Synthesis, DFT Analyses, Antiproliferative Activity, and Molecular Docking Studies of Curcumin Analogues

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Abstract: With 19.3 million new cases and almost 10 million deaths in 2020, cancer has become a leading cause of death today. Curcumin and its analogues were found to have promising anticancer activity. Inspired by curcumin’s promising anticancer activity, we prepared three semi-synthetic analogues by chemically modifying the diketone function of curcumin to its pyrazole counterpart. The curcumin analogues (3a–c) were synthesized by two different methods, followed by their DFT analyses to study the HOMO/LUMO configuration to access the stability of compounds (ΔE = 3.55 to 3.35 eV). The curcumin analogues (3a–c) were tested for antiproliferative activity against a total of five dozen cancer cell lines in a single (10 µM) and five dose (0.001 to 100 µM) assays. 3,5-Bis(4-hydroxy-3-methoxystyryl)-1H-pyrazole-1-yl-(phenoxy)ethanone (3b) and 3,5-bis(4-hydroxy-3-methoxystyryl)-1H-pyrazole-1-yl-(2,4-dichlorophenoxy)ethanone (3c) demonstrated the most promising antiproliferative activity against the cancer cell lines with growth inhibitions of 92.41% and 87.28%, respectively, in a high single dose of 10 µM and exhibited good antiproliferative activity (%GIs > 68%) against 54 out of 56 cancer cell lines and 54 out of 60 cell lines, respectively. The compound 3b and 3c demonstrated the most potent antiproliferative activity in a 5-dose assay with GI50 values ranging between 0.281 and 5.59 µM and 0.39 and 0.196 and 3.07 µM, respectively. The compound 3b demonstrated moderate selectivity against a leukemia panel with a selectivity ratio of 4.59. The HOMO-LUMO energy-gap (ΔE) of the compounds in the order of 3a > 3b > 3c, was found to be in harmony with the anticancer activity in the order of 3c ≥ 3b > 3a. Following that, all of the curcumin analogues were molecular docked against EGFR, one of the most appealing targets for antiproliferative activity. In a molecular docking simulation, the ligand 3b exhibited three different types of interactions: H-bond, π-π-stacking and π-cationic. The ligand 3b displayed three H-bonds with the residues Met793 (with methoxy group), Lys875 (with phenolic group) and Asp855 (with methoxy group). The π-π-stacking interaction was observed between the phenyl (of phenoxy) and the residue Phe997, while π-cationic interaction was displayed between the phenyl (of curcumin) and the residue Arg841. Similarly, the ligand 3c displayed five H-bonds with the residue Met793 (with methoxy and phenolic groups), Lys845 (methoxy group), Cys797 (phenoxy oxygen), and Asp855 (phenolic group), as well as a halogen bond with residue Cys797 (chloro group). Furthermore, all the compound 3a-c demonstrated significant binding affinity (−6.003 to −7.957 kcal/mol) against the...
active site of EGFR. The curcumin analogues described in the current work might offer beneficial therapeutic intervention for the treatment and prevention of cancer. Future anticancer drug discovery programs can be expedited by further modifying these analogues to create new compounds with powerful anticancer potentials.

**Keywords:** antiproliferative activity; curcumin analogues; pyrazole; anti-EGFR

1. Introduction

The development of new antiproliferative agents with greater efficacy and a smaller amount of side effects remains a modern scientific research challenge. Over the last few decades, there has been a surge in interest in using natural products for therapeutic purposes. Although this concept appears to be new, our forefathers have used natural compounds/extracts to treat illness since time immemorial. The development of synthetic pharmaceuticals over the last century not only revolutionized modern medicine, but also accrued the undesirable properties and side effects associated with these drugs. The unfavorable properties of synthetic drugs have prompted a search for natural alternatives, with the hope that naturally occurring compounds will be better tolerated and safer than their synthesized counterparts [1,2]. Curcumin, one of the curcuminoids obtained from the powdered root of turmeric (*Curcuma longa* Linn.), belongs to the family Zingiberaceae is a β-diketone that showed an antiproliferative effect on various panels of cancer cell lines [3]. Medicinal chemists have identified four main sites to bring about chemical modification in curcumin to form semi-synthetic congeners. The four main sites including the active methylene (-CH2-), aryl side chain, diketone group, and carbon-carbon double bonds (-CH=CH-) to create a number of semi-synthetic curcumin analogues with improved bioactivity [4]. In this study, we report on the modification of the diketone group to pyrazole heterocycle and their antiproliferative activity. The chemical modification is outlined in Figure 1. The structural modification was discovered to improve biological activities by increasing stability, decreasing rotational freedom, and minimizing metal-chelation properties [5]. Curcumin analogues have previously been shown to have anticancer, antimalarial, and anti-HIV activities, according to our research team [6–10]. However, a wide range of biological activities have been reported, including antibacterial, anticancer, antioxidant, antimalarial, anti-inflammatory, anti-Alzheimer’s, and anti-HIV activities [11–17]. Curcumin analogues have also been identified as epidermal growth factor receptor (EGFR) inhibitors [18,19].

