding mutation or gene fusion. The *FGFR1OP-FGFR1* fusion ultimately results in non-receptor type kinases (Modified from Katoh, 2016)

1.3.5. *APAF1*: a regulator of apoptosome formation

Proteins derived from the Apoptotic Peptidase Activating Factor 1 (*APAF1*) gene transcripts are associated with the process of programmed cell death, due to their role in formation of the apoptosome apparatus. Hypoxic conditions induce the exclusion of exon 17a from APAF1 mRNA during the splicing process in cancer cells, and the resulting APAF1 protein isoform does not facilitate apoptosome formation (Benedict *et al*., 2000; Bao *et al*., 2006; Pečiulienė *et al*., 2022).

1.3.6. *CDC42BPA*: a regulator of cell migration

Cell division cycle 42 (CDC42) binding protein kinase alpha (CDC42BPA) proteins induce the formation of actin-based structures, known as filopodia. Moreover, these proteins are associated with cell migration processes as well as cytoskeleton organisation and cell growth (Nobes *et al*., 1995; Pečiulienė *et al*., 2022). *CDC42BPA* and B-Raf Proto-Oncogene, Serine/Threonine Kinase (*BRAF*) fusion is associated with cancer development in desmoplastic infantile astrocytoma/ganglioglioma (Guinle *et al*., 2024).

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium hydrogen phosphate (Sigma-Aldrich, USA).

Fetal bovine serum (FBS); penicillin / streptomycin; Trypsin / EDTA (Biochrom, Germany).

Dimethyl sulfoxide (DMSO); McCoy's 5A medium (Thermo Fisher Scientific, USA).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Merck, Germany).

Isopropyl alcohol (Carl Roth, Germany).

Agarose, Ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (Tris), Ethanol (Roth, Germany).

Boric acid, ethidium bromide (Sigma-Aldrich, USA).

Deoxynucleotide triphosphate (dNTP) (Thermo Fisher Scientific, USA),

MassRuler DNA Ladder Mix (#SM0403) (Thermo Fisher Scientific, USA),

DNA Gel Loading Dye (6X) (Thermo Fisher Scientific, USA).

2.1.2. Solutions

Tris-borate-EDTA (TBE) solution: 89.8 mM Tris, 90 mM Boric acid, 1.96 mM EDTA (pH 8).

Ethidium bromide solution: 0.5 μg/ml ethidium bromide.

PBS buffer: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.

Agarose gel: 1.5% agarose in TBE buffer solution.

2.1.3. Cell growth media

Cell growth medium: McCoy's 5A medium, 10 % FBS, 100 U penicillin and 0.1 mg/ml streptomycin solution.

Cell freezing medium: 10 % DMSO in fetal bovine serum.

2.1.4. Kits and enzymes.

RNA purification was done with Quick-RNA™ Miniprep Kit, in which the enzyme DNase I was included (Zymo Research, USA).

Synthesis of complementary DNA (cDNA) was accomplished with RevertAid First Strand cDNA Synthesis Kit (#K1621), which includes the enzymes: RiboLock RNase Inhibitor and RevertAid M-MuLV RT reverse transcriptase (Thermo Fisher Scientific, USA).

DreamTaq DNA polymerase was used in target DNA fragment amplification by PCR (Thermo Fisher Scientific, USA).

Trypsin solution (0.5 μg/ml) (Sigma-Aldrich, USA)

2.1.5. Primers used for DNA amplification by PCR

Primers used for DNA amplification by PCR are shown in Table 1.

Table 1. Utilised primers

Primer	Sequence	Annealing temperature
18S (Dir.)	5' - AACTCACTGAAGATGAGGTG - 3'	60 °C
18S (Rev.)	5' - CAGACAAGGCCTACAGACTT - 3'	
FAS (Dir.)	5' - GTGAACACTGTGACCCTTGC - 3'	59 °C
FAS (Rev.)	5' - CCTTGGTTTTCCTTTCTGTGC - 3'	
FGFR1OP (Dir.)	5' - CTGTGGGTGGACCCTTATTATTAG - 3'	63 °C
FGFR1OP (Rev.)	5' - GACACTTGTATCACTCTGATTGCC - 3'	
PUF60 (Dir.)	5' - GCCAAGAAGTACGCCATGG - 3'	64 °C
PUF60 (Rev.)	5' - GTAGACGCGGCACATGATG - 3'	
APAF1 (Dir.)	5' - GTGAAGTGTTGTTCGTGGTCTG - 3'	62 °C
APAF1 (Rev.)	5' - CATCACACCATGAACCCAAC - 3'	
CDC42BPA (Dir.)	5' - GCTAATGCTGTGAGGCAAGAAC - 3'	65 °C
CDC42BPA (Rev.)	5' - GCTCACTCTGTTCACGTAGCTT - 3'	
CASP9 (Dir.)	5' - GCTCTTCCTTTGTTCATCTCC – 3'	60 °C
CASP9 (Rev.)	5' - CATCTGGCTCGGGGTTACTGC – 3'	

2.1.6. Used Cell line

The Human colorectal carcinoma HCT116 cell line was provided by the VU LSC department of immunology and cell biology, acquired from ATCC.

2.1.7. Bioinformatical analysis tools

Half-maximal inhibitory concentration is calculated with GraphPad Prism 10 (Version 10.4.1, GraphPad Software Inc, 2024). Isoform expression results were quantified with AzureSpot Pro image analysis software (Azure biosystems, USA). Statistical significance of PCR results was determined with R studio (Build 561, Posit PBC, 2023), Students t-test, results: *$p<0.05$

2.2. Methods

2.2.1. HCT116 cell cultivation

HCT116 cells were cultivated with McCoy's medium with 10% fetal bovine serum (FBS) and 100 U penicillin and 0.1 mg/ml streptomycin solution (p/s). The cell culture was grown in a CO_2 incubator (21% O_2, 5% CO_2) at 37 °C and 95% humidity.

When adhesive cells covered approximately 90% of the surface of the cultivation flask, the cells were split. They were washed with phosphate buffered saline (PBS) and then incubated with trypsin (TRP) for 5-10 min at 37°C. HCT116 cells were resuspended in growth medium. Cell count was assessed according to the manufacturer's instructions with the Cedex HiRes Analyzer (F. Hoffmann-La Roche AG) cell analysis system.

2.2.2. Preparation of cells for MTT assay

The cells were seeded in a 96-well cell culture plate, at a concentration of 5000 cells in one well and grown for 24 hours. Following this, the growth medium was removed from the cells. Then 100 µL of McCoy's medium with different analysed compounds at a final concentration of 100 µM were added to each respective experimental point (3 wells per compound). Additional well triplets served as positive control (100 µL McCoy's medium, with cells), negative control (100 µL McCoy's medium, without cells). Vehicle control (100 µL McCoy's with 100 µM DMSO, with cells) was used to verify the effect of DMSO, in which the compounds were dissolved, on cell viability. Cells were incubated for 72 hours at 37°C.

2.2.3. Cell viability assessment via the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay

After cell incubation, the media was removed from the cells and 0.2 mg/mL of MTT reagent with McCoy's medium without fetal bovine serum was added to the wells, then the cells were maintained under these conditions for 2 hours. After incubation, the majority of the MTT solution was removed, and isopropyl alcohol was added. Absorption was measured at 570 nm to determine cell viability. This is due to formazan, a product of the MTT reduction reaction that occurs in viable cells, absorbing most effectively at this wavelength.

Measured optical density values depend on formazan concentration, which reflects MTT reagent reduction in viable cells. Viability was calculated with the following formula:

$$\left(\frac{A_{treatment} - A_{blank}}{A_{control} - A_{blank}}\right) \times 100\%$$

$A_{treatment}$ – *Absorption of cells treated with the analysed compounds.*

A_{blank} – *Positive control, growth media only.*

$A_{control}$ – *Negative control, absorption of cells treated with DMSO.*

2.2.4. Determining half-maximal inhibitory concentration.

Cells were prepared for incubation with different concentrations of compounds IT 123, IT 126 and IT 135 in McCoy's medium, increasing by increments of 10 μM, producing a concentration gradient ranging from 10 μM to 100 μM. Each concentration was tested in triplicate. The half-maximal inhibitory concentration was determined with the same MTT assay.

2.2.5. Cell freezing and thawing

After the removal of cell growth medium, the cells were washed with PBS buffer and incubated in a trypsin solution (0.5 μg/ml) for 5-10 min at 37 ºC. The cells were collected and then centrifuged for 5 min at a speed of 1000 × g. The supernatant was discarded; the cells were resuspended in cell freezing medium (10% DMSO in fetal bovine serum) and then the cells were distributed into cryovials. The cryovials were then stored in a freezer at a temperature of –70 °C, where they cooled at a rate of 1 °C/min. Following this, they were placed in cryogenic storage liquid nitrogen tanks.

If the frozen cells were required to be thawed for further analysis, they were kept at a temperature of 37 °C for 1 min, after which they were immediately seeded in a sterile flask with growth media.

