Triazolo-Thiadiazole Derivatives

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The fusion of 1,2,4-triazole and 1,3,4-thiadiazole rings results in a class of heterocycles compounds with an extensive range of pharmacological properties. A series of 1,2,4-triazolo[3,4-b]-1,2,4-thiadiazoles was synthesized and tested for its enzyme inhibition potential and anticancer activity. The results show that 1,2,4-triazolo[3,4-b]-1,2,4-thiadiazoles display potent anticancer properties in vitro against a panel of cancer cells and in vivo efficacy in HT-29 human colon tumor xenograft in CB17 severe combined immunodeficient (SCID) mice.

1 4-triazolo[3 4-b]-1 2 4-thiadiazole inhibitor of Akt phosphorylation anticancer MTT assay HT-29 human colon tumor xenograft ATP binding site

1. Introduction

Akt, termed protein kinase B (PKB), is a serine/threonine kinase composed of three isoforms: Akt1, Akt2, and Akt3. All three isoforms share very similar amino acid sequences, with their expression level differentiated; Akt1 and Akt2 are abundantly expressed, while Akt3 is particularly detected in the brain, heart, and kidneys [1][2]. All Akt isoforms display the same basic structure comprised of three regions: (1) an amino terminal pleckstrin homology (PH) domain that interacts with membrane phospholipids such as phosphatidylinositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol 4,5-bisphosphate (PIP2); (2) a central kinase domain that contains the threonine regulatory residue, Thr308, in which phosphorylation activates Akt; and (3) a carboxyl-terminal regulatory domain that consists of a hydrophobic region of 40 amino acids including the serine regulatory residue (Ser473) [3]. The structural elements, PH domain, and the regulatory residues Thr308 and Ser473 play critical roles in the activation of Akt. Two events are required in Akt's activation: (a) PH-domain-dependent translocation to the plasma membrane and (b) phosphorylation at the Thr308 and Ser473 residues. The first step includes the interaction of the PH domain with PIP3, which is followed by the translocation of Akt to the plasma membrane. Afterwards, Akt adopts a new conformation such that the Thr308 residue would be phosphorylated by phosphoinositide-dependent kinase-1 (PDK1). This signaling event leads to phosphorylated Ser473 via the mechanistic targeting of rapamycin (mTOR)C2 [4][5][6].

Phosphatidylinositol 3-kinase (PI3K), an upstream signaling molecule, along with Akt constitute the PI3K/Akt signaling transduction pathway through which cellular survival and growth are induced in response to extracellular signal [7]. Among all protein components of the PI3K/Akt pathway, the inhibition of Akt has been widely explored due to its association with tumor progression and aggressiveness [8]. Significant alterations have been demonstrated in the expression levels of Akt isoforms in certain malignancies, for instance, Akt1 is particularly

elevated in breast, prostate, and gastric tumors whilst Akt2 is overexpressed in prostate, ovarian, breast, pancreatic, and colorectal cancers [9][10][11]. Thus far, Akt inhibitors are divided in four basic categories: (1) ATP-competitive inhibitors, (2) allosteric inhibitors, (3) lipid-based inhibitors, and 4) PH domain inhibitors [12]. The first category of ATP-competitive inhibitors (CCT128930 as an Akt2 inhibitor and BAY-1125976 as an Akt1/2 inhibitor), including pan-Akt kinase inhibitors (afuresertib, GSK690693, AZD5363, GDC-0068, and AT7867 as inhibitors of all Akt isoforms), targets the kinase domain and precisely binds to the ATP-binding pocket [13]. The high degree of homology of the ATP-binding site among different serine/threonine kinases as well as the extensive conservation of this domain within the AGC kinase family (protein kinase A, G, and C families) contributes to a low specificity of ATP-competitive inhibitors. The development of such molecules can be further obstructed due to the lack of strong efficacy against tumors in vivo, with toxicities to normal tissues being recorded at the same time [14][15]. Regarding allosteric inhibitors, for example, the MK-2206 compound, are associated with the Akt kinase domain. Compared with ATP-competitive inhibitors that show efficacy only against cancer cell lines with Akt mutations, allosteric inhibitors display broader anticancer activity as compounds of this type are potent against cancer cell lines with PI3KCA mutations or loss of phosphatase and tensin homologue (PTEN) activity [16]. The third category of lipid-based inhibitors (PX-866 and perifosine) blocks the interaction of Akt with PIP3 since this type of molecule inhibits PI3K and therefore prevents the production of PIP3 from PIP2. Finally, PH domain inhibitors (triciribine and PX-316) inactivate Akt via interactions with the PH-domain, interrupting membrane translocation, which is required for activating Akt [17].