![Figure 1. Structural modification of diketone function into pyrazole.](image-url)
Cancer is a disease in which a few of the body’s cells grow out of control and spread to other parts of the body. With 19.3 million new cases and almost 10 million deaths in 2020, cancer has become a leading cause of death today. Today, breast cancer is the most commonly diagnosed cancer, followed by lung cancer. Breast, lung, colorectal, liver, and stomach cancers account for 11.7, 11.4, 10, 7.3, and 5.6 percent of all new cancer cases, respectively [20]. There are over 100 different forms of cancer. According to World Health Organization (WHO) reports, lung cancer is the main cause of cancer-related death in men, while breast cancer is the leading cause of cancer-related death in women. Chronic infections, mostly caused by the human papillomavirus (HPV) and hepatitis B virus (HBV), cause around 20% of all malignancies worldwide, and are avoidable with very efficient vaccines [21]. Tobacco use alone is responsible for 25% of cancer deaths worldwide [22]. Eliminating or reducing exposure to risk factors can prevent one-third to one-half of all malignancies [21]. The treatments of cancer nowadays involve surgical removal and radiation of large accumulated masses followed by systemic chemotherapy [23]. Chemotherapy continues to be a fundamental regime in clinical handling of all types of cancer, although it contains a high risk of toxicity and multidrug resistance (MDR) against anticancer agents [24,25]. As a result, our reliance on nature became more rational, as active constituents of natural origin would be assumed to be safe. Natural products (NPs) derived from plants, marine organisms, and microorganisms account for the vast majority (more than 60%) of anticancer drugs currently in clinical use [23,26]. Plant-derived anticancer drugs such as camptothecin, etoposide, paclitaxel, vinblastine, and vincristine are a few examples that are currently being used to treat a variety of cancers [27]. Many prospective phytoconstituents have received a lot of attention in the literature for treating conditions including cancer (breast and skin), oxidative stress, inflammation, etc. [28,29]. Curcumin, a β-diketone derived from the powdered root of turmeric, has been shown to have an antiproliferative effect on a variety of cancer cell lines; however, bioavailability is a major issue [3,30,31]. Chemical modification of functional groups could be used to alter curcumin bioavailability, and we converted the diketone function into its pyrazole counterpart in the current study to prepare their semi-synthetic analogues. Several studies have shown that this type of modification increases biological activity [5].

The EGFR is a key anticancer target and the most studied receptor in the tyrosine kinase super-family [32,33]. Curcumin and their semi-synthetic congeners were found to have anti-EGFR activity [7,18]. We previously reported that the bulky nature of curcumin analogues allows them to fit well within the active site of EGFR and exhibit a variety of interactions such as H-bonding, π-π-stacking, and π-cationic. They demonstrated interaction with important EGFR active site residues such as Cys797, Met793, and Arg841 [7]. We investigated the antiproliferative and anti-EGFR activities of newer curcumin analogues in this study.

2. Results

2.1. Chemistry

The curcumin analogues (3a–c) synthesized in the present investigation are summarized in Scheme 1 and two different methods (Method A and Method B) were adopted for the synthesis. 3,5-Bis-4-hydroxy-3-methoxystyryl)-N-(2-methoxyphenyl)-1H-pyrazole-1-carboxamide (3a) was prepared by heating an equimolar mixture of curcumin (1) (0.001 mol; 368 mg) and N-(2-methoxyphenyl)hydrazine carboxamide (2a) (0.001 mol; 181 mg) in glacial acetic acid at 80 °C. Similarly, 1-(3,5-bis((E)-4-hydroxy-3-methoxystyryl)-1H-pyrazol-1-yl)-2-(substituted phenoxy)ethan-1-ones (3b–c) were prepared by heating an equimolar mixture of curcumin (1) (0.001 mol; 368 mg) and substituted phenoxy-acetohydrazide (2b–c) in glacial acetic acid at 80 °C. In this method (Method A), the reaction mixture was heated with continuous stirring for 10 h to complete the reaction while thin layer chromatography (TLC) in n-hexane: ethylacetate (6:4) was used to monitor the reaction throughout. The curcumin analogues were also prepared by another method (Method B). The method B glycerol-water system (1:2) was taken as solvent and the reaction was charged at 60–80 °C.
with continuous stirring for 6 h. The yields and time taken in the reaction are compared in Table 1. The method B was found to be more promising for the synthesis of curcumin analogues (3a–c). The synthetic protocol is summarized in Scheme 1. The intermediates 2a, and 2b–c were prepared using the methods described elsewhere [34,35]. The structure of curcumin analogues (3a–c) were confirmed by spectroscopic techniques. The IR spectra of curcumin analogues 3b–c showed acyl carbonyl (-CH$_2$C=O) peak at 1650 and 1600 cm$^{-1}$, while pyrazole C=N of compounds 3a–c was observed at 1544 to 1548 cm$^{-1}$. The phenolic function (ArOH) of compounds 3a–c, while the phenoxy (-O-) functions of compounds 3b–c were observed ranging between 3427–3431 and 1276–1280 cm$^{-1}$, respectively. The peak of the C-Cl function of 3c was observed at 680 cm$^{-1}$. The $^1$H NMR showed a singlet for the six protons the methoxy function (OCH$_3$) of curcumin in compounds 3a–c at $\delta$ 3.82–3.83 ppm, while the methoxy function (OCH$_3$) present in the phenyl ring of compound 3a at $\delta$ 3.69 ppm. The methylene bridge (-CH$_2$-) function of compounds 3b and 3c were observed at $\delta$ 4.60 and 4.80 ppm, respectively. The pyrazole CH of compounds 3a–c was observed as a singlet at 6.61 ppm and a broad singlet for the two protons of phenolic function (ArOH) at $\delta$ 9.14–9.91 ppm. The secondary amine peak of compounds 3a was observed as a singlet at $\delta$ 10.56 ppm. Two doublets were observed at $\delta$ 6.73–6.77 ppm corresponding to the -CH=CH- with coupling constant ($J$) of 12.4–14.4 Hz confirming the trans couplings. The aromatic protons of compounds 3a–c were observed as a singlet, and multiplet depending upon the nature of protons at $\delta$ 7.80–7.56 ppm. The $^{13}$C NMR of the prototype compound 3b displayed different carbon peaks at $\delta$ 160.71, 151.32, 147.46, 144.89, 137.31, 133.41, 129.83, 128.81, 123.6, 121.18, 120.19, 116.80, 114.31, 112.101, 107.72, 72.09, and 56.41 ppm. The ES-MS spectra of compound 3a showed a peak at $m/z$ 294.1 and 499.1 corresponding to M$^+$ and (M + 1)$^+$ corresponding to the molecular formula C$_{29}$H$_{26}$N$_{2}$O$_6$.