2.2.6. RNA isolation from HCT116 cells and cDNA synthesis

RNA from HCT116 cells, which were cultivated in growth media with selected triazole-based compounds (IT123, IT126, IT135), at their half-maximal inhibitory concentrations for 72 hours under normoxia or hypoxia, was isolated and purified with the RNA purification kit "Quick-RNA™ Miniprep Kit" according to the manufacturer's instructions. RNA concentration was determined with NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA).

cDNA synthesis was conducted with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions.

2.2.7. DNA fragment amplification by PCR and visualisation using electrophoresis

cDNAs were amplified using primers provided in Table 1, as outlined in the program shown in Table 2. Components for the PCR reaction mix are provided in Table 3.

Table 2. DNA fragment amplification by PCR program

Step	Step 1	Step 1	Step 2	Step 3	Step 1	Step 2
Temperature	95 °C	95 °C	Primer annealing temperature	72 °C	72 °C	4 °C
Time	2 min.	20 s.	20 s.	30 s.	5 min.	∞
Cycle	1 cycle	30-35 cycles			1 cycle	

Table 3. PCR components for one sample

DreamTaq buffer	1/10 (v/v)
dNTP	0.2 mM
Fw. primer	0.2 µg
Rev. primer	0.2 µg
DreamTaq DNA polymerase	1U
Template DNA	1 µg
H_2O	Up to 20 µl

PCR products were analysed using agarose gel (1.5%). Samples were mixed with DNA Gel Loading Dye and loaded onto the gel. Electrophoresis was carried out in TBE buffer at 125 V for 1-2 hours. The agarose gel was dyed with ethidium bromide for 10 minutes, after which the amplified DNA fragments were visualised under UV light with an Azure 280 imaging system (Azure biosystems, USA).

3. Results

3.1. Assessment of the effects of triazole-based compounds on HCT116 cell viability

The initial concentration of the tested compounds (100 μM) was chosen based on earlier studies using breast cancer MDA-MB-231 and glioblastoma U-87 cell lines (Šermukšnytė *et al*., 2024). The structures and abbreviations of the compounds used in this study are provided in Appendix 1., the exact structures of two synthesised compounds, IT-129 and IT-133, aren't provided, as the person who synthesised these compounds did not disclose them to us.

Analysis of the effects of the triazole-based compounds on colorectal cancer cell (HCT116) viability, via the MTT assay, revealed that the compounds IT 123, IT 126 and IT 135 had the greatest effect on cell viability (Figure 11). These compounds reduced HCT116 cell viability to 17%, 8% and 22%, respectively. Compounds IT 124, IT 125, IT 129, IT 130, IT 131, IT A-16 also showed cytotoxic effects, which reduced cell viability to 29%, 32%, 32%, 40%, 43% and 45%, respectively. The other examined compounds did not have any notable effect on cell viability and did not reduce cell viability by more than 50% (Figure 11).

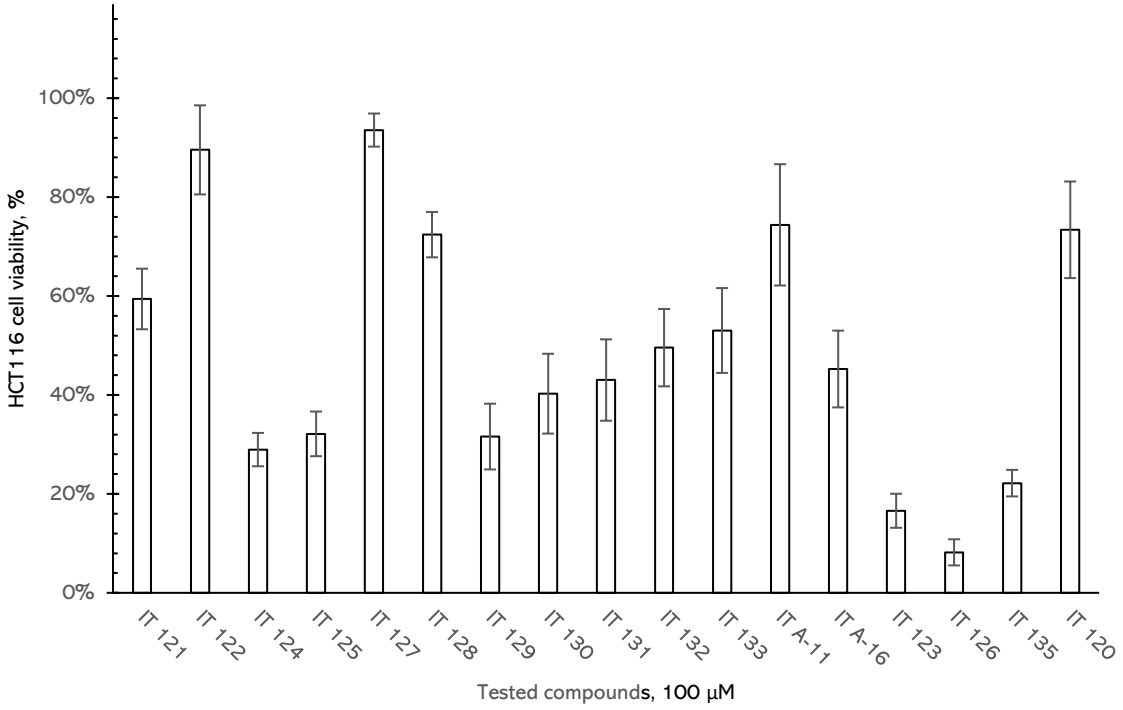

Figure 11. Colorectal cancer (HCT116) cell viability. HCT116 cells were treated for 72 hours with 100 μM concentration triazole-based compounds (IT 121, IT 122, IT 124, IT 125, IT 127, IT 128, IT 129, IT 130, IT 131, IT 132, IT 133, IT A-11, IT A-16, IT 123, IT 126, IT 135, IT 120). Viability was determined with the MTT assay, represented by the columns. Results are shown as the mean and standard deviation of three technical replicates

These MTT results indicate that in the case of HCT116 cells, at a concentration of 100 μM, the greatest cytotoxic effects were exhibited by compounds IT 123, IT 126 and IT 135 (Table 4).

Table 4. Colorectal cancer (HCT116) cell viability, after treatment with triazole-based compounds

Compound	IT 121	IT 122	IT 124	IT 125	IT 127	IT 128	IT 129	IT 130	IT 131
Viability, %	59%	90%	29%	32%	94%	72%	32%	40%	43%
Compound	IT 132	IT 133	IT A-11	IT A-16	IT 123	IT 126	IT 135	IT 120	
Viability, %	50%	53%	74%	45%	17%	8%	22%	73%	

These three compounds were selected for further analysis of their effectiveness by evaluating their half-maximal inhibitory concentration (IC_{50}).

Assessment of the viability of HCT116 cells depending on dose of tested compounds suggested that compounds IT 123 and IT 135 had similar effects on cell viability (Figure 12 A, C). The determined half-maximal inhibitory concentrations of compounds IT 123, IT 126 and IT 135 on HCT116 cell viability is shown in Table 5.

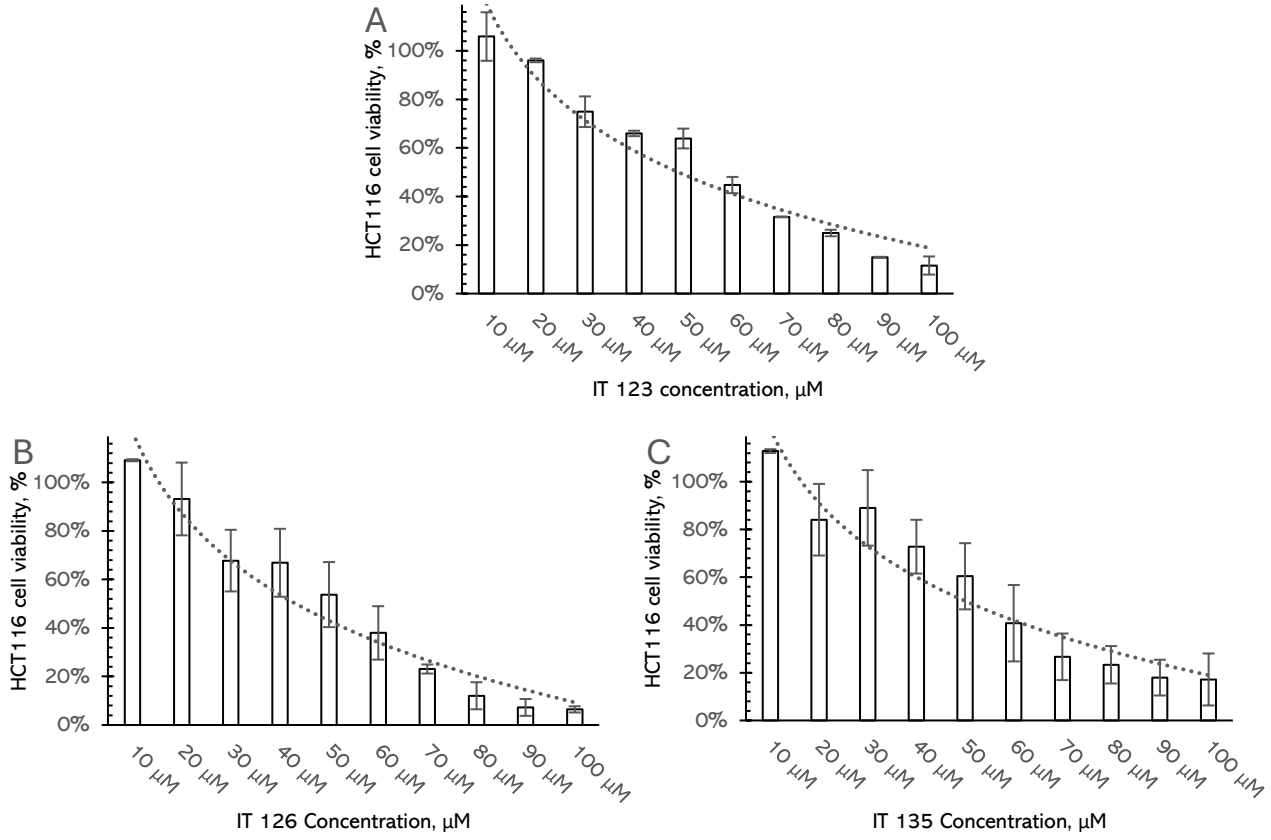

Figure 12. The half-maximal inhibitory concentration of compounds IT 123 (A), IT 126 (B) and IT 135 (C) was determined by assessing HCT116 cell viability at different concentrations. Dotted line represents logarithmic curve of the response to dose. Data represents mean and standard deviation of three technical replicates