The fusion of 1,2,4-triazole and 1,3,4-thiadiazole rings results in a class of heterocyclic compounds with an extensive range of pharmacological properties including antifungal, antibacterial, antiviral, anti-inflammatory, analgesic, and anthelmintic properties [18]. Earlier studies indicated that previous 3,6-disubstituted 1,2,4-[3,4-*b*]thiadiazoles induced potent anti-inflammatory activity along with a minimal ulcerogenic effect and lipid peroxidation compared to ibuprofen and flurbiprofen. Some of these compounds demonstrated moderate to weak antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* while their antifungal activity against *Candida albicans* was quite weak [19][20][21]. Furthermore, 1,2,4-triazolo[3,4-*b*][1,3,4]thiadiazole derivatives containing 3-methyl or benzyl moiety showed moderate anti-HIV-1 activity at subcytotoxic concentrations [22]. In previous work, we have shown that triazolo[3,4-*b*]thiadiazole derivatives show potent in vitro antiproliferative activities [23]. Our studies resulted in the identification of three bioactive compounds (KA39, KA25, and KA26) against three human colorectal cancer cell lines (Figure 1). Among them, KA39 was the most potent anticancer agent and inhibitor of topIIα phosphorylation at Ser-1106 as well. Additional molecular docking studies revealed that KA39 can occupy the same binding site as etoposide and can interact with the ATPase domain of topIIα, but the exact mechanism of inhibition of topIIα phosphorylation by KA39 is still unclear. As part of our ongoing work on the assessment of the biological activity of triazolo[3,4-*b*][1,3,4]thiadiazoles, we report the synthesis, structural characterization, and evaluation of inhibitory effects of fifteen new derivatives. In particular, we examined the introduction of ethyl or propyl substituents on sulfonamide combined with different substituents on C-6 of the triazolo[3,4-*b*][1,3,4]thiadiazole template. In the current work, the anticancer activities of all derivatives were primarily assessed in vitro while the most active molecules KA39, KA25, and KA26, according to in vitro screening, were tested in vivo. Further studies were carried out to investigate whether the most potent antitumor compound

blocks the phosphorylation of Akt1 and Akt2 kinases. The molecular modeling studies indicated that 1,2,4-triazolo[3,4-*b*]-1,2,4-thiadiazoles bind well to the ATP binding site in Akt1 and Akt2.