**Scheme 1.** Protocol for the synthesis of semi-synthetic curcumin analogues (3a–c).

**Table 1.** The percentage yield and reaction time for the synthesis of curcumin analogues (3a–c).

| S. No. | Compound | Method   | Reaction Condition | Yield (%) | Reaction Time (in h) |
|-------|----------|----------|--------------------|-----------|---------------------|
| 1     | 3a       | Method A | Stirring at 80 °C in AcOH | 72        | 10                  |
| 2     | 3b       |          |                    | 78        | 10                  |
| 3     | 3c       |          |                    | 74        | 10                  |
Table 1. Cont.

| S. No. | Compound | Method                  | Reaction Condition                  | Yield (%) | Reaction Time (in h) |
|--------|----------|-------------------------|-------------------------------------|-----------|----------------------|
| 4      | 3a       | Method B                | Stirring at 60–80 °C in Glycerol-water system (1:2) | 75        | 6                    |
| 5      | 3b       | Method B                |                                     | 80        | 6                    |
| 6      | 3c       |                         |                                     | 78        | 6                    |

2.2. DFT Studies

Density functional theory (DFT) studies are critical for comprehending intermolecular dynamics and designing molecules with desired pharmaceutical characteristics. This tool may be used to identify a molecule’s fundamental properties such as its frontier molecular orbital energy levels, chemical reactivity, and stability, among others [36,37]. To further understand the impact of the molecular fragments, DFT calculations were conducted using the B3LYP/6-311G(dp) basis set. The DFT-optimized conformations of the compounds 3a–c have been presented in Figure S1 [Supplementary Materials].

As shown in the Figure S2 [Supplementary Materials], HOMO is mostly confined to one of the 4-hydroxy-3-methoxyphenyl fragments attached to the pyrazole functionality, with appreciable contributions from the latter. LUMO, on the other hand, is mostly restricted to the pyrazole ring. However, the aromatic ring appended through the amide bond in the pyrazole ring was not covered by either HOMO or LUMO in the DFT-optimized molecules. The HOMO and LUMO energies and relevant global reactivity descriptors are presented in Table 2. The HOMO-LUMO gap for the compounds 3a–c was determined to be 3.55, 3.40 and 3.35 eV, respectively. This is a typical value for tiny organic cores and corresponds to the prior study [38].

Table 2. DFT results for compounds 3a–c in terms of global reactivity descriptors.

| Parameters               | 3a   | 3b   | 3c   |
|--------------------------|------|------|------|
| HOMO                     | −5.242 | −5.445 | −5.540 |
| LUMO                     | −1.693 | −2.049 | −2.193 |
| HOMO-LUMO gap            | 3.549 | 3.396 | 3.347 |
| Hardness (η)             | 1.775 | 1.698 | 1.674 |
| Softness (S)             | 0.564 | 0.589 | 0.598 |
| Chemical potential (μ)   | −3.468 | −3.747 | −3.867 |
| Electronegativity (χ)    | 3.468 | 3.747 | 3.867 |
| Electrophilicity index (ω)| 3.388 | 4.134 | 4.467 |

2.3. Antiproliferative Activity

The antiproliferative activity of the curcumin analogues was carried out against five dozen cancer cell lines derived from nine diverse panels including, breast, colon, CNS, leukemia, melanoma, non-small cell lung, ovarian, renal, and prostate cancer cell lines at one dose (10 μM) and five dose assay (0.01, 0.10, 1.00, 10, and 100 μM), as per the National Cancer Institute US [39–42]. The anticancer activity is represented as growth percent (GP) and percent growth inhibition (%GI), and are related as: %GI = 100 − GP. In single dose assay the compound 3a displayed less antiproliferative activity and was found to displayed moderate activity against NCI-H522 and HT29 with growth percent (GP) of 73.19 and 80.40 percent, respectively. The compound 3b displayed maximum anticancer activity with growth percent (GP) 7.59 percent (percent growth inhibition; %GI = 92.41%) followed by compound 3c (GP = 12.72%; %GI = 87.28%) and 3a (GP = 99.22%; %GI = 0.78%). The curcumin analogues 3b and 3c were found to be highly sensitive against all the leukemia cancer lines. The antiproliferative data of curcumin was retrieved from the NCI data base with NSC 32982 [39]. The compound is supposed to be active on particular cell lines if the GP is found to be 32% or less (i.e., %GI = 68% or more) [40–44]. The compound
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The compounds 3b and 3c were found to be active against all the cell lines of renal, prostate and breast cancer cell panels and Figure S4 (melanoma, ovarian, renal prostate and breast cancer panels) [Supplementary Materials]. The antiproliferative activity of all the compounds (3a–c) in term %GIs is given in the Table 3, while the antiproliferative activity of all the compounds (3a–c) in term GP is given in the Table S1 [Supplementary Materials]. Furthermore, the compound 3a demonstrated maximum sensitivity against NCI-H522 with GP of 73.19% (%GI = 24.81%) and least sensitivity against IGROV1 with GP of 109.32% (%GI = 9.32%). The compound 3b demonstrated maximum sensitivity against RXF 393 with GP of −44.52% (%GI = 144.52%) and least sensitivity against COLO205 with GP of 79.25% (%GI = 20.75%). Similarly, the compound 3c demonstrated maximum sensitivity against NCI-H522 with GP of −28.71% (%GI = 128.71%) and least sensitivity against COLO205 with GP of 82.37% (%GI = 17.63%). The curcumin analogues 3b and 3c displayed promising antiproliferative activity in the one dose assay (10 µM). The compound 3b exhibited good inhibitions against 54 cancer cell lines out of 56 cancer cell lines, similarly the compound 3c exhibited good inhibitions against 54 cancer cell lines out of 60 cancer cell lines at 10 µM. The antiproliferative data of curcumin analogues (3a–c) in a single dose assay at 10 µM are given in Figures S5–S7 (Supplementary Materials). Because the compounds (3b and 3c) demonstrated promising antiproliferative activity in a single dose assay, they qualified for 5-dose assay testing [40–44].