Table 5. IC_{50} values of the tested compounds in HCT116 cells

Compound	IT 123	IT 126	IT 135
IC_{50}	51.39 μM	41.23 μM	53.09 μM

3.2. Effect of triazole-based compounds on mRNA isoform formation of cancer-associated genes

The established IC_{50} concentrations of compounds IT123, IT126 and IT135 were used in further analysis of their effects, on the mRNA isoform formation of selected cancer-associated genes in cells cultured under normal (21% O_2) or hypoxic conditions (1% O_2). A hypoxic microenvironment is a common feature in solid tumours (Chen et al., 2023).

3.2.1. Triazole-based compound facilitated changes in FAS pre-mRNA splicing

In cells cultured under normal oxygen conditions the ratio of exon 6-retaining FAS (FAS isoform) and exon 6-skipping (sFAS isoform) soluble FAS isoforms, was approximately 4:1 (Figure 13, lane 1). In cells treated with compound IT123 this ratio is approximately 2:1 (Figure 13, lane 2). This result indicates that compound IT123 has a slight effect on the formation of the sFAS mRNA isoform under normal oxygen conditions. Compounds IT126 and IT135 slightly increased FAS mRNA formation (Figure 13, lanes 3-4). In cells cultured under hypoxic conditions, the ratio of FAS and sFAS mRNA isoforms is approximately 1:2 (Figure 13, lane 5). This result indicates that cellular hypoxia influences exon 6 inclusion/exclusion in FAS mRNA in HCT116 cells. Treatment of cells cultivated under hypoxia, with compounds IT126 and IT135 slightly encouraged FAS mRNA isoform formation, nearly altering the ratio to 1:1 (Figure 13, lanes 7-8). Compound IT123 showed little effect on FAS mRNA formation (Figure 13, lane 6).

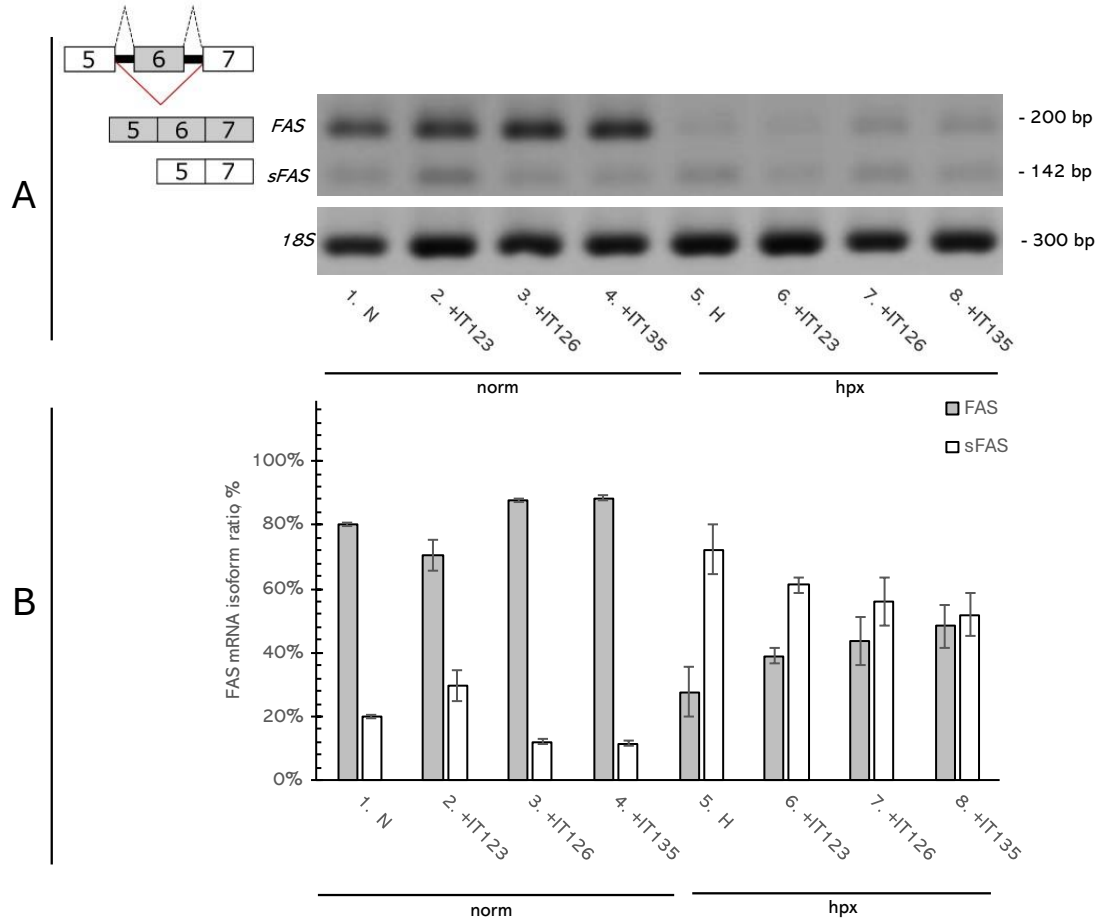

Figure 13. Change in FAS mRNA isoform expression. (A) Schematic depiction of FAS pre-mRNA and exon 6-including/excluding mRNA isoform variants, as well as PCR results of FAS mRNA isoform expression in HCT116 cells under normal (21% O_2; norm) and hypoxic (1% O_2; hpx) conditions. 18S served as loading control. (B) Quantified expression ratio of two FAS isoforms under normal (norm; lanes 1-4) and hypoxic (hpx; lanes 5-8) conditions. FAS – exon 6-retaining FAS mRNA isoform, sFAS – exon 6-excluding soluble FAS mRNA isoform, N – isoform ratio in cells cultivated under normoxia (21% O2), H – isoform formation in cells cultivated under hypoxia (1% O2), +IT123/+IT126/+IT135 – isoform formation in cells cultivated in media with 51.39 µM of IT 123, 41.23 µM of IT 126 and 53.09 µM of IT 135 respectively, under either normoxia or hypoxia. Total FAS mRNA was labelled as 100%. Data represents mean and standard deviation of three technical replicates

3.2.2. Triazole-based compound facilitated changes in FGFR1OP pre-mRNA splicing

In untreated cells cultured under normal oxygen conditions, the ratio of mRNA isoforms that include/exclude exon 7 was 1:1.5 (Figure 14, lane 1). HCT116 cells that were cultured under normal oxygen conditions, treated with compounds IT123, IT 126 and IT 135, did not result in any meaningful change in the ratio of exon 7 inclusion/exclusion in FGFR1OP mRNA (Figure 14, lanes 2-4). The ratio of exon 7 inclusion/exclusion in cells cultured under hypoxic conditions, was approximately 1:2 (Figure 14, lane 5). This indicates that hypoxia influences exon 7 inclusion/exclusion in FGFR1OP mRNA (Figure 14, lane 5). Cells cultured under hypoxic

conditions, treated with compounds IT 123, IT 126 and IT 135, did not experience any meaningful change in the expression of these two mRNA isoforms (Figure 14, lanes 6-8).