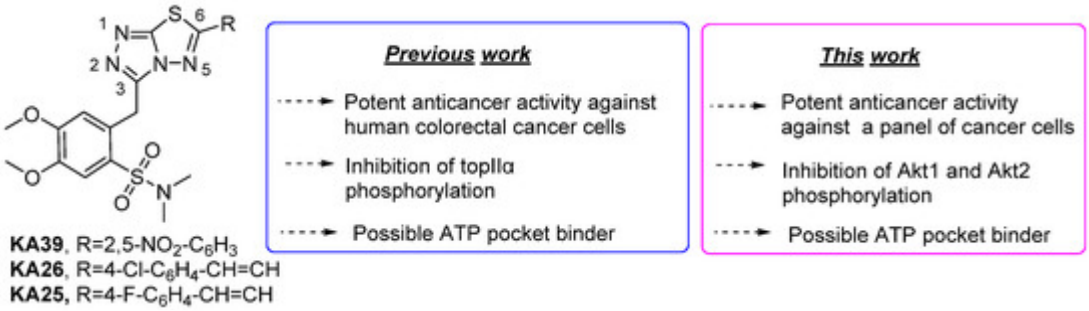
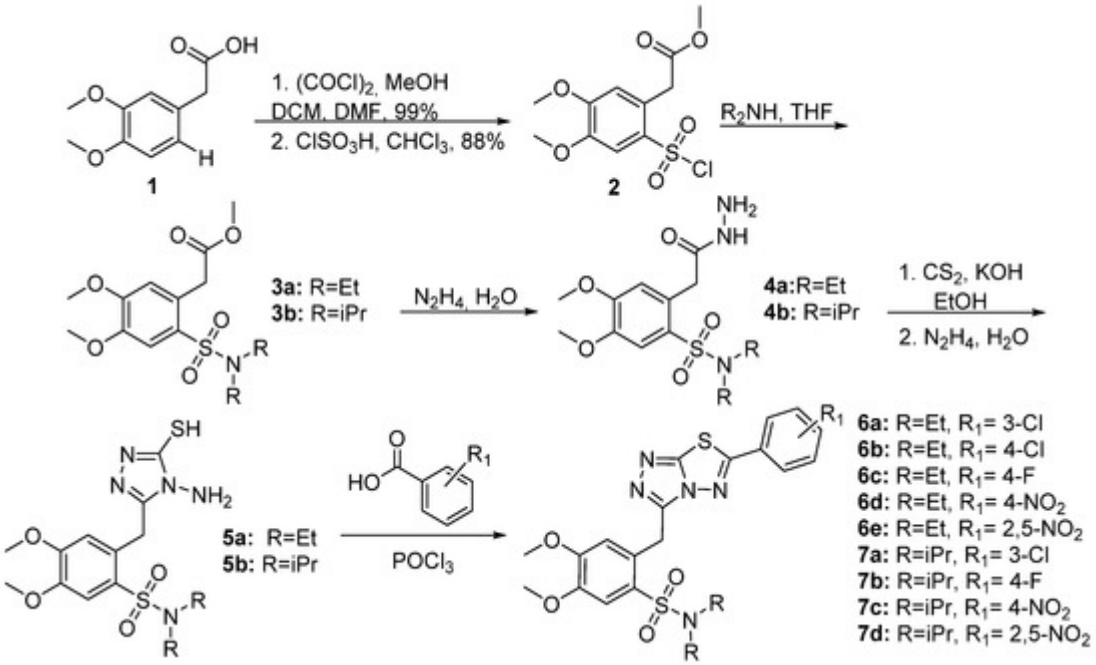


Figure 1. Structures of active triazolo[3,4-*b*][1,3,4]thiadiazoles and overview of their activity.

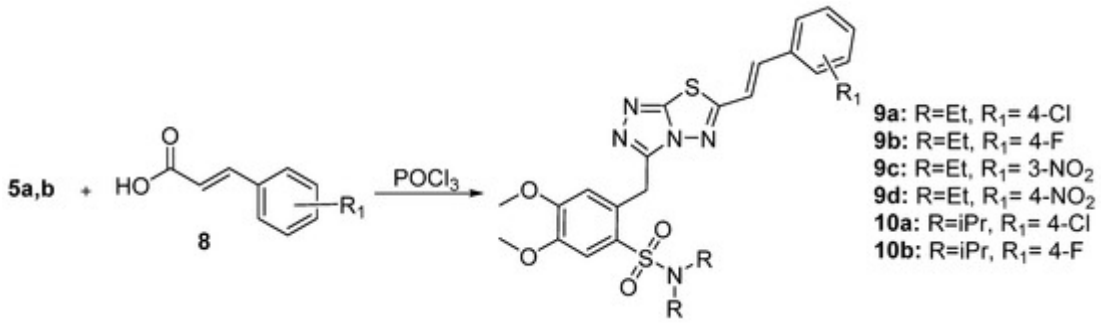
2. Synthesis of triazolo[3,4-b]thiadiazole Derivatives