In the 5-dose assay the compound 3b demonstrated promising antiproliferative activity against 60 NCI cancer cell lines with GI50 values ranging between 0.281 and 5.59 µM, TGI values ranging between 0.49 and >100 µM, and LC50 values 4.48 and >100 µM. Similarly, the compound 3c exhibited promising antiproliferative activity with GI50 values ranging between 0.224 and 3.82 µM, TGI values ranging between 0.837 and >100 µM, and LC50 values 11.4 and >100 µM. The antiproliferative activity of compounds 3b and 3c against 60 NCI cancer cell lines in the 5-dose screening in terms of GI50, TGI, and LC50 are given in Table 3. The
compound 3b displayed the most promising antiproliferative activity against CCRF-CEM (GI50 = 0.281 µM), while the compound 3c displayed the most promising activity against SR (GI50 = 0.224 µM) among the leukemia cell lines. The compound 3b displayed the most promising antiproliferative activity against HOP-62 (GI50 = 1.08 µM), while the compound 3c displayed the most promising activity against NCI-H460 (GI50 = 0.517 µM) among the non-small lung cancer cell line. The compound 3b displayed the most promising antiproliferative activity against HCT-116 (GI50 = 0.386 µM), while the compound 3c displayed the most promising activity against HCT-15 (GI50 = 0.342 µM) among the colon cancer cell line. The compounds 3b and 3c displayed the most promising antiproliferative activity against the CNS cancer cell line SF-539 with GI50 values of 0.354 and 0.38 µM, respectively. The compounds 3b and 3c displayed the most promising antiproliferative activity against the melanoma cancer cell line MDA-MB-435 with GI50 values of 0.21 and 0.243 µM, respectively. The compounds 3b and 3c displayed the most promising antiproliferative activity against the ovarian cancer cell line OVCAR-3 with GI50 values of 0.552 and 0.511 µM, respectively. The compound 3b and 3c displayed the most promising antiproliferative activity against the renal cancer cell line UO-31 with GI50 values of 0.302 and 0.362 µM, respectively. The compounds 3b and 3c displayed the most promising antiproliferative activity against the prostate cancer cell line DU-145 with GI50 values of 1.37 and 0.833 µM, respectively. The compounds 3b and 3c displayed the most promising antiproliferative activity against the breast cancer cell line MCF7 with GI50 values of 0.343 and 0.336 µM, respectively. The compound 3b exhibited less selectivity against all the eight panels (except leukemia) of cancer cell lines with a selectivity ratio (SR) ranging between 0.59 and 1.63, similarly the compound 3c exhibited less selectivity against all the panels of cancer cell lines with SR ranging between 0.80 and 2.94 (Table 3). The mean GI50 for an individual panel was calculated for each curcumin analogues (3b and 3c) (Table 3; Subpanel MID) and compared with that of the curcumin (Figure 2). The curcumin analogues 3b and 3c displayed superior activity to curcumin (Figure 2). The curcumin analogues displayed superior antiproliferative activity to curcumin. Furthermore, the compounds 3b exhibited moderate selectivity against the leukemia panel of cancer cell lines with a selectivity ratio of 4.59. The value of SR > 6 showed higher selectivity, SR with 3–6 value showed moderate selectivity, while the SR value less than 3 showed less selectivity against a particular panel of cancer cell lines [45,46]. The anticancer data for compounds 3b and 3c in terms of TGI and LC50 in µM concentrations is given in Table S1 (Supplementary Materials) and a graph plot between GP and Log10 concentrations are given Figure S8 (for compound 3b) and Figure S9 (for compound 3b) (Supplementary Materials).

### Table 3. Mean GI50 values of curcumin analogues against cancer cell lines

| Cancer Panel | Mean GI50 (µM) |
|--------------|---------------|
| Leukemia     | 0.281         |
| Non-small cell lung cancer | 0.224         |
| Colon cancer | 0.833         |
| CNS cancer   | 0.386         |
| Melanoma     | 0.517         |
| Ovarian cancer | 0.354     |
| Renal cancer | 1.37          |
| Prostate cancer | 0.123    |
| Breast cancer | 0.343       |

**Figure 2.** The mean GI50 (in µM) for curcumin (Cu) and curcumin analogues 3b and 3c.
According to the frontier-orbital theory, the two major parameters that influence bioactivities are the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) [47–49]. Thus, the study of the frontier-orbital energies may be helpful to the investigation of anticancer activity. The HUMO-LUMO gaps (\( \Delta E \)) of the three compounds followed as \( 3a > 3b > 3c \). The narrow HUMO-LUMO gap (\( \Delta E \)) implies a high chemical reactivity as well as biological activity [50]. This suggested that compound \( 3c \) might possess a relatively high anticancer activity. The anticancer activity of \( 3b,c \) was found to be promising in a single dose assay with %GIs of 92.41 and 87.28, respectively, at 10 \( \mu \)M, while the mean GI\(_{50}\) was calculated as 1.241 and 1.149 \( \mu \)M, respectively, in the five dose assay. Both the compounds were found to be active against 54 cancer cell lines. Comparing the anticancer activity of the three compounds, the order followed as \( 3c \geq 3b > 3a \) (Figure 3).