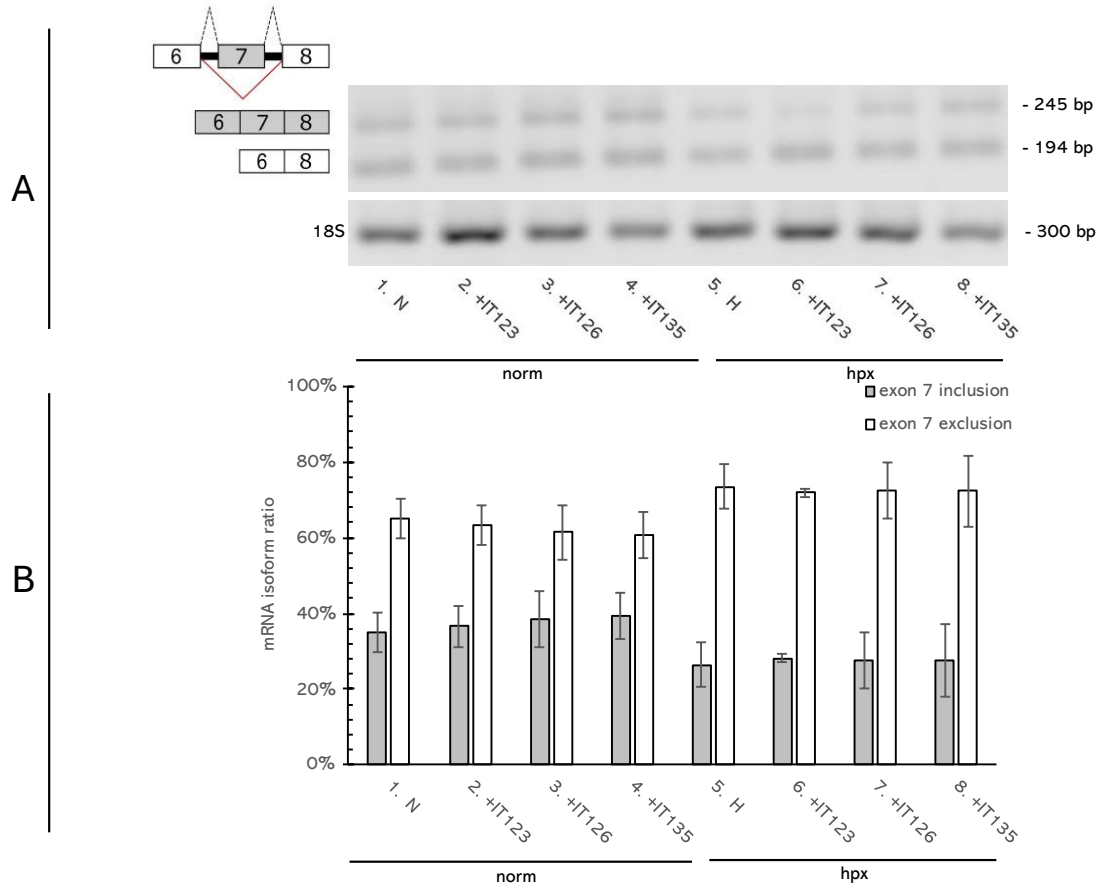

Figure 14. Alteration in FGFR1OP mRNA isoform expression. (A) Schematic representation of FGFR1OP pre-mRNA and exon 7-including/excluding mRNA isoforms, as well as PCR results of FGFR1OP mRNA isoform expression in HCT116 cells under hypoxic (1% O_2; hpx) and normoxic (21% O_2; norm) conditions. 18S was used as loading control. (B) Quantified expression ratio results of the two FGFR1OP isoforms under normoxic (norm; lanes 1-4) and hypoxic (hpx; lanes 5-8) conditions. The exon 7-retaining mRNA isoform is shown in grey, while the exon 7-excluding mRNA isoform is shown in white, N – isoform ratio in cells cultivated under normoxia (21% O2), H – isoform formation in cells cultivated under hypoxia (1% O2), +IT123/+IT126/+IT135 – isoform formation in cells cultivated in media with 51.39 µM of IT 123, 41.23 µM of IT 126 and 53.09 µM of IT 135 respectively, under either normoxia or hypoxia. Total FGFR1OP mRNA was labelled as 100%. Data represents mean and standard deviation of three technical replicates

3.2.3. Triazole-based compound facilitated changes in PUF60 pre-mRNA splicing

In cells cultivated under normal oxygen conditions, the ratio of mRNA isoforms that include exon 5 and isoforms that exclude exon 5 is approximately 1.2:1 (Figure 15, lane 1). In cells treated with compound IT 123 the ratio of exon 5 inclusion/exclusion is reversed (Figure 15, lane 2). This suggests that compound IT 123 slightly influences the formation of these isoforms. The other tested compounds did not have any effect on exon 5 inclusion/exclusion in PUF60 mRNA under normal oxygen conditions (Figure 15, lanes 3-4). Hypoxic conditions promoted exon 6 inclusion in PUF60

mRNA, and the ratio of exon 5 inclusion/exclusion was 1:4 (Figure 15, lane 5). This suggests that hypoxic conditions noticeably influence the inclusion/exclusion of this exon in forming PUF60 mRNA. HCT116 cells that were cultured under hypoxic conditions, treated with compounds IT123, IT 126 and IT 135, did not result in any meaningful change in the ratio of exon 5 inclusion/exclusion in PUF60 mRNA (Figure 15, lanes 6-8).

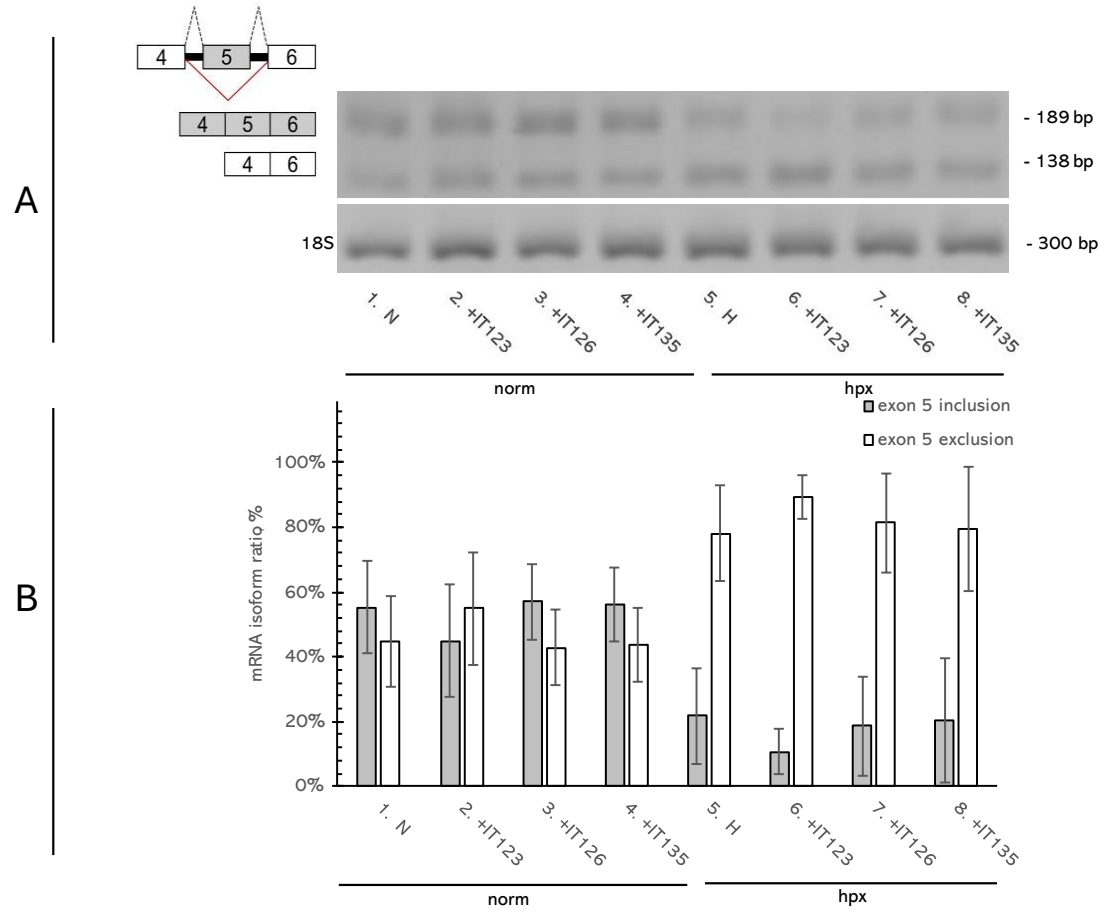

Figure 15. Change in PUF60 mRNA isoform expression. (A) Schematic representation of PUF60 pre-mRNA and exon 5-including/excluding mRNA isoforms, as well as PCR results of PUF60 mRNA isoform expression in HCT116 cells under normoxia (21% O_2; norm) and hypoxia (1% O_2; hpx). 18S was used as loading control. (B) Quantified expression ratio of two PUF60 isoforms under normal (norm; lanes 1-4) and hypoxic (hpx; lanes 5-8) conditions. The exon 5-retaining mRNA isoform is shown in grey, while the exon 5-excluding mRNA isoform is shown in white, N – isoform ratio in cells cultivated under normoxia (21% O2), H – isoform formation in cells cultivated under hypoxia (1% O2), +IT123/+IT126/+IT135 – isoform formation in cells cultivated in media with 51.39 µM of IT 123, 41.23 µM of IT 126 and 53.09 µM of IT 135 respectively, under either normoxia or hypoxia. Total PUF60 mRNA was labelled as 100%. Data represents mean and standard deviation of three technical replicates

3.2.4. Triazole-based compound facilitated changes in APAF1 pre-mRNA splicing

The ratio of 17a-including/excluding APAF1 mRNA isoforms, under normal oxygen conditions was approximately 1.2:1 (Figure 16, lane 1). In cells treated with compound IT 123, the ratio of exon 17a inclusion/exclusion is 1:1.8 (Figure 16, lane 2). This indicates that compound IT

123 slightly encourages the skipping of exon 17a. The other tested compounds, under normal oxygen conditions, did not influence the inclusion/exclusion of exon 17a in APAF1 mRNA. The ratio of 17a-including/excluding APAF1 mRNA isoforms, under hypoxic conditions was approximately 1:1.9 (Figure 16, lane 5). This indicates that hypoxic conditions greatly influenced the exclusion of exon 17a in APAF1 mRNA. Treatment of HCT116 cells that were cultivated under hypoxic conditions, with compounds IT123, IT126 and IT135, had no influence on exon 17a inclusion/exclusion (Figure 16, lanes 6-8).