2.1. Synthesis of 3,6-disubstituted 1,2,4-triazolo-[3,4-*b*]-[1,3,4]-thiadiazoles

Synthesis of the 1,2,4-triazolo-[3,4-*b*]-[1,3,4]-thiadiazoles was accomplished according to the synthetic methodology developed by our group (Scheme 1 and Scheme 2) [23]. After a reaction with oxalyl chloride, 2-(3,4-dimethoxyphenyl)acetic acid **1** was esterified and the corresponding methyl ester reacted with chlorosulfonic acid in CHCl3 to give methyl 2-(2-(chlorosulfonyl)-4,5-dimethoxyphenyl)acetate **2** in 88% yield. Subsequently, sulfonyl chloride **2** furnished the corresponding sulfonamides 3a,b after reaction with Et2NH or iPr2NH. Furthermore, these compounds reacted with hydrazine hydrate to give hydrazides 4a,b that reacted with KOH and CS2 in EtOH to give the potassium thiocarbamates. The latter products were cyclized in the presence of hydrazine hydrate to triazoles 5a,b [24]. The desired 1,2,4-triazolo[3,4-*b*]-[1,2,4]-thiadiazoles (6a–e, 7a–d, 9a–d, and 10a,b) were finally prepared via the reaction of triazoles 5a,b with POCl3 and various benzoic or cinnamic acids.



Scheme 1. Synthesis of benzoic acid derivatives.



Scheme 2. Synthesis of cinnamic acid derivatives.

2.2. In Vitro Anticancer Activity

The fifteen novel triazolo-thiadiazole derivatives and the previously described KA25, KA26, and KA39 were tested in vitro in two well-established human cancer cell lines: PC-3 and SKOV-3 cells. According to the first drug screening results, the 13 derivatives were inactive at the tested concentrations while the most active compounds (KA25, KA26, KA39, 6e, and 7d) were further tested in vitro in colorectal, ovarian, and prostate cancer cell lines. As Table 1, Table 2 and Table 3 show, the most potent anticancer activities in all human cancer cell lines were induced by the KA39, 6e, and 7d compounds ($p < 0.001$, two tailed paired *t*-test). All these compounds bear the 2,5-dinitrophenyl substituent on C-6 of the triazolo[3,4-*b*][1,3,4]thiadiazole core. Nevertheless, the triazolo[3,4-*b*]thiadiazole KA39 was significantly more active than 6e and 7d, which displayed less anticancer potency (Table 1, Table 2 and Table 3). Concerning KA25 and KA26 derivatives, both exhibited cytostatic activity in all human cancer cell lines, whereas KA25 induced cytotoxic effects only in ovarian and prostate cancer cells.

Table 1. Growth inhibition/cytostatic (GI50 and TGI) and cytocidal/cytotoxic (IC50) effects induced by triazolo[3,4-*b*]thiadiazole derivatives KA25, KA26, and KA39 on nine human cancer cell lines. GI_{50}: 50% growth inhibition; TGI: Total growth inhibition; IC_{50}: the concentration that causes 50% cell death.

Cell Line	KA25			KA26			KA39		
	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)
SKOV-3	15 ± 0.5	50 ± 1.5	85 ± 2.1	20 ± 0.5	>100	>100	7 ± 0.2	12 ± 0.8	25 ± 1.4
UWB 1.289	2 ± 0.5	5 ± 0.7	62 ± 1.2	29 ± 0.7	53 ± 1	70 ± 1.4	6 ± 0.2	10 ± 0.8	22 ± 1.2
UWB1.289+BRCA1	4.0 ± 0.5	13 ± 0.8	38 ± 1.1	42 ± 0.5	65 ± 0.7	>100	6 ± 0.5	11 ± 0.7	94 ± 2.3
HT-29	1 ± 0.3	>100	>100	2 ± 0.76	>100	>100	11.5 ± 0.8	15.9 ± 0.55	19.5 ± 0.9