Table 3. 60 NCI cancer cell lines-based antiproliferative activity of curcumin analogues \( 3a–c \) in single dose (10 \( \mu \)M) and 5-dose assay (0.001–100 \( \mu \)M) of curcumin analogues \( 3b–c \).

| Panel              | Cell Line  | Single Dose Assay (10 \( \mu \)M) | Five Dose Assay |  |
|-------------------|------------|----------------------------------|----------------|---|
|                   |            | %GI\(a\) | %GI | %GI | GI\(_{50}\) | MID\(^b\) | SR | GI\(_{50}\) | MID\(^b\) | SR |
| Leukemia          | CCRF-CEM   | 4.17    | 93.21 | 95.65 | 0.281 | 0.27 | 4.59 |           | 0.39 | 2.94 |
|                   | HL-60(TB)  | 14.61   | -    | 118.70 | 0.282 | 0.314 |           | 0.381 |           | 0.425 | 0.569 |
|                   | K-562      | -6.41   | -    | 95.59 | 0.366 | 0.27 | 4.59 |           | 0.39 | 2.94 |
|                   | MOLT-4     | 7.64    | -    | 100.33 | 0.368 | 0.381 |           | 0.425 |           | 0.569 | 0.224 |
|                   | RPMI-8226  | -2.92   | 103.54 | 106.80 | 0.377 | 0.39 | 2.94 |           | 0.39 | 2.94 |
|                   | SR         | 1.88    | -    | 99.99 | 0.318 | 0.39 | 2.94 |           | 0.39 | 2.94 |
| Non-Small Cell Lung Cancer | A549/ATCC | 6.89    | 91.32 | 89.42 | 2.42 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | EKVX       | -0.71   | 71.88 | 61.65 | 2.53 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | HOP-62     | 8.37    | 91.61 | 88.72 | 1.08 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | HOP-92     | -4.08   | 85.63 | 87.45 | 3.13 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | NCI-H226   | 0.94    | 90.04 | 73.48 | 3.15 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | NCI-H23    | 1.79    | 98.40 | 85.92 | 1.33 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | NCI-H322M  | 0.78    | 69.16 | 59.18 | 1.78 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | NCI-H460   | -4.28   | 92.40 | 90.72 | 1.25 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | NCI-H522   | 24.81   | 139.28 | 128.71 | 0.304 | 1.54 | 0.81 | 1.43 | 0.80 |
| Colon Cancer      | COLO 205   | 2.26    | 20.75 | 17.63 | 1.96 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | HCC-2998   | 2.95    | 65.24 | 61.52 | 2.27 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | HCT-116    | -       | 96.32 | 96.52 | 0.386 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | HCT-15     | 8.77    | 90.36 | 90.48 | 0.417 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | HT29       | 19.60   | 133.23 | 97.07 | 2.66 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | KM12       | 0.07    | 91.04 | 90.07 | 0.501 | 1.54 | 0.81 | 1.43 | 0.80 |
|                   | SW-620     | -5.14   | 92.65 | 83.27 | 0.608 | 1.54 | 0.81 | 1.43 | 0.80 |
| CNS Cancer        | SF-268     | 1.27    | 88.05 | 79.78 | 0.651 | 1.26 | 0.98 | 1.36 | 0.84 |
|                   | SF-295     | 1.78    | 84.50 | 76.89 | 2.78 | 1.26 | 0.98 | 1.36 | 0.84 |
|                   | SF-539     | 3.08    | 110.32 | 91.36 | 0.354 | 1.26 | 0.98 | 1.36 | 0.84 |
|                   | SNB-19     | -1.82   | 85.25 | 71.02 | 1.36 | 1.26 | 0.98 | 1.36 | 0.84 |
|                   | SNB-75     | -0.46   | 77.26 | 94.88 | 0.822 | 1.26 | 0.98 | 1.36 | 0.84 |
|                   | U251       | 1.88    | 91.19 | 83.69 | 0.564 | 1.26 | 0.98 | 1.36 | 0.84 |
**Table 3.** Cont.