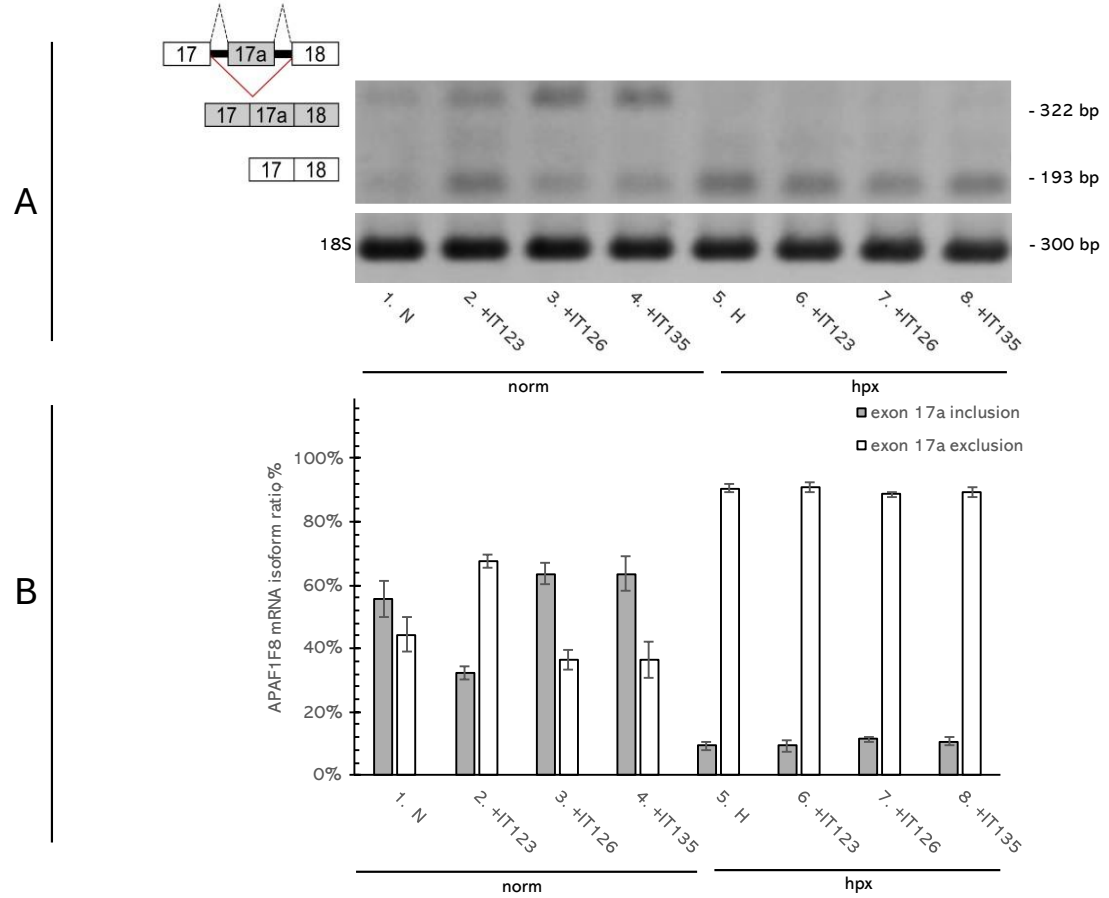

Figure 16. Change in APAF1 mRNA isoform expression. (A) Schematic representation of APAF1 pre-mRNA and exon 17a-including/excluding mRNA isoforms, as well as the PCR results of APAF1 mRNA isoform expression in HCT116 cells under normoxic (21% O_2; norm) and hypoxic (1% O_2; hpx) conditions. 18S was used as loading control. (B) Quantified expression ratio of two APAF1 isoforms under normal (norm; lanes 1-4) and hypoxic (hpx; lanes 5-8) conditions. The exon 17a-retaining mRNA isoform is shown in grey, while the exon 17a-excluding mRNA isoform is shown in white, N – isoform ratio in cells cultivated under normoxia (21% O2), H – isoform formation in cells cultivated under hypoxia (1% O2), +IT123/+IT126/+IT135 – isoform formation in cells cultivated in media with 51.39 µM of IT 123, 41.23 µM of IT 126 and 53.09 µM of IT 135 respectively, under either normoxia or hypoxia. Total APAF1 mRNA was labelled as 100%. Data represents mean and standard deviation of three technical replicates

3.2.5. Triazole-based compound facilitated changes in CDC42BPA pre-mRNA splicing

In untreated cells cultivated under normal oxygen conditions, the ratio of inclusion/exclusion of exon 13 in CDC42BPA mRNA was approximately 1.6:1 (Figure 17, lane 1). The treatment of cells with compounds IT126 and IT135, slightly encouraged exon 13 skipping in forming CDC42BPA mRNA. Hypoxic conditions encouraged exon 13 inclusion in CDC42BPA mRNA. The ratio of exon 13 inclusion/exclusion was approximately 4:1 (Figure 17, lane 5). Treatment of HCT116 cells cultivated under hypoxic conditions, with compounds IT123, IT126 and IT135, did not influence exon 13 inclusion/exclusion in CDC42BPA mRNA (Figure 17, lanes 6-8)

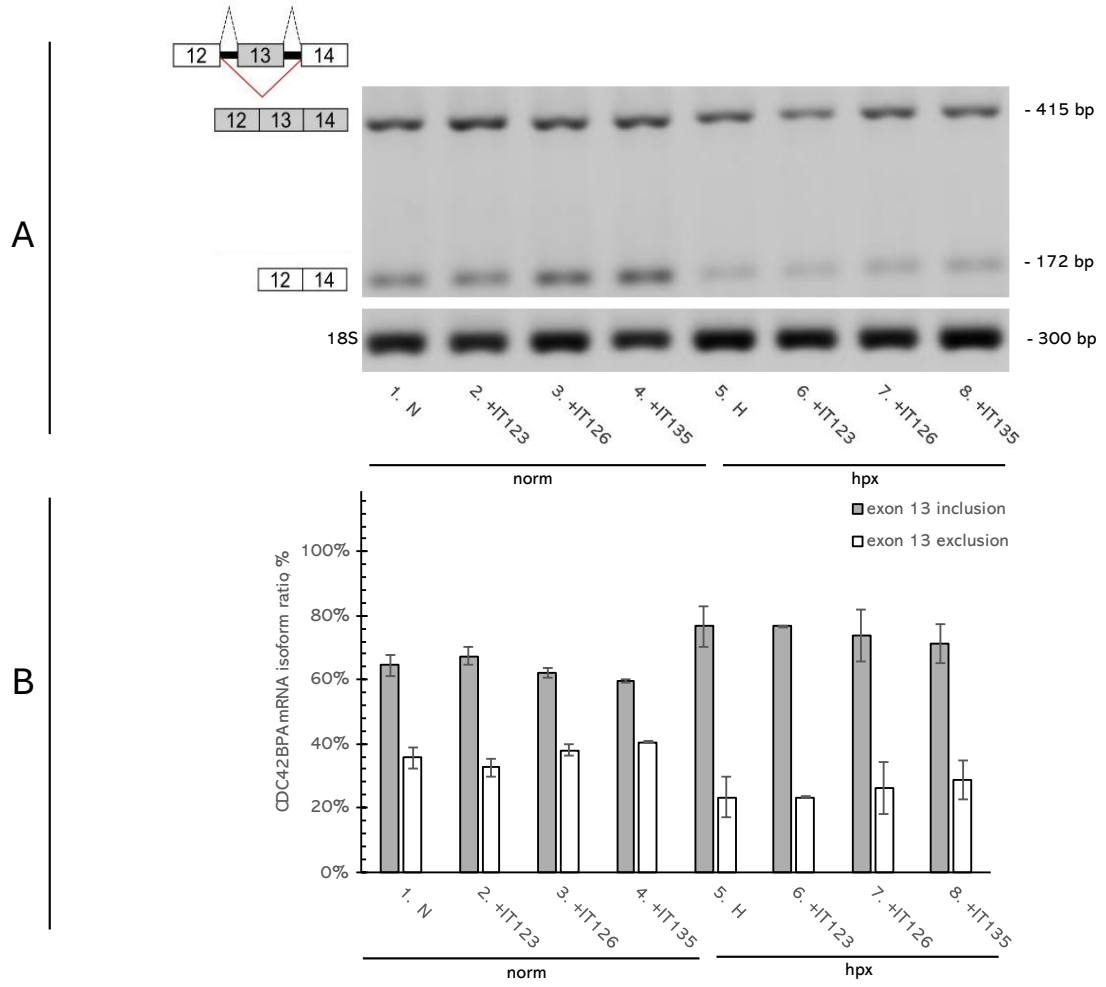

Figure 17. Alteration in CDC42BPA mRNA isoform expression. (A) Schematic representation of CDC42BPA pre-mRNA and exon 13-including/excluding mRNA isoforms, as well as the PCR results of CDC42BPA mRNA isoform expression in HCT116 cells cultured under normoxic (21% O_2; norm) and hypoxic (1% O_2; hpx) conditions. 18S was used as loading control. (B) Quantified expression ratio of two CDC42BPA isoforms under normal (norm; lanes 1-4) and hypoxic (hpx; lanes 5-8) conditions. The exon 13-retaining mRNA isoform is shown in grey, while the exon 13-excluding mRNA isoform is shown in white, N – isoform ratio in cells cultivated under normoxia (21% O2), H – isoform formation in cells cultivated under hypoxia (1% O2), +IT123/+IT126/+IT135 – isoform formation in cells cultivated in media with 51.39 µM of IT 123, 41.23 µM of IT 126 and 53.09 µM of IT 135 respectively, under either normoxia or hypoxia. Total CDC42BPA mRNA was labelled as 100%. Data represents mean and standard deviation of three technical replicates