Cell Line	KA25			KA26			KA39		
	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)
LS174T	9.3 ± 2.0	100 ± 0.1	>100	8 ± 1.5	>100	>100	12 ± 1.5	17 ± 1.3	21.5 ± 1.5
SW403	4.8 ± 0.8	>100	>100	11 ± 0.8	>100	>100	5.2 ± 0.2	7.9 ± 0.7	10 ± 0.76
LoVo	8 ± 0.76	13.8 ± 0.52	>100	15 ± 0.8	>100	>100	2.2 ± 0.2	5.5 ± 0.1	10.5 ± 0.15
PC-3	14 ± 1.0	22 ± 0.8	>100	42.5 ± 1.5	>100	>100	5 ± 0.15	8 ± 0.1	12 ± 0.1

Compound	PC-3			SKOV-3		
	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)
9b	42 ± 1.0	>100	>100	>100	>100	>100
10b	31 ± 1.1	>100	>100	50 ± 2.0	>100	>100
9a	11 ± 1.0	>100	>100	84 ± 2.0	>100	>100
10a	2 ± 0.5	84 ± 2.0	>100	76 ± 6.0	>100	>100
6b	32 ± 0.4	56 ± 0.6	104 ± 0.8	36 ± 0.1	60 ± 0.8	>100
6a	25 ± 1.4	112 ± 2.5	>100	6 ± 0.3	14 ± 2.5	>100
7a	20 ± 0.8	>100	>100	16 ± 0.8	46 ± 1.5	>100
6c	90 ± 2.0	>100	>100	>100	>100	>100
7b	25 ± 0.5	80 ± 1	>100	80 ± 0.8	>100	>100
6d	50 ± 0.5	>100	>100	80 ± 0.5	>100	>100
7c	23 ± 0.8	102 ± 1.2	>100	15 ± 1.4	28 ± 2.2	66 ± 2.5
9c	38 ± 2.0	>100	>100	>100	>100	>100
9d	30 ± 0.5	>100	>100	>100	>100	>100

…een newly …azolo[3,4-*b*]thiadiazole derivatives 6e and 7d on five human cancer cell lines.

Cell Lines	6e			7d		
	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)	GI_{50} (μM)	TGI (μM)	IC_{50} (μM)
SKOV-3	18 ± 0.7	24 ± 1.2	38 ± 1.8	13 ± 0.5	17 ± 1.0	23 ± 1.4
PC-3	11 ± 1.0	17 ± 1.0	26.5 ± 0.9	8 ± 1.0	11 ± 1.04	22 ± 0.3
DU-145	13 ± 0.5	18.3 ± 0.2	28 ± 0.2	6.8 ± 0.8	14 ± 0.5	21.3 ± 0.9
DLD-1	25 ± 2.0	37 ± 2.0	53.6 ± 1.05	7.3 ± 0.75	12 ± 0.5	22 ± 1.0
HT-29	18 ± 1.0	29 ± 0.5	44 ± 1.0	17.9 ± 2.51	26 ± 1.52	40 ± 1.0

3. Discussion

Aberrations in the PI3K/Akt signal transduction pathway are a very common phenomenon in human carcinogenesis, with over half of tumors showing irregular Akt activation [25]. More precisely, the overexpression of certain oncogenes or a lack of tumor suppressor genes influences the biological activity of the PI3K/Akt network, stimulating cancer cell growth. For instance, mutations of Epidermal Growth Factor Receptor, EGFR/PI3K, loss of the tumor suppressor protein phosphatase and tensin homologue (PTEN), and mutations or amplifications of Akt itself enhance Akt's signaling in cancer cells [26][27]. Amplified and mutated Akt isoforms play crucial roles in tumorigenesis, while Akt2 is particularly involved in EMT transition and metastasis. Regarding mutations, Akt1 is the most frequently mutated isoform compared with Akt2, in which mutations do not occur at high frequency and cannot be considered as activating [28][29]. The most prevalent mutation in Akt1, E17K, is located within the PH domain and is found in several solid tumors including breast (3.3%), colorectal (1.06%), lung (1.0%), and ovarian cancers (0.5%) as well as in melanoma (0.5%) [30][31]. Akt1E17K mutation, induced by the substitution of lysine to glutamic acid at amino acid 17, leads to a pathological translocation of Akt1 to the plasma membrane and to alterations in PIP specificity as binding to PIP2 seems to be highly increased. Given that Akt1 normally binds to PIP3, further utilization of PIP2 in the case of Akt1E17K enhances Akt1's signaling [31][32][33].