| Panel       | Cell Line | Single Dose Assay (10 µM) | Five Dose Assay |  |  |
|-------------|-----------|---------------------------|-----------------|---|---|
|             |           | 3a | 3b | 3c | %GI | %GI | %GI | GI50 | MID b | SR | GI50 | MID b | SR |
| LOX IMVI    |           | 1.23 | 96.77 | 96.21 | 0.55 |      |      |      | 0.497 |      |      |      |    |
| MALME-3M    |           | –0.37 | 92.79 | 87.91 | 0.585 |      |      |      | 0.488 |      |      |      |    |
| M14         |           | –4.24 | 93.25 | 95.66 | 0.343 |      |      |      | 0.349 |      |      |      |    |
| MDA-MB-435  |           | –4.44 | 91.16 | 112.15 | 0.21 |      |      |      | 0.243 |      |      |      |    |
| SK-MEL-2    |           | 12.95 | 118.73 | 104.36 | 0.661 |      |      |      | 0.76  | 1.63 | 0.302 | 0.72  | 1.59 |
| SK-MEL-28   |           | –8.09 | 81.67 | 74.42 | 1.12 |      |      |      | 1.19  |      |      |      |    |
| SK-MEL-5    |           | –7.15 | 128.80 | 112.95 | 0.834 |      |      |      | 0.661 |      |      |      |    |
| UACC-257    |           | 3.56 | 69.55 | 58.09 | 2.21 |      |      |      | 2.19  |      |      |      |    |
| UACC-62     |           | –2.07 | 95.12 | 94.13 | 0.326 |      |      |      | 0.552 |      |      |      |    |
| IGROV1      |           | –9.32 | 91.57 | 87.46 | 0.919 |      |      |      | 1.99  |      |      |      |    |
| OVCAR-3     |           | –4.55 | 104.46 | 86.01 | 0.552 |      |      |      | 0.511 |      |      |      |    |
| OVCAR-4     |           | –7.52 | 74.40 | 68.58 | 0.845 |      |      |      | 1.29  |      |      |      |    |
| OVCAR-5     |           | –6.96 | 77.89 | 43.27 | 2.75 |      |      |      | 2.71  |      |      |      |    |
| OVCAR-8     |           | –0.06 | 96.74 | 93.48 | 2.52 |      |      |      | 1.59  |      |      |      |    |
| NCI/ADR-RES |           | –0.40 | 79.56 | 78.50 | 5.59 |      |      |      | 0.538 |      |      |      |    |
| SK-OV-3     |           | 7.73 | 128.58 | 100.16 | 1.53 |      |      |      | 1.26  |      |      |      |    |
| 786-0       |           | –3.54 | 98.73 | 86.31 | 1.06 |      |      |      | 0.634 |      |      |      |    |
| A498        |           | 4.70 | 88.83 | 82.44 | 2.68 |      |      |      | 3.82  |      |      |      |    |
| ACHN        |           | –4.23 | 82.04 | 81.30 | 0.49 |      |      |      | 0.476 |      |      |      |    |
| Caki-I      |           | –4.75 | 75.80 | 85.21 | 0.657 |      |      |      | 0.818 |      |      |      |    |
| RXF 393     |           | –2.31 | 144.52 | 109.98 | 1.00 |      |      |      | 1.27  |      |      |      |    |
| SN 12C      |           | –0.55 | 84.29 | 91.91 | 0.799 |      |      |      | 0.935 |      |      |      |    |
| TK-10       |           | 12.38 | 87.27 | 79.48 | 2.91 |      |      |      | 1.79  |      |      |      |    |
| UO-31       |           | 15.23 | 99.52 | 91.28 | 0.302 |      |      |      | 0.362 |      |      |      |    |
| PC-3        |           | –1.38 | 91.51 | 89.77 | 1.60 |      |      |      | 1.48  | 0.84 | 1.80  | 1.31  | 0.87 |
| DU-145      |           | –5.84 | 90.91 | 83.46 | 1.37 |      |      |      | 0.833 |      |      |      |    |
| MCF7        |           | –4.53 | 88.70 | 95.79 | 0.343 |      |      |      | 0.336 |      |      |      |    |
| MDA-MB-231  |           | –9.02 | 88.67 | 89.85 | 2.38 |      |      |      | 2.38  |      |      |      |    |
| HS 578F     |           | 1.56 | 77.50 | 89.78 | 1.12 |      |      |      | 1.20  |      |      |      |    |
| BT-549      |           | –4.15 | 103.68 | 98.59 | 0.571 |      |      |      | 0.526 |      |      |      |    |
| T-47D       |           | 11.07 | 87.57 | 85.02 | 1.62 |      |      |      | 1.25  |      |      |      |    |
| MDA-MB-468  |           | –6.31 | 101.76 | 87.50 | 1.71 |      |      |      | 1.99  |      |      |      |    |
| Mean        |           | 0.78 | 92.41 | 87.28 |      |      |      |      | 1.241 |      |      |      |    |

(-) Indicates activity not tested; a The average sensitivity of all cell lines toward the test agent in µM; b The average sensitivity of all cell lines of a particular subpanel toward the test agent in µM; Bold font shows the good percent growth inhibition (%GI (≥68%) in one dose assay; %GI = 100 – GP; MID a and MID b are the mean GI50 of 60 NCI cancer cell lines and the individual cancer cell line panels; Selectivity ratio (SR) = MID a / MID b; GI50, stands for 50% growth inhibition.
The anti-EGFR activity of curcumin and curcumin analogues was well documented in the literature [7,18]. The molecular docking against EGFR (PDB ID: 3W2R) was carried out as a putative mechanism of anticancer activity of the curcumin analogues investigated in the current work as per the reported protocol [51]. The molecular docking score, 2D interaction images, and types of interactions of curcumin analogues and curcumin are given in Table 4. All the curcumin analogues displayed efficient binding against the active site of EGFR with a binding affinity of $-6.003$ to $-7.957$ kcal/mol, while curcumin displayed a binding affinity of $-7.391$ kcal/mol. The ligand 3a displayed two H-bonds one between the phenolic function and the residue Leu718 and another between the carbonyl function and residue Thr854. The ligand 3c displayed four H-bonds and a halogen bond, the two $\pi$-cationic interactions. The ligand 3b displayed three H-bonds, one H-bond between the methoxy function and the most important residue Met793, one H-bond between another methoxy function and the residue Lys875 and one H-bond between the phenolic function and the residue Asp855. The $\pi$-$\pi$-stacking interaction was observed between phenyl (of phenoxy) and the residue Phe997, while $\pi$-cationic interaction was displayed between phenyl (of curcumin) and the residue Arg841. The 3D interactions of ligand 3b against the active site of EGFR are shown in Figure 5. The curcumin displayed two types of interactions: H-Bond interaction with the residues Phe856, Ala743, and Lys745 and $\pi$-Cationic with the residue Arg858.

**Figure 3.** DFT-optimized structures, HOMO/LUMO visualization, anticancer activity and DFT correlation of compounds 3a–c.