3.2.6. Triazole-based compound facilitated changes in CASP9 pre-mRNA splicing

In cells cultured under normal oxygen conditions, the ratio of the exon 3, 4, 5, 6 cassette including caspase-9a mRNA isoform and the exon cassette excluding caspase-9b mRNA isoform was approximately 5:1 (Figure 18, lane 1). The treatment of cells with compound IT123, IT126 and IT135 did not influence the inclusion/exclusion of the exon cassette in caspase-9 mRNA (Figure 18, lanes 2-4). Hypoxic conditions slightly reduced the expression of the caspase-9b mRNA isoform (Figure 18, lane 5). Treatment of HCT116 cells, with compounds IT123, IT126 and IT135 did not influence the inclusion/exclusion of the exon cassette in caspase-9 mRNA (Figure 18, lanes 6-8).

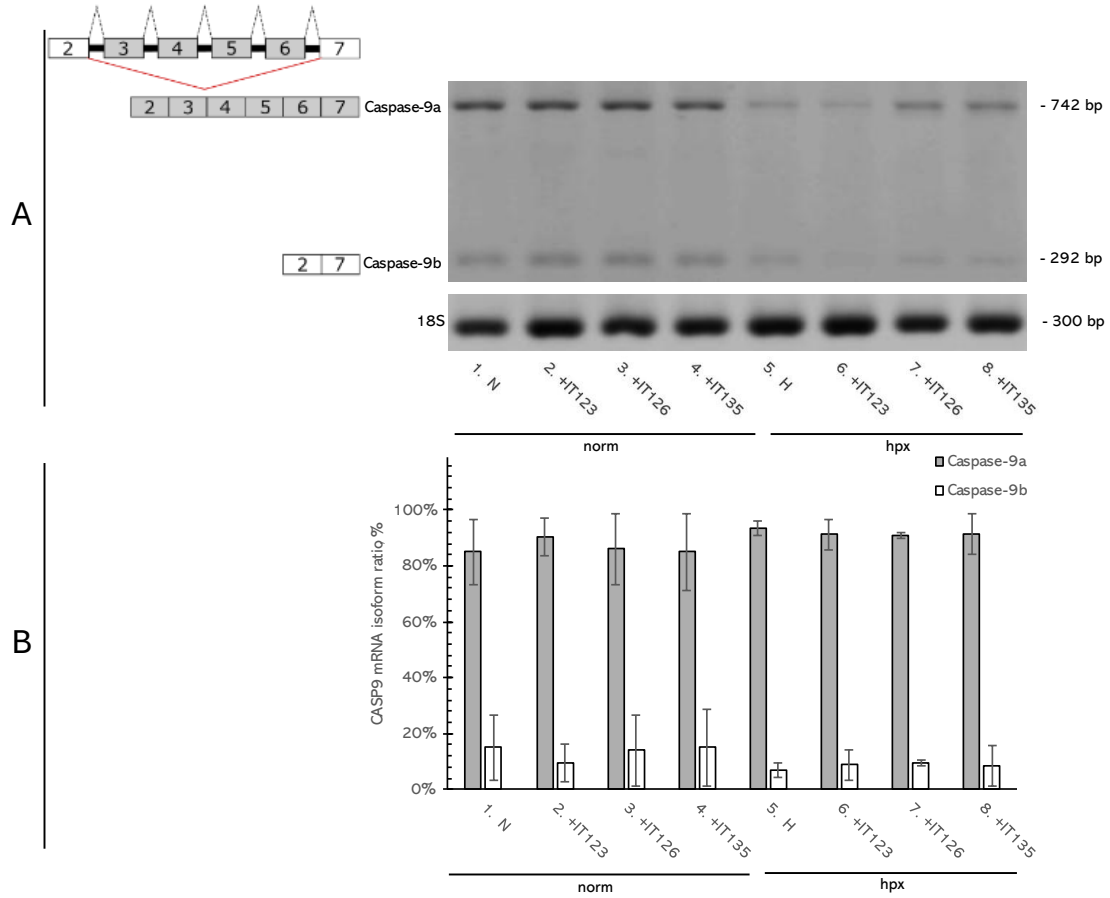

Figure 18. CASP9 mRNA isoform expression alterations. (A) Schematic representation of CASP9 pre-mRNA and exon cassette-including/excluding mRNA isoforms, as well as the PCR results of CASP9 mRNA isoform expression in HCT116 cells cultured under normal oxygen (21% O_2; norm) and hypoxic (1% O_2; hpx) conditions. 18S was used as loading control. (B) Quantified expression ratio of two CASP9 isoforms under normal (norm; lanes 1-4) and hypoxic (hpx; lanes 5-8) conditions. Caspase-9a – exon cassette-retaining caspase-9 mRNA isoform shown in grey, caspase-9b – exon cassette-excluding caspase-9b mRNA isoform shown in white, N – isoform ratio in cells cultivated under normoxia (21% O2), H – isoform formation in cells cultivated under hypoxia (1% O2), +IT123/+IT126/+IT135 – isoform formation in cells cultivated in media with 51.39 µM of IT 123, 41.23 µM of IT 126 and 53.09 µM of IT 135 respectively, under either normoxia or hypoxia. Total caspase-9 mRNA was labelled as 100%. Data represents mean and standard deviation of three technical replicates

4. Discussion

Evaluation of the effects of potential anticancer drugs on cell viability is the initial step in a typical anti-cancer drug evaluation process. An earlier study that screened the same compounds using the MTT assay, was done, using breast cancer and glioblastoma cells. In this study, we show the effect of these compounds on cell viability using the human colorectal carcinoma HCT116 cell line.

Compounds IT 123, IT 126 and IT 135 had the greatest effect on breast cancer and glioblastoma cells. These compounds reduced breast cancer cell viability to 19.9 ± 0.82%, 18.5 ± 1.04%, and 13.6 ± 0.18%, respectively. While compounds IT 123, IT 126 and IT 135 reduced glioblastoma cell viability to 20.8 ± 2.98%, 12.5 ± 1.73%, and to 12.5 ± 1.81%, respectively (Šermukšnytė *et al*., 2024). In this master's thesis, of the 17 screened triazole-based compounds, IT 123, IT 126 and IT 135 were also recorded to have the greatest effect on HCT116 cell viability reducing it to 16.59 ± 3.44%, 8.18 ± 2.64% and 22.17 ± 2.68%, respectively. Compounds IT 123 and IT 126 had a greater cytotoxic effect on HCT116 cells than breast cancer and glioblastoma, while IT 135 had a lesser effect. The results of IT 124, IT 125, IT 129, IT 130, IT 131, IT A-16 compound screening (29-45%) suggested moderate cytotoxic effects, these results are comparable to results obtained by Šermukšnytė *et al*. (2024) who discovered that these compounds reduce breast cancer and glioblastoma cell viability to 20-50%. In this master's thesis, all other compounds screened using HCT116 cells did not reduce cell viability by more than 50%, a result that is comparable to those obtained from screening with breast cancer and glioblastoma cells.

The most effective compounds, IT 123, IT 126 and IT 135 were selected for further analysis by evaluation of their half-maximal inhibitory concentrations against HCT116 cells. IT 126 had the lowest IC_{50} value (41.23 μM), while IT 123 (51.39 μM) and IT 135 (53.09 μM) had similar values. Comparison of these results with the results obtained by Šermukšnytė *et al*. (2024) who evaluated the IC_{50} values of these compounds against breast cancer and glioblastoma cells, indicates that compound IT 135, had lesser selectivity against HCT116 cells compared to breast cancer cells (IC_{50} value of 39.2 μM), but had similar results to glioblastoma cells (IC_{50} value of 48.9 μM). IT 123, had similar results against breast cancer cells (IC_{50} value of 49.5 μM), and glioblastoma cells (IC_{50} value of 51.1 μM). IT 126 displays selectivity against HCT116 cells compared to breast cancer cells (IC_{50} value of 47 μM), and glioblastoma cells (IC_{50} value of 47.8 μM).