Tumor aggressiveness as well as poor survival rates are particularly linked to Akt2's amplification and overexpression [34]. Accordingly, earlier studies' mutations in the Akt2 isoform are quite rare while amplifications in the isoform have been detected in 16% of pancreatic cancers; 16% of uterine cancers; 13% of breast cancers; and

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The present study attempts to explore the impact of KA25 and KA39 on Akt1 and Akt2 phosphorylation in colorectal cancer cell lines (HT-29, LoVo, and SW403) with specific PIK3CA and KRAS statuses (Table 4). More specifically, two of the referred cell lines, HT-29 and SW403, harbor mutations in PIK3CA, p.P449T and p.Q546K, respectively, while LoVo cells retain a wild-type PIK3CA (https://cancer.sanger.ac.uk/cosmic, accessed on 4 September 2020). With regard to PIK3CA mutations, most of them are concentrated in hotspots within two domains, the helical (exon 9) and kinase (exon 20) [36]. Nonetheless, four additional missense mutations have been detected including p.P449T, which belongs to gain-of-function mutations and contributes to enhanced PIK3CA kinase activity (>2-fold) compared to the wild-type PIK3CA. In general terms, gain-of-function mutations in PIK3CA are known for triggering the PI3K kinase activity and consequently activating its downstream molecules such as Akt [37]. Further studies have indicated that rare PIK3CA mutants, including p.Q546K, possess a high oncogenic potency as well as hyperactivate PI3K/Akt downstream signaling cascade [38]. Due to the fact that HT-29 and SW403 cell lines contain gain-of-function mutations in PIK3CA, the levels of phosphorylated Akt may be elevated in both cell lines in comparison to the wild-type PIK3CA expressed by the LoVo cell line.

Table 4. Description of histotypes and special characteristics of the three human colorectal cancer cell lines in which the inhibition of Akt (Akt1/2) phosphorylation, induced by KA25 and KA39, was investigated.

Cancer Type	Human Cell Line Designation	KRAS Status	PIK3CA Status	References
Colorectal adenocarcinoma	HT-29	wild-type	p.P449T	[39][40]
Colorectal adenocarcinoma, Duke's type C, grade IV	LoVo	p.G13D	wild-type	[39][40]
Colorectal adenocarcinoma Dukes' type C, grade III	SW403	p.G12V	p.Q546K	[41][42][43]

p.G12V mutations, respectively (Table 6). Experimental studies using these cell lines showed that neither p.G12V nor p.G13D KRAS mutations stimulate the phosphorylation of Akt to a greater extent than wild-type KRAS cells. Moreover, it has been also demonstrated that Akt phosphorylation was decreased in KRAS-G12V cells compared to KRAS wild-type cell lines [44][45][46]. Consequently, the LoVo and SW403 cell lines (KRAS-mutant) probably express similar or lower levels of phosphorylated Akt than HT-29 cells (KRAS wild-type). As far as cell sensitivity to Akt inhibitors is concerned, recent studies support that cell lines with PI3K and/or PTEN mutations display a higher susceptibility to this type of inhibitor than cells with KRAS and/or v-raf murine sarcoma viral oncogene homolog B (BRAF) mutations [47]. More specifically, Akt inhibitors show greater selectivity and potency in cells with increased Akt kinase activity resulting from mutations in PI3K or PTEN. Later studies implied that cell sensitivity to allosteric

Akt1/2 inhibitors is significantly associated with Akt phosphorylation in human cancer cell lines, leading to the conclusion that cell lines with elevated levels of phosphorylated Akt are more susceptible to the pharmacologic inhibition of Akt1/2. However, the certain lineage may affect the pharmacologic sensitivity to Akt1/2 inhibition, for instance, PIK3CA-mutant breast cancer cell lines are more sensitive than PIK3CA-mutant colon cancer cell lines. Altogether, high levels of phosphorylated Akt signify AKT dependency in PIK3CA-mutant cancer cells [48][49].

On the other hand, Akt inhibitors, utilized in the inhibition of downstream effector pathways, have not shown an impressive antitumor efficacy on KRAS-mutant tumors [50]. As observed, cells with mutated KRAS and/or BRAF are less sensitive to Akt inhibitors since no alterations in the activation of MAPK pathway have been seen [47]. A relevant example that correlates PIK3CA and RAS mutations with sensitivity to Akt inhibitors concerns the AZD5363 compound; PIK3CA and PTEN mutations are strongly associated with sensitivity to this Akt inhibitor, regardless of RAS status [51]. Additionally, tumors bearing an overactivated PI3K/Akt pathway without any EGFR, RAS, or BRAF mutations depend more on this pathway and become more sensitive to selective Akt inhibitors [52]. It is noteworthy that both of the referred to KRAS mutations (p.G12V and p.G13D) are associated with resistance to anti-EGFR therapy via intracellular pathways, restricting the administration of anti-EGFR monoclonal antibodies (mAbs) to patients bearing KRAS wild-type tumors [53][54]. Based on PIK3CA and KRAS statuses, we could put forward the hypothesis that KA25- and KA39-induced inhibition of Akt1 and Akt2 phosphorylation might be more effective in HT-29 (PIK3CA-mutant; KRAS wild-type) than those of LoVo cells (PIK3CA wild-type; KRAS-mutant). Regarding SW403 cells, in which PIK3CA and KRAS mutations concur, Akt1 and Akt2 phosphorylation could be impeded similarly to either HT-29 or LoVo cells. Nevertheless, both methods of estimation (% inhibition and ratios correlated with control) indicate that both triazolo[3,4-*b*]thiadiazole derivatives exhibited significant inhibitory activity against Akt1and Akt2 phosphorylation in all three cancer cell lines. It is notable that the inhibition of Akt1 and Akt2 phosphorylation, induced by KA25 and KA39, was fairly similar in all three cell lines carrying PIK3CA and/or KRAS mutations. Nonetheless, it could be suggested that the reduction in phosphorylated Akt (Akt1/2) was slightly greater in LoVo (mutated KRAS) and SW403 (mutated KRAS and PIK3CA) cells compared to the HT-29 cell line (mutated PIK3CA).

Furthermore, pharmacokinetic studies on the tested compounds are currently under investigation. Premature experimental data showed that these compounds have poor water solubility (log mol/L = −4.4 to −3.5), high $CaCO_2$ permeability (log Papp in 10^{-6} cm/s = −0.135 to 0.965), and high intestinal absorption (94–100%). These compounds present low distribution volumes with high binding to serum proteins (72–92%), are unable to penetrate the CNS, and are poorly distributed to the brain. They are not substrates of P-glycoprotein but they inhibit both glycoproteins I and II. They metabolize in liver and they are substrates of CYP3A4 but inhibit CYP2C19 and CYP2C19. Their total clearance in rats was 2.2–5.7 mL/min/kg.

It is interesting to note that a large number of small molecules are proven to inhibit Akt in vitro and in vivo, though only a limited number of these have entered clinical trials [40]. Ongoing clinical studies indicate that the tested Akt inhibitors are not effective as single agents, displaying rather low therapeutic responses. Furthermore, even if there is increasing knowledge concerning Akt functions and activation, there are no Akt inhibitors approved for oncologic use. For example, miltefosine, the only Akt inhibitor approved, is intended for treating visceral and cutaneous

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