### 2.4. Molecular Docking Studies

The anti-EGFR activity of curcumin and curcumin analogues was well documented in the literature [7,18]. The molecular docking against EGFR (PDB ID: 3W2R) was carried out as a putative mechanism of anticancer activity of the curcumin analogues investigated in the current work as per the reported protocol [51]. The molecular docking score, 2D interaction images, and types of interactions of curcumin analogues and curcumin are given in Table 4. All the curcumin analogues displayed efficient binding against the active site of EGFR with a binding affinity of $-6.003$ to $-7.957$ kcal/mol, while curcumin displayed a binding affinity of $-7.391$ kcal/mol. The ligand 3a displayed two H-bonds one between the phenolic function and the residue Leu718 and another between the carbonyl function and residue Thr854. The ligand 3c displayed four H-bonds and a halogen bond, the two $\pi$-cationic interactions. The ligand 3b displayed three H-bonds, one H-bond between the methoxy function and the most important residue Met793, one H-bond between another methoxy function and the residue Lys875 and one H-bond between the phenolic function and the residue Asp855. The $\pi$-$\pi$-stacking interaction was observed between phenyl (of phenoxy) and the residue Phe997, while $\pi$-cationic interaction was displayed between phenyl (of curcumin) and the residue Arg841. The 3D interactions of ligand 3b against the active site of EGFR are shown in Figure 5. The curcumin displayed two types of interactions: H-Bond interaction with the residues Phe856, Ala743, and Lys745 and $\pi$-Cationic with the residue Arg858.
Table 4. The molecular docking results of curcumin analogues against EGFR.

| S. No. | Compound | 2D Molecular Docking Score | Glide Emodel | Interaction |
|--------|----------|-----------------------------|--------------|-------------|
| 1      | 3a       | -7.957                      | -80.016      | H-Bond (Leu718); H-Bond (Thr854) |
| 2      | 3b       | -7.778                      | -87.340      | H-Bond (Met793); H-Bond (Asp855); H-Bond (Lys875); π-π-Stacking (Phe997); π-Cationic (Arg841) |
| 3      | 3c       | -6.003                      | -75.919      | H-Bond (Met793); H-Bond (Asp855); H-Bond (Lys875); H-Bond (Cys797); Halogen bond (Cys797) |
| 4      | Curcumin | -7.391                      | -85.784      | H-Bond (Phe856); H-Bond (Ala743); H-Bond (Lys745); π-Cationic (Arg858) |
3. Discussion

The anticancer activity of six 3,5-bis(4-hydroxy-3-methylstyryl)-N-(substitutedphenyl)-1H-pyrazole-1-carboxamides has previously been reported, and their antiproliferative properties in terms of GPs ranged between 7.23 and $-19.19$ percent, which were found to be higher than that of compound 3a, which showed a mean percent GP of 99.22 percent [7]. The curcumin analogues reported earlier showed superior antiproliferative activity to curcumin in the one dose (10 $\mu$M) as well as the five dose assay [7,52]. The curcumin analogue displayed maximum anticancer activity with IC$_{50}$ of 7.1 $\mu$M against MCF7 cell line [53]. The curcumin pyrazole reported by other researchers displayed cytotoxicity against PC-3, MCF-7, MDA-MB-231 with IC$_{50}$ values of 5.6 $\pm$ 2.0, 5.9 $\pm$ 0.6 and 6.6 $\pm$ 1.9 $\mu$M, respectively [54]. The curcumin analogues reported displayed anticancer activity against Hep-G2, HCT-116, and QG-56 cell lines with IC$_{50}$ values ranging between 12.5 and 50 $\mu$M [55]. The cucumin analogue showed maximum cytotoxicity against LNCaP and PC-3 with IC$_{50}$ values of 54.8 $\pm$ 2.5 and 52.1 $\pm$ 4.8 $\mu$M, respectively [56]. The curcumin analogue showed
cytotoxicity against MCF-7, MDA-MB-231, HeLa, DU-145, and SKNSH with IC50 values of 5.31, 8.33, 7.69, 8.62, and 8.19 µM and also inhibited Akt and STAT3 phosphorylation and increased ERK phosphorylation [57]. The curcumin analogue inhibited tubulin with an IC50 value of 16 µM [58]. The curcumin analogues (3b and 3c) in the current investigation showed superior antiproliferative activity to curcumin in the one dose as well as the five dose assay. The curcumin analogues (3b,c) displayed maximum sensitivity against the leukemia cell line panel with GI50 values ranging between 0.224 and 0.569 µM (Table 3), while the sensitivity ratios were found to be 4.59 and 2.94, respectively. A series of four curcumin analogues have also been reported by our research team that showed antiproliferative activity with a mean GP ranging between 0.92 and −16.09, while compounds (3b,c) displayed mean GPs of 7.59 and 12.72, respectively [52]. The chemical modifications of a diketonic function into pyrazole and dihydropyrimidinone analogues were always found to be more promising when compared with the chemical modification of a diketonic function into bigenelli type curcumin analogues [7,10,55,59,60]. The curcumin analogues reported in another published work demonstrated cytotoxicity on the CCGF-CEM cell line with IC50 ranging between 3.13 and 93.40 µM, while compounds 3b (GI50 = 0.281 µM) and 3c (GI50 = 1.34 µM) in the current investigation showed superior activity against the same cell lines [61]. The compounds 3,5-Bis(4-hydroxy-3-methoxystyryl)-1H-pyrazole-1-yl-(substituted phenox)ethanones (3b,c) were reported for the first time in the current investigation and they demonstrated promising antiproliferative activity against 5 dozen cancer cell lines derived from nine diverse panels. Some of the curcumin analogues reported earlier showed IC50 values of 16.71 (curcumin pyrazole) and 5.85 µM (curcumin semicarbazide) in an SRB assay against the HCT 116 cell line, while our compounds 3b and 3c displayed superior anticancer activity with GI50 values of 0.386 and 0.416 µM, respectively [62]. The curcumin analogue 3b was found to be moderately selective against the leukemia panel with an SR of 4.59. In our previous work the curcumin analogues were found to be non-selective towards the cancer cell line panels [7]. In the current study, it was discovered that the curcumin analogues demonstrated better antiproliferative activity than previously reported curcumin analogues [63,64]. Since the reported curcumin analogues (3a–c) in the current investigation showed encouraging binding affinity against the EGFR, this information may also help to highlight the biological significance of curcumin analogues.