In contrast to this earlier study with breast cancer and glioblastoma cells, we additionally compared mRNA isoform formation of selected cancer-associated genes in colorectal carcinoma

HCT116 cells under normal oxygen and hypoxic conditions, as well as investigate the effects of selected triazole-based compounds on mRNA isoform formation under the same conditions.

Comparative analysis of FAS, FGFR1OP, PUF60, APAF1, CDC42BPA and CASP9 mRNA isoform expression in HCT116 cells cultured under normoxic and hypoxic conditions, indicates that the hypoxic microenvironment affects the pre-mRNA splicing of all six screened genes in HCT116 cells. These results were expected as previous studies found that hypoxic conditions increase the expression of soluble FAS mRNA isoform, caspase-9a mRNA isoform, as well as the exon 7-skipping FGFR1OP mRNA isoform, exon-5 skipping PUF60 mRNA isoform, exon 17a-skipping APAF1 mRNA isoform and exon 13-retaining CDC42BPA mRNA isoform, in various cancer cells (Bowler et al., 2018; Pečiulienė et al., 2022).

Comparison of mRNA isoform expression of selected genes in HCT116 cells that were untreated, and cells treated with compounds IT 123, IT 126 and IT 135, under either normal oxygen conditions or hypoxic conditions, demonstrated that these compounds have gene-specific and oxygen dependent effects.

Soluble FAS proteins which result from sFAS mRNA translation function as decoy receptors that inhibit apoptosis signals and enable cancer cell avoidance of programmed cell death (Cheng *et al*., 1994). Under normal oxygen tension IT123 encouraged sFAS mRNA isoform formation, the soluble FAS proteins derived from this mRNA isoform promote cancer cell survival. However, sFAS mRNA isoform formation under normal oxygen conditions was downregulated by compounds IT126 and IT135, and under hypoxic conditions compounds IT126 and IT135 also downregulated sFAS mRNA isoform formation, reducing cancer cell ability to avoid apoptosis. IT123 did not demonstrate any meaningful effects under hypoxic conditions.

The *PUF60* gene is associated with the regulation of RNA splicing and transcription, moreover the exclusion of exon 5 results in an isoform that functions as a c-myc (Proto-Oncogene C-Myc – gene associated with cell growth, proliferation and apoptosis) repressor (Pelengaris et al., 2002; Bowler *et al*., 2018; Chen *et al*., 2025). Under normal oxygen conditions, compound IT123 slightly encouraged exon 5 exclusion form PUF60 mRNA, but had no effect under hypoxia. Other tested compounds had no effect under either normoxia or hypoxia on PUF60 mRNA isoform formation. Promoted exclusion of exon 5 by IT123 encourages the formation of an isoform that is associated with c-myc repression.

The *APAF1* gene is associated with the the formation of the apoptosome. Exclusion of exon 17a from APAF1 mRNA results in a APAF1 protein isoform that does not facilitate apoptosome formation (Benedict *et al*., 2000; Bao *et al*., 2006; Pečiulienė *et al*., 2022). Under normal oxygen conditions, compound IT123 promoted exon 17a exclusion form APAF1 mRNA but had no effect

under hypoxia. Compounds IT126 and IT135 had no effect under either normoxia or hypoxia on APAF1 mRNA isoform formation. Encouraged exon 17a exclusion by compound IT123 would encourage apoptosome formation inhibition, promoting cell survival.

The *CDC42BPA* gene is associated with the formation of filopodia, cell migration, cytoskeleton organisation and cell growth (Nobes et al., 1995; Pečiulienė et al., 2022). According to Bowler et al. (2018), the exon 13-excluding CDC42BPA isoform has no currently known function. Under normal oxygen conditions, compounds IT126 and IT135 slightly encouraged exon 13 exclusion from CDC42BPA mRNA, while IT123 had no effect. All three compounds had no effect under hypoxia on CDC42BPA mRNA isoform formation. Compounds IT126 and IT135 by encouraging the skipping of exon 13, potentially disrupt the invasive and cell growth potential of cancer cells.

FGFR1OP is a gene associated with ciliogenesis, cell growth, cellular motility, centrosome mediation and cell cycle progression (Acquaviva *et al*., 2009). However, none of the tested compounds had any effect on the formation of FGFR1OP mRNA isoforms under either normoxia or hypoxia, thus the compounds do not alter cancer cell growth or cell cycle progression.

Caspase-9 pre-mRNA splicing can result in two mRNA isoforms with opposite functions, the pro-apoptotic caspase-9a isoform and the anti-apoptotic caspase-9b isoform. The caspase-9b protein inhibits caspase-9a functioning by excluding caspase-9a binding to the apoptosome (Shultz et al., 2010). Unfortunately, none of the tested compounds had any effect on the formation of caspase-9 mRNA isoforms under either normoxia or hypoxia, suggesting that these compounds do not inhibit the ability of cancer cells to avoid apoptosis by alterations in caspase-9 pre-mRNA splicing.

In conclusion, compounds IT123, IT126 and IT135 have the greatest effect on HCT116 cell viability. It is also possible to conclude that the hypoxic microenvironment influences mRNA isoform formation in HCT116 cells and that compound IT123 influences FAS, PUF60 and APAF1 mRNA isoform formation, while compounds IT126 and IT135 influence FAS and CDC452BPA mRNA isoform formation.

Conclusions

1. The analysis of the effects on HCT116 cell viability of the seventeen triazole-based compounds, using the MTT assay, indicated that compounds IT123 (IC_{50} value of 51.39 μM), IT126 (IC_{50} value of 41.23 μM) and IT135 (IC_{50} value of 53.09 μM), had the greatest effect on cell viability, reducing it to 8-22%.
2. Comparative analysis of FAS, FGFR1OP, PUF60, APAF1, CDC42BPA and CASP9 mRNA isoform formation in HCT116 cells cultivated under hypoxic conditions compared to those cultivated under normal oxygen tension, showed that in cells cultured under hypoxic conditions, there was an increase in sFAS, caspase-9a mRNA isoform formation, as well as an increase in exon 7 exclusion in FGFR1OP, exon 5 exclusion in PUF60, exon 17a exclusion in APAF1 and exon 13 inclusion in CDC42BPA mRNAs.
3. Comparative analysis of selected gene pre-mRNA splicing in HCT116 cells cultivated under normal oxygen tension and treated with selected compounds showed that compound IT123 promoted sFAS mRNA isoform formation, exon 17a exclusion from APAF1 mRNA and slightly encouraged exon 5 exclusion from PUF60 mRNA, IT126 slightly encouraged exon 13 exclusion from CDC42BPA mRNA and slightly promoted FAS mRNA isoform formation, IT135 encouraged FAS mRNA isoform formation and slightly promoted exon 13 exclusion from CDC42BPA mRNA. HCT116 cells, cultivated under hypoxia and treated with the selected compounds showed that compounds IT126 and IT135 encouraged FAS mRNA isoform formation.

Author's Personal Contribution

Formulation of overarching research goals and aims was realised by dialogue between the author and the supervisor Dr. Arvydas Kanopka. The author performed all the procedures outlined in this work. The author applied statistical analysis techniques on the study data and performed the visualisation of results. Result interpretation and the conclusions formulation were carried out with the supervision of the supervisor. This master's thesis was written by the author.

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VILNIAUS UNIVERSITETAS
GYVYBĖS MOKSLŲ CENTRAS

Aidan Linas Marmion

Magistro baigiamasis darbas

Triazolo junginių poveikis pre-iRNR splaisingui HCT116 ląstelėse

SANTRAUKA

Vėžinės ligos pasižymi nekontroliuojama ląstelių proliferacija. Nepaisant didelių pastangų, vėžys tebėra viena aktualiausių visuomenės sveikatos problemų, ir prognozuojama, kad jo atvejų skaičius toliau kils. Storosios žarnos vėžys buvo viena dažniausiai nustatytų vėžio formų 2022 metais, ir sudarė net 9.6% visų vėžio atvejų. Dauguma šiuo metu naudojimui patvirtintų chemoterapinių vaistų pasižymi įvairiais šalutiniais poveikiais. Chemoterapijai galėtų padėti naujos efektyvesnės ir saugesnės chemoterapinės medžiagos.

Šio darbo tikslas buvo ištirti grupės triazolo junginių poveikį su vėžių susiejusių genų pre-iRNR splaisingui HCT116 ląstelėse. Darbo uždaviniai buvo įvertinti traizolo junginių poveikį ląstelių gyvybingumui, palyginti su vėžiu susijusių iRNR izoformų susidarymą normokisnėmis ir hipoksinėmis salygomis ir įvertinti parinktų triazolo junginų įtaką iRNR izofromų susidarymui tomis pačiomis sąlygomis.

HCT116 ląstelės buvo kultivuotos normoksinėmis ir hipoksinėmis salygomis (72 val.). Poveikis ląstelių gyvybingumui buvo įvertintas MTT metodu. Buvo nustatyta atrinktų triazolo junginių pusiau maksimalaus inhibavimo koncentracija (IC_{50}). Buvo izoliuota parinktų genų iRNR, bei susintetinta komplimentari DNR amplifikuota PGR metodu. Rezultatai buvo vizualizuoti elektroforeze ir įvertinti bioinformatinės analizės įrankiais.