4. Materials and Methods

4.1. Preparation of Curcumin Analogue 3a

An equimolar mixture of curcumin (1) (1 mmol; 368 mg) and N-(2-methoxyphenyl)-hydrazine carboxamide (2a) (1 mmol; 181 mg) in glacial acetic acid was stirred continuously at 80 °C in a sand bath for 10 h. The reaction mixture was then concentrated under a vacuum to remove excess solvent before being poured into crushed ice, filtered, dried, and recrystallized with ethanol to yield the compound 3a. The intermediate 2a was prepared as per the reported method in two steps starting from o-anisidine [34]. Method A used glacial acetic acid as a solvent, whereas Method B used a green solvent system glycerol-water (1:2), which was found to be faster and yielded slightly more.

4.2. Preparation of Curcumin Analogue 3b,c

An equimolar mixture of curcumin (1) (1 mmol; 368 mg) and substituted phenoxyacetohydrazide (2b–c) (1 mmol) in glacial acetic acid was stirred continuously at 80 °C in the sand bath for 10 h. The reaction mixture was then concentrated under a vacuum to remove excess solvent before being poured into crushed ice, filtered, dried, and recrystallized with ethanol to yield compound 3b–c. The intermediates 2b–c were prepared as per the reported method in two steps starting from substituted phenol as per the reported method [35]. Method A used glacial acetic acid as a solvent, whereas Method B used a green solvent system glycerol-water (1:2), which was found to be faster with slightly higher yields.
4.3. DFT Analyses

The ligand’s 2D structure was created in Marvin Sketch (Marvin, 21.14, 2021, ChemAxon, http://www.chemaxon.com/ accessed on 24 July 2022), then transformed to a 3D structure and saved in xyz format. The DFT calculations were carried out in the gas phase using the Orca 5.03 package [65,66] and the B3LYP/6-311G (dp) basis set [67]. Chemcraft (https://www.chemcraftprog.com accessed on 24 July 2022) and Avogadro programs were used for analysis of the DFT results [68,69].

4.4. Antiproliferative Activity

The antiproliferative activity of the curcumin analogues (3a–c) was evaluated against nine diverse panels of 60 cancer cell lines at 10 µM according to the National Cancer Institute (NCI US) protocol [39–42]. The antiproliferative activity was calculated as growth percent (GP) and percent growth inhibition (%GI) at one dose assay at 10 µM. In the five dose assay, the curcumin analogues (3b–c) were treated against the cell lines in the given concentrations of 0.001 to 100 µM and there different parameters viz. GI₅₀, TGI and LC₅₀ were calculated for each cell line [43,44]. The parameters GI₅₀, TGI and LC₅₀ are the molar concentration producing 50% growth inhibition, total growth inhibition (TGI) and a 50% cellular death, respectively.

4.5. Molecular Docking Studies

The ligands 3a–c were molecular docked against EGFR. The protein data bank provided the EGFR (PDB: 3W2R) X-ray crystal structure with a resolution of 2.05 Å; R-value 0.220 (observed) (https://www.rcsb.org/structure/3W2R accessed on 24 July 2022) [70]. The ligands 3a–c were saved as mol files, and docking was performed according to the reported protocol [7,51].

5. Conclusions

We have prepared and reported the antiproliferative activity of three curcumin analogues (3a–c). The antiproliferative activity of compounds 3b and 3c showed promising anticancer activity in a one dose as well as a five dose assay. The compound 3b showed the most promising antiproliferative activity among the series of curcumin analogues and showed moderate selectivity against leukemia with an SR value of 4.59. The curcumin analogues (3b,c) demonstrated superior antiproliferative activity to curcumin. HOMO-LUMO gaps (ΔE) from DFT analysis and anticancer activities were discovered to be in agreement. All the curcumin analogues (3a–c) demonstrated significant binding affinity against the EGFR, a potential anticancer target. Because the compounds demonstrated promising anticancer activity and significant binding affinity against the EGFR, the current report has the potential to enhance the anticancer research development program in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11212835/s1, Characterization of compounds 3a–c; Figure S1: The DFT-optimized conformations of the compounds 3a–c; Figure S2: Visualization of HOMO/LUMO of compounds 3a–c; Figures S3 and S4: Antiproliferative profile of compound 3a–c and curcumin (Cu), Figures S5–S7: Anticancer data of compounds 3a–c in single dose assay; Figures S8 and S9 Anticancer data of compounds 3b,c in the five dose assay; Table S1: 60 NCI cancer cell lines based antiproliferative activity of curcumin analogues 3a–c in single dose (10 µM) and 5-dose assay (0.001–100 µM) of curcumin analogues 3b–c.

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Abbreviations
CNS Central nervous system
DFT Density functional theory
EGFR Epidermal growth factor receptor
ES-MS Electrospray Ionization Mass Spectrometry
eV Electron volt
GI Growth inhibition
GP Growth percent
HOMO highest occupied molecular orbital
IR Infrared
LC Lethal concentration
LUMO lowest unoccupied molecular
NCI National Cancer Institute
NMR Nuclear magnetic resonance
NP Natural product
TGI Total growth inhibition
TLC Thin layer chromatography

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