Įvertinus 17 triazolo junginių poveikį HCT116 ląstelių gyvybingumui, paaiškėjo, kad junginiai IT123, IT26 ir IT135 buvo efektyviausi ir sumažino ląstelių gyvybingumą iki 8-22%. Šių junginių IC_{50} vertės buvo nustatytos atitinkamai 51.39 μM, 41.23 μM ir 53.09 μM. Palyginus FAS, FGFR1OP, PUF60, APAF1, CDC42BPA ir CASP9 pre-iRNR splaisingą HCT116 ląstelėse kultivuotose normoksinėje arba hipoksinėje aplinkoje, paaiškėjo, kad hipoksija įtakoja iRNR izoformų susidarymą. Įvertinus atrinktų junginių poveikį pre-iRNR splaisingui ląstelėse kultivuotose normaliomis ir hipoksinėmis sąlygomis, paaiškėjo, kad junginiai turėjo skirtingus poveikius skirtingų genų pre-iRNR splaisingui skirtingomis sąlygomis. Normoksinėmis sąlygomis FAS pre-iRNR splaisingas buvo paveiktas visų junginių, bet tuo tarpų hipoksinėmis sąlygomis tik IT126 ir IT135. PUF60 ir APAF1 pre-iRNR splaisingas buvo paveiktas tik IT123 normoksijoje, o CDC42BPA iRNR izoformų formavimasis buvo paveiktas tik IT126 ir IT135 normoksijoje. Visi trys junginiai neturėjo jokio poveikio FGFR1OP ir CASP9 pre-iRNR splaisingui.

VILNIUS UNIVERSITY
LIFE SCIENCES CENTER

Vardas Pavardė

Master's thesis

Effect of triazole-based compounds on pre-mRNA splicing in HCT116 cells

ABSTRACT

Cancer is a disease marked by uncontrolled proliferation of cells, which in spite of great efforts remains one of the most urgent public health issues and is predicted to increase in occurrence. Colorectal cancer in particular was one of the most frequently reported forms of cancer in 2022, making up 9.6% of cases. Many of the currently approved chemotherapeutic agents exhibit adverse effects. Consequently, chemotherapy can be greatly bolstered by the discovery of more effective and safe chemotherapeutic agents.

This work aimed to investigate the effects of a set of triazole-based compounds on the pre-mRNA splicing of tumour-associated genes in HCT116 cells, including the evaluation of the effects of the triazole-based compounds on the viability of cells. Also, to compare tumour-associated mRNA isoform formation under normoxia and hypoxia, and to evaluate the effects of selected triazole-based compounds on the mRNA isoform formation under the same conditions.

The HCT116 cells in normoxia or hypoxia (72h) were cultivated. The effects on cell viability were assessed using the MTT assay. The half-maximal inhibitory concentrations (IC_{50}) of selected compounds was determined. mRNAs of selected genes were isolated, complimentary DNA was synthesised and then amplified by PCR. These results were visualised via electrophoresis and examined using bioinformatical analysis tools.

Assessment of the effects on HCT116 cell viability of 17 triazole-based compounds, revealed that compounds IT123, IT126 and IT135 were the most effective, reducing cell viability to 8-22%. Their IC_{50} values were determined to be 51.39 μM, 41.23 μM and 53.09 μM, respectively. Analysis of FAS, FGFR1OP, PUF60, APAF1, CDC42BPA and CASP9 pre-mRNA splicing in cells cultured under either normoxia or hypoxia, indicated that hypoxia influences mRNA isoform formation. Assessment of the effects of selected compounds on pre-mRNA splicing in cells cultured either under normoxia or hypoxia, showed that these compounds have distinct effects on pre-mRNA splicing of different genes. In normoxia FAS pre-mRNA splicing was altered by all compounds, while under hypoxia only by compounds IT126 and IT135. PUF60 and APAF1 pre-mRNA splicing was affected only by IT123 under normoxia, while CDC42BPA mRNA isoform formation was only affected by IT126 and IT135 under normoxia. All three compounds had no effect on FGFR1OP and CASP9 isoform formation.

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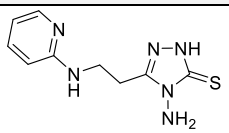
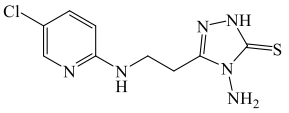
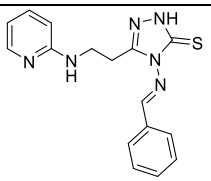
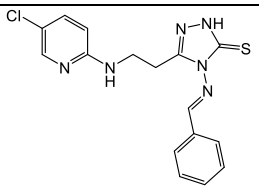
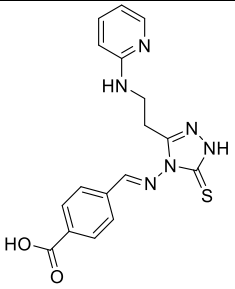
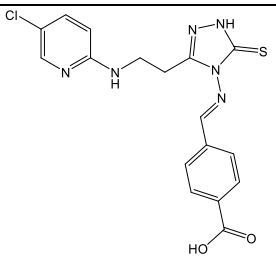
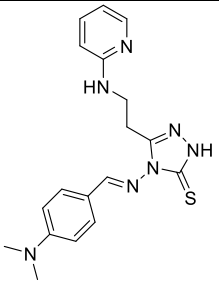
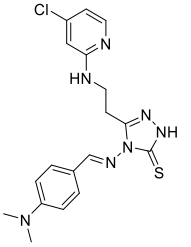
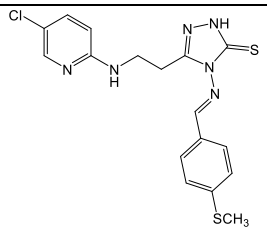
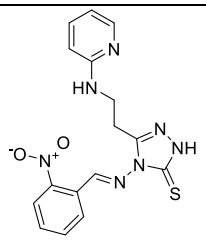
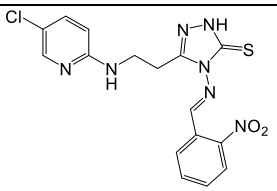
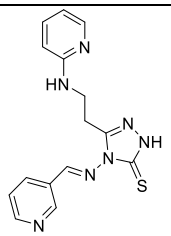
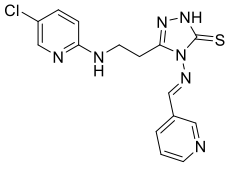
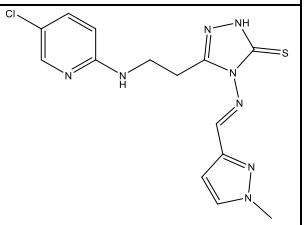
Appendices

Appendix 1. Analysed triazole-based compounds.

Compound abbreviation	Analysed compound	Compound structure
IT 127	4-amino-5-(2-(pyridin-2-ylamino)ethyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
IT 120	4-amino-5-(2-((5-chloropyridin-2-yl)amino)ethyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
IT 128	4-(benzylideneamino)-5-(2-(pyridin-2-ylamino)ethyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
IT 121	4-(benzylideneamino)-5-(2-((5-chloropyridin-2-yl)amino)ethyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
IT 132	4-(((3-(2-(pyridin-2-ylamino)ethyl)-5-thioxo-1,5-dihydro-4*H*-1,2,4-triazol-4-yl)imino)methyl)benzoic acid	
IT 122	4-(((3-(2-((5-chloropyridin-2-yl)amino)ethyl)-5-thioxo-1,5-dihydro-4*H*-1,2,4-triazol-4-yl)imino)methyl)benzoic acid	
IT 131	4-((4-(dimethylamino) benzylidene)amino)-5-(2-(pyridin-2-ylamino)ethyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	

Appendix 1 continued. Analysed triazole-based compounds.

Compound abbreviation	Analysed compound	Compound structure
IT 124	5-(2-((4-chloropyridin-2-yl)amino)ethyl)-4-((4-(dimethylamino)benzylidene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
IT 123	5-(2-((5-chloropyridin-2-yl)amino)ethyl)-4-((4-(methylthio)benzylidene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
IT 130	4-((2-nitrobenzylidene)amino)-5-(2-(pyridin-2-ylamino)ethyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
IT 125	5-(2-((5-chloropyridin-2-yl)amino)ethyl)-4-((2-nitrobenzylidene)amino)-2,4-dihydro-3H-1,2,4-triazole-3-thione	
IT 135	5-(2-(pyridin-2-ylamino)ethyl)-4-((pyridin-3-ylmethylene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
IT 126	5-(2-((5-chloropyridin-2-yl)amino)ethyl)-4-((pyridin-3-ylmethylene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	
A-11 (IT 153)	5-(2-((5-chloropyridin-2-yl)amino)ethyl)-4-(((1-methyl-1*H*-pyrazol-3-yl)methylene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	

Appendix 1 continued. Analysed triazole-based compounds.

Compound abbreviation	Analysed compound	Compound structure
A-16 (IT 154)	5-(2-((5-chloropyridin-2-yl)amino)ethyl)-4-((2-hydroxybenzylidene)amino)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione