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Synthesis of 5-fluorouracil co-crystals with novel organic acids as co-formers and its anticancer evaluation against HCT-116 colorectal cell lines

Farhat Jubeen[1], Aisha Liaqat[1], Fiza Amjad[1], Misbah Sultan[2], Sania Zafar Iqbal[1], Imran Sajid[3], Muhammad Bilal Khan Niazi[4], Farooq Sher[5,*]

1. Department of Chemistry, Government College Women University, Faisalabad 38000, Pakistan
2. Institute of Chemistry, University of the Punjab, Lahore 54590, Pakistan
3. Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore 54590, Pakistan
4. School of Chemical and Materials Engineering, National University of Sciences and Technology, Islamabad 44000, Pakistan
5. School of Mechanical, Aerospace and Automotive Engineering, Coventry University, Coventry CV1 5FB, UK

*Corresponding author:
Email: Farooq.Sher@coventry.ac.uk; Tel: +44 (0) 24 7765 7688

Abstract

5-fluorouracil (5-FU) being a mainstream anticancer drug is under keen and detailed investigation for prodrugs formulations in order to minimize the associated side effects. Co-crystallization of 5-FU is an innovative technique for the synthesis of 5-FU prodrugs to improve its anticancer effectiveness. The present study is based on the synthesis of 5-FU supramolecular synthons with four co-formers; Succinic acid, cinnamic acid, malic acid and benzoic acid utilizing acetone as a solvent. Solid state grinding followed by slow evaporation solution method was applied. Colorless clear crystals were obtained in all the cases. The co-crystal formation was supported with the help of FTIR and PXRD. Through FTIR, the main peaks of interest in the spectrum of 5-FU, frequency (ν) of N-H (3409.02 cm^{-1}) and of carbonyl group (1647.80 cm^{-1}), were prominently shifted in all the spectra of co-crystal demonstrating the replacement as well as the development of already present interactions with new ones. For 5-FU-Cinnamic acid co-crystals, the anticipated peaks

were observed at 1673.13 cm^{-1} (–C=O) and 3566.89 cm^{-1} (N-H) manifesting significant change in comparison to 5-FU. With the help of powdered XRD characterization, the representative peak of 5-FU was recorded at $2\theta=28.80^{o}$. The shifting of this peak and development of many new ones in the spectra of co-crystals proved the development of new structural entities. Finally, the anticancer activity of all the co-crystals was evaluated in comparison to that of API. All the co-crystals manifest significantly greater growth inhibition potential than the main API. 5-FU-Cinnamic acid (3C) was the one which proved to be the most potent anticancer agent at all the four concentrations; 4.82% (12 µg/mL), 34.21% (25 µg/mL), 55.08 % (50 µg/mL) and 67.29% (100 µg/mL). In short, this study proved to be a true example to enhance the anticancer potential of 5-FU following fairly easy fabrication requirements of co-crystallization phenomenon. After the successful synthesis of these supramolecular synthons and subsequent enhancement of growth inhibition potential of 5-FU, these co-crystals can further be evaluated for *in vivo* trials and membrane crossing potentials in future.

Keywords: 5-fluorouracil; co-crystallization; solid state grinding method; MTT assay and supramolecular interactions.

1 Introduction

5-FU which is simply a uracil derivative is in the exploration of many other biological activities in addition to anticancer potential e.g., antimicrobial, anti-fungal, against hypertonic scars, keloids and actinic keratosis etc. It is an antimetabolite of uracil obtained by the replacement of hydrogen at C-5 of uracil molecule with a fluorine atom, a member of pyrimidine-based antimetabolites. 5-FU is among the most common chemotherapeutic agents, introduced in 1958 [1], used against a variety of solid cancers by acting as thymidylate synthase inhibitor e.g., breast, colorectal, gastric, head and neck cancers etc. [2, 3]. Cancer is not a single disease but a syndrome containing hundreds

of diseases involving rapid and uncontrolled cell growth with the potential to invade or spread to the other parts of the body through the blood circulation. It is a serious public health issue around the globe[4, 5]. The modern mean of cancer therapies, chemotherapy and radiation have, adversarial side effects in the individuals suffering from cancer and require much dedication and work to minimize the adverse outcomes[6]. The activity of 5-FU is manifested after several enzymatic transformations for its binding to thymidylate synthase and its inhibition, followed by interference in the synthesis of nucleic acids [7, 8]. Frequent undesirable side effects associated with 5-FU chemotherapy are gastrointestinal symptoms, alopecia, cardio-toxicity and neutropenia, while leuko-encephalopathy, depression, organic mental disorder [9] have limited its clinical use; only tegafur, Floxuridine, Carmofur [10] are potential drugs in use.

Chemical modification of an active drug in order to minimize its drawbacks and to bring unchanged at the targeted site is a contemporary phenomenon to obtain the desired outcomes of a drug. In this regard, innumerable approaches for 5-FU structural modifications have been devised and tested. 5-FU formulations in the form of nanoparticles [11, 12, 13, 14, 15], combination with macromolecules [4, 16], attachment with target selective pro moieties like DNA intercalator molecules [17, 18] certainly incorporated and enhanced many vital properties of 5-FU. Following these strategies; half-life of 5-FU, target selectivity and sustained blood level is enhanced. However; in none of the above-mentioned approaches, synthesis methodologies are free from inadequacies. There is not even a single protocol in the above-mentioned strategies which reveals combined advantages of single step synthesis, easy synthesis requirements, no need for isolation and purification of end products. Co-crystallization is an advanced phenomenon which combined all the above-mentioned synthesis requirements in a single methodology[19].

5-FU Co-crystals were successfully synthesized with some aromatic compounds; 3-hydroxybenzoic acid, 4-aminobenzoic acid and cinnamic acid following grinding method. All three synthesized co-crystals manifest improved membrane permeability. However, the reasons behind the selection of these co-formers were not specified [20]. Besides this, cocrystals of 5-FU are also reported with heterocyclic compounds; Acridine, Phenazine and 4,4-bispyridylethene [21] and benzoic acid derivatives; 4-methylbenzoic acid and 3-aminobenzoic acid following solution method. Again in none of these studies, the effectiveness of co-formers was mentioned [22]. Although, studies are reported about the successful formation of 5-FU co-crystal with a variety of co-formers with the help of their structural evidence. However, data is lacking in their biological effectiveness; like, in none of these the anticancer potential of the synthesized pro moieties was evaluated. In a very recent study, supramolecular synthons of 5-FU were synthesized with two dihydroxybenzoic acid compounds (gentisic acid and 3,4-dihydroxybenzoic acid) and a pyridine derivative (p-aminopyridine)[23]. No doubt, the study was comprehensive in lieu of physical and biological characterization of synthesized co-crystals. However, ethanol was used as a solvent which itself is strongly capable of hydrogen bonding interactions. In a very innovative study of drug plus drug co-crystals of 5-FU with 5-fluorocytocine, water was used as a solvent to develop co-crystals. Water is itself capable of developing strong hydrogen bonding interactions [24, 25]. In short, some of the studies in this domain are focused only on the successful formation of co-crystals without their biological evaluation. Also, in majority of the studies the reason behind the selection of co-formers or the effectiveness of co-formers is not mentioned. Moreover, the solvents utilized for the development of co-crystals in almost all the reported studies are capable of participating in the formation of hydrogen bonding interactions.

In contrast to all reported studies; the present study aimed to utilise a solvent not having any hydrogen bond donating or accepting groups. 5-FU Co-crystals are being reported with four diverse co-formers; cinnamic acid (Cn), succinic acid (Sc), malic acid (Ml) and benzoic acid (Bn). All the co-formers were selected by keeping their individual anticancer, antioxidant and anti-inflammatory properties [26, 27, 28, 29] in consideration which could be resulted in the augmentation of the overall effectiveness of 5-FU after co-crystallization. In all the reported studies in this domain, methanol or water solvent was used to facilitate the development of supramolecular interactions. However, in the present study, acetone, a nonpolar, aprotic solvent is used to ascertain the hydrogen bonding interactions only between API and co-formers. Although acetone has partial negative oxygen atom and partially positive terminal hydrogens however it does not form intramolecular hydrogen bonds however intermolecular hydrogen bonding is reported but only with water molecules[30]. The co-crystal formation is analyzed with the help of FTIR and PXRD. Besides this anticancer potential of all the synthesized co-crystal is evaluated with the help of MTT assay. This study is a true example to further develop supramolecular interactions (non-covalent interactions) of 5-FU with a variety of effective co formers and further studies can be designed following the same approach utilizing a variety of suitable solvents.

2 Material and methods

2.1 Materials

5-FU was purchased by Sigma-Aldrich. Other chemicals in the present study were cinnamic acid (VWR, 99%), succinic acid (Avandol, ultrapure), malic acid (BDH Chemicals Ltd, 99.5%), benzoic acid (Duksan pure chemicals, 99.5%) and acetone (Merck KGaA, 99.5%). Co-crystals of 5-FU were synthesized with cinnamic acid, succinic acid, malic acid and benzoic acid following solid-state grinding followed by slow evaporation solution method [31,32, 33].

2.2 Preparation of co-crystals

2.2.1 Mechanical grinding method

The equimolar quantities of API (0.572 g) and co-former (Cn, 0.162 g; Sc, 0.129 g; Ml, 0.1474 g and Bn, 0.1343 g), 4.4 mM, were ground strongly using motor and pestle for about 30 minutes. The method employed in the study[32], was followed with some modifications. The ground mass was then dissolved in about 10 mL of acetone to form a solution. Afterwards, it was heated to form a clear solution. Finally, the vials were cooled to room temperature and placed for a few days for evaporation to observe crystal formation. In all cases, Colourless crystals were obtained.

2.3 Characterization

2.3.1 FTIR and PXRD

The development of supramolecular interactions as a consequence of practical changes in vibrational modes of anticipated functional groups were evaluated with the help of FTIR analysis. This characterization was done at ATR-FTIR spectrophotometer (Shimadzu IR prestige-21, USA). Transmission mode in the range of 400 cm^{-1} to 4000 cm^{-1} was applied to record the spectra. The study of all the Spectra was done in comparison to the API spectrum alone and the difference absorption frequencies of -N-H and -C=O groups from the observed place and shape (position at which the peak was observed in the spectrum of API alone) were evaluated in order to study the formation of non-covalent interactions for the synthesis of co-crystals [34]. For further confirmation of new hydrogen bonding interactions, co-crystals were analysed through PXRD [20, 34, 35]. PXRD analysis is based on constructive interference between homochromatic X-rays and crystalline samples. During this procedure, X-rays were formed by cathode ray tube. These rays were then

filtered to get homochromous rays, accumulated and then focused towards the sample. Finally, the bio-evaluation of the as prepared co-crystals was done through MTT assay [36, 37].

2.3.2 MTT assay *In vitro*

For the evaluation of antitumor potential of synthesized co-crystals, Human colorectal cancer cell lines (HCT 116) ATCC®CCL-247™ [(catalogue no: 91091005-1VL) Sigma Aldrich] were utilized. Cultivation of cells in the form of a monolayer was performed in T-75 flasks Costar and the subculturing was done two times a week. The conditions were; 37 °C in CO_2 (5%) and 100% relative humidity provided incubator. The passage number was kept low, from 5 to 20, for the accomplishment of this process. The medium applied for the cultivation of HCT 116 was McCoy's 5A Gibco Glasgow, complemented with fetal bovine serum (FBS) (10%), Gibco, Glasgow, UK and antibiotics (1%) (streptomycin, penicillin)[38].

Washing of the anchorage-dependent cells at an exponential growth phase was performed with PBS (phosphate buffered saline) about 2 mL. Subsequently, the separation was done with the help of 0.5 mL of 1X trypsin and reared for 2–5 minutes at 37 °C in the incubator. About 100 µL of complete growth media was poured into 96-well flat-bottom microplates per well. Afterwards, the calculation of cells was performed for anticipated densities with the help of staining using trypan blue and tallied with a hemacytometer. Densities of 1,000–100,000 cells per well were injected[37]. Subsequently, cells were treated with a range of concentrations of synthesized co-crystals; 12, 25, 50 and 100 mg/mL. The experiments were performed in triplicates to avoid errors.

Further in each experiment Background control wells having the same volume of complete culture medium were involved accompanied by a positive control comprising Triton X-100 and negative

controls as well. Then the plate was cultivated at 37 ºC for 24 hours in CO_2 supplied moistened incubator[39]. 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (10 µL) was directly supplemented in the culture media of each well after 24 hours. Then incubation of the plate was performed for about 4 hours at 37 ºC in 5% CO_2 incubator. After incubation, the separation of culture media was done softly so that the cell`s monolayer remains intact. Afterwards, each well was) supplemented with 100 µL volume of dimethyl sulfoxide (DMSO) in each well and plate was shaken to homogenize formazan[40]. Absorbance was evaluated with the help of spectrophotometer at 570 nm wavelength. The anticancer potential was found out by the calculation of inhibition rate and graphs were plotted against all concentrations. Finally, for each extract. IC_{50} values were calculated. The growth inhibition rate was calculated using Eq. (1) [41]:

$$\text{Percentage Mortality (\%)} = \frac{\text{O.D (Control well)} - \text{O.D (Treated well)}}{\text{O.D (Control well)}} \times 100 \quad (1)$$

where O.D is optical density.

3 Results and discussion

3.1 Verification of supramolecular interactions

This phenomenon is based on the fact, that different chemical bonds and functional groups having several vibrational modes absorb in the infrared region and have characteristic absorption peaks. FTIR analysis was done to observe the variations in the vibrational modes of functional groups as a consequence of new supramolecular interactions. Spectra of co-crystals of API and co- former were studied comparatively to the spectrum of API alone and shifting of different peaks such as N-H and C=O, from peaks of API spectrum were due to involvement of these groups in hydrogen bonding between them. Absorption frequencies of 5-FU, all the co formers and co-crystals are

represented in Table 1. In the IR spectrum of unsubstituted or non-derivatized 5-FU, a rounded peak at 3409.02 cm^{-1} can be accredited to the absorption frequency of (N-H) group (stretching) and a wide sharp band of high intensity at wavenumber, 1647.77 cm^{-1} can be assigned to *ν*(C=O) groups (stretching) [42, 43].

3.1.1 5-FU-Cinnamic acid (5-FU-Cn)

For the development of 5-FU supramolecular synthones with Cinnamic acid; the already present –N-H…N-H interactions were supposed to be replaced by –N-H…O-H or –N-H…O=C as shown in Figure 2. The larger molecule of cinnamic acid is associated with enhanced steric hindrance. This leads to weaker supramolecular interactions. These weak intermolecular interactions result in strong intramolecular interactions, therefore; it was expected that –N-H groups will absorb at an increased stretching frequency. Noninvolvement of molecules in the supramolecular interactions are reduced stretching of intramolecular bonds and in turn increase strength. The observation of 3432.03, 3566.89 cm^{-1} peaks in the spectrum of co-crystal with a significant blue shift, as clear from Figure 1, is supporting the successful synthesis of co-crystals and in an agreement with the results of Nadzri *et al*.,[33]. This observation is further supported by the shift of -C-N absorption frequency towards shorter wavelength (blue shift). In the spectrum of 5-FU, the *ν* (-C-N) was observed at 1242.00 cm^{-1} which shifted hypochromially at 1252.47 in the spectrum of 5-FU-Cn supramolecular synthons[38]. On the other hand, the trend of absorption frequency of -C=O groups at 1673.13 cm^{-1} is according to the trend reported in the literature[33] following hypochromic shift. Again, the increased strengthening of –C=O bond may be the consequence of increased steric hindrance and bigger molecular size of co-former also proposed in Figure 2. This is also proved true after the peak at 1179.10 cm^{-1} (-C-O) [42] in the 5-FU spectrum diminished in the co-crystal

spectrum due to the vanished single bond properties of carbonyl groups in the course of co-crystal formation.

3.1.2 5-FU-Succinic acid (5-FU-Sc)

The –N-H absorption frequency for 5-FU-Sc co-crystals was observed at 3543.53 cm^{-1} clear from Figure 4 that manifesting the same blue shift as discussed for the above case exactly in accordance with Nadzri *et al.*,[33]. Moreover, this observation can also be justified by the same trend of a blue shift in the absorption frequencies of -C-N groups; 1346.62cm^{-1} (5-FU) and 1437.33 cm^{-1} (5-FU-Sc)[38].

The absorption frequency of carbonyl groups was observed at 1644.51 cm^{-1}. This frequency seems to be almost at the same frequency in comparison to that of un-substituted API and bathochromially shifted as compared to the co-former [23] as shown in Figure 3. Moreover, the absorption frequencies of -C-O were also observed at almost same positions; 1179.10 cm^{-1} (5-FU) and 1174.75 cm^{-1} (5-FU-Sc) [42]. This suggests the efficient involvement of carbonyl groups of co-former in the development of hydrogen bonding interaction as compared to 5-FU. This may be the result of more steric hindrance of the cyclic structure of 5-FU than the aliphatic structure of succinic acid as depicted in Figure 4.

3.1.3 5-FU-Maleic acid (5-FU-Ml)

Following the usual hypochromic shift [20, 34], the –N-H absorption frequency of 5-FU-Ml co-crystals was detected at 3492.20 cm^{-1}, observed from the spectra Figure 5, clearly demonstrating the development of new supramolecular interactions as anticipated in Figure 6. As the maleic acid has almost the same structure as succinic acid, therefore, a same trend of absorption of carbonyl was observed. This frequency of carbonyl groups was recorded at 1669.96 cm^{-1} (Figure 5); strongly

red shifted as compared to co-former and reverse is true in comparison to API. This observed trend is also affirmed by the observation of -C-O absorption frequencies of API and co-crystals at the shift of less than 10 cm^{-1} (non-significant); 1179.10 cm^{-1} (5-FU) and 1080.89 cm^{-1} (5-FU-Ml) [42]. This suggests the strong disturbance and involvement of carbonyl groups of co-former molecules while the very less involvement of carbonyl groups of API in the formation of non-covalent interactions between API and Co-former for the development of co-crystals. This could be explained by the bigger molecular size of malic acid that generates high steric hindrance while forming hydrogen bonds with the consecutive NH-CO-NH groups of 5-FU as manifested in Figure 6.

3.1.4 5-FU-Benzoic acid (5-FU-Bn)

The absorption frequency of –N-H group was observed at 3431.72 cm^{-1} with regular hypochromic shift and the frequency of –C=O was found at 1672.37 cm^{-1} (Figure 7). The hypochromic shift of –N-H group was further tallied with the same shift of -C-N group frequency. Absorption frequency of -C-N in the 5-FU spectrum was observed at 1242.00 cm^{-1} while this group in the co-crystal spectrum shown their IR absorption at 1284.12 cm^{-1} [38]. On the other hand, the carbonyl group frequency of 5-FU at 1647.77 cm^{-1} is missing in the co-crystal spectrum. As the 1672.37 cm^{-1} is matching with v(C=O) of co-former (1674.98cm^{-1}), therefore; it could be proposed that the double bond character of the carbonyl group of 5-FU was diminished as a result of strong involvement of this group for the development of supramolecular interactions. Both these frequencies followed the same trend as discussed above for all the other co-crystal forms. The proposed hydrogen bonding interactions which could be developed are represented in Figure 8. In short, in all the co-crystal forms, significant shifts in absorption frequencies of anticipated peaks with almost the same

trends were observed. The observed trends in all the cases are in the favour of an effective formation of new non-covalent interactions.

3.2 Distinction of API and co-crystals` structure by PXRD

Once the development of supramolecular interactions was clearly recognized through FTIR analysis, the formation of co-crystals was supplementarily indorsed with the help of PXRD. The intensity, 2θ values, FWHM (full width at half maximum) values of most protruding peaks and crystal size of API and all the co-crystals are arranged in Table 2. The values of intensity are representative of preferred orientation as well as crystallinity of molecules. FWHM not only provides the comparison of data distribution under the curve of most prominent peaks but is also important to find out the crystal size. The characteristic peaks in the spectrum of 5-FU are clearly different from the respective peaks in the co-crystal spectra in all the above-mentioned aspects as well as crystal size. These differences are observable with respect to both the shapes and the intensity of the peaks as manifested in Figure 9. These differences are obviously suggestive of the changes in the structural properties of API as a consequence of the changes in supramolecular interactions with different co-formers [34, 44].

It is observable from the API and co-crystals` stacked graph in Figure 9, the peak observed at 2θ =28.80 °, precisely the same as stated in [34], is the most strong representative peak of 5-FU. This illustrative value of 5-FU appeared to be prominently different from the values observed in all the graphs of co-crystals [23]. For 5-FU-Cn co-crystals the most prominent, intense peak appeared at 27.78°. The intensity of this peak is lesser than the most prominent peak of API manifesting decreased preferred orientation and in turn, reduced crystallinity. Similarly, in the case of 5-FU-Sc co-crystals the intensity of most prominent peak is very low in comparison to that of API

demonstrating the very small preferred orientation and lessened crystallinity. This finding is also supported by the strongest (-N-H) stretching frequencies of 5-FU-Cn and 5-FU-Sc co-crystals with the help of FTIR analysis that manifests the strengthening of intramolecular interactions as a consequence of weakening of intermolecular interactions as a result of the intervention of co-former molecules. This resulted in the disturbance of already present supramolecular interactions in 5-FU crystal structure due to the development of interactions between 5-FU and co-former molecules. Moreover, the crystal size of 5-FU-Sc and 5-FU-Cn co-crystals is smaller than the size of API manifesting the formation of co-crystals in the amorphous form [45].

While if we Consider 5-FU-Ml and 5-FU-Bn co-crystals, both have their most prominent peaks at 28.72 º and almost matching and very high intensities. This observation is revealing much enhanced crystallinity as a result of very high preferred orientation. The crystal size of both of these co-crystals is much bigger than the 5-FU crystal size. The observation of almost similar peaks in 5-FU-Ml and 5-FU-Bn co-crystals may be as a consequence of the same type of groups responsible for hydrogen bonding interactions in the same directions. Moreover; the crystal size of both the co- formers are almost the same. Besides these facts, there are many other peaks in the spectrum of 5-FU which are missing in all the spectra of co-crystals also clear from Figure 9. Furthermore, in all the four spectra of co-crystals, many new peaks are found which are indicative of the formation of entirely new and different structural entities than 5-FU [34]. From all the four co-crystal forms; 5-FU-Ml and 5-FU-Bn proved to be the entities with the highest crystallinity, having bigger crystallite size than the other two co-crystals.

3.3 Assessment of Anticancer potential of co-crystals through MTT assay

Table 3 incorporates the values of percent growth inhibition of actinomycetes *in vitro* at four different concentrations in HCT-116 colorectal cell lines, after application of 5-FU alone and all the four co-crystals of 5-FU[37]. For the evaluation of the most effective growth inhibiting agent, values are represented comparatively in Figure 10 to Figure 13. It is clear from the observed values that as the concentration of actinomycetes is increased, the value of percent growth inhibition is also increased and maximum growth inhibition is observed at 100 ug/mL for API and all the co-crystals. This trend is quite rational as the increased concentration of actinomycetes provides increased substrate sites for the applied agents to execute and subsequently the numerical value of growth inhibition is also increased.

After a comparison of all the co-crystals, it is clear that 5-FU-Cn co-crystals proved to be the highest growth inhibiting agents at all the four concentrations. At 100 µg/mL concentration, the growth inhibiting potential of 5-FU-Cn co-crystals is 67.30%, highest among all the synthesised supramolecular synthons. The reason behind this observation may be the anticancer potential of cinnamic acid itself. Cinnamic acid derivatives were widely studied and employed as potent antitumor agents. The α,β-unsaturated carbonyl moiety in cinnamic acid is considered as an active site utilized for the design of anticancer drugs [46]. The other most important reason also evident from the FTIR and PXRD analysis is the loose bonding (FTIR) and less crystallinity (PXRD) of 5-FU-Cn co-crystals, resulted in easy release of API and co-former molecules and their prompt action. The second most potent anticancer agent as clear from the values of Table 3 are 5-FU-Bn co-crystals at all the four concentrations; 66.92% growth inhibition at 100 µg/mL. While the 5-FU-Ml and 5-FU-Sc cocrystals have mixed trend. However, at 100 µg/mL, 5-FU-Ml acid has

greater growth inhibition, 44.55% than 5-FU-Sc (38.91%). The reason behind this variable end observed for 5-FU-Sc and 5-FU-Ml co-crystals is not clear. Dissimilar growth inhibiting strengths of API and all the co-crystals may be attributed to a different structural characteristic of co-formers and diverse interactions between API and co-formers.

4 Conclusion

Four different co-crystals were synthesized following the solid state grinding followed by normal slow evaporation solvent method. The solvent used in this study to facilitate supramolecular interactions was acetone; not used before in any study on a similar concept. Co-crystals were successfully formed in all four cases at room temperature. FTIR and PXRD analysis of API and cocrystals clearly indicated the development of hydrogen bonding interactions between API and all the co-formers. In the FTIR spectrum of 5-FU, the absorption frequencies of main anticipated peaks that is N-H and –C=O were found at 3409.02 cm^{-1} and 1647.77 cm^{-1} respectively. Both of these peaks were clearly shifted in all the spectra of co-crystals exactly according to the trends reported in the literature. These shifts were clearly indicative of alterations in the intermolecular interactions of 5-FU molecules as a consequence of intervention of co-former molecules. Moreover, in the PXRD spectrum, the most prominent peak of 5-FU was recorded at $2\theta = 28.80$. This peak was not detected in any spectrum of co-crystals; further, in all the spectra of co-crystals many new peaks were recorded manifesting the development of entirely different moieties after co-crystallization. After the successful formation of co-crystals in all the four cases, their anticancer effectiveness was evaluated utilizing HCT-116 colorectal cell lines at four different concentrations of synthesized co-crystals. All the four co-crystals proved to be more effective than API alone at all the four concentrations. Moreover, 5-FU-Cn (3C) co-crystals at 100 ug/mL proved to be the most potent anticancer agent in this study. In short, this study proved the formation of

compelling prodrugs of 5-FU following very easy synthesis procedure and conditions. As this method is quite captivating from the point of view of its tireless synthesis requirements and diligent outcomes; these co-crystals can be further optimized for their *in vivo* safety. Moreover; many other potentially effective co-formers could also be studied in future.

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Table 1. FTIR absorption frequencies of the main peaks of interest.

Sample ID	*v* (C=O) cm^{-1}	*v* (N-H) str cm^{-1}
5-FU	1647.77	3409.02
5-FU-Cn (3C)	1673.13	3432.03, 3566.89
Cn (3)	1668.29	-
5-FU-Sc (4C)	1644.51	3543.53
Sc (4)	1676.04	-
5-FU-Ml (5C)	1669.96	3492.20
Ml (5)	1688.89	-
5-FU-Bn (6C)	1672.37	3431.72
Bn (6)	1674.98	-

Table 2. 2θ, FWHM and crystallite size of 5-FU and co-crystals using Scherrer equation.

Sample ID	**Intensity In counts**	**2θ in Degrees**	**Θ**	**θ in Radians**	**Cos θ**	**FWHM**	**Crystallite Size (A^o)**
5-FU	1156	28.80^o	14.40	0.25	0.969	0.24	5.96
5-FU-Cn (3C)	1086.54	27.78^o	13.89	0.24	0.971	0.32	4.41
5-FU-Sc (4C)	787.92	28.78^o	14.39	0.25	0.969	0.25	5.79
5-FU-Ml (5C)	4544.86	28.72^o	14.36	0.25	0.969	0.16	8.74
5-FU-Bn (6C)	4529.28	28.72^o	14.36	0.25	0.969	0.17	8.74

Table 3. Percentage growth inhibition in HCT-116 cell lines using different concentrations of API and Synthesized co-crystals.

Sample ID	**Concentrations of actinomycetes**			
	12 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
5FU	7.89±0.64	16.21±0.20	39.53±0.56	54.28±0.56
3C	24.81±0.42	34.21±0.41	55.08±0.53	67.29±1.73
4C	14.85±0.57	18.42±0.42	32.89±0.50	38.91±0.51
5C	9.97±0.90	26.13±0.53	31.02±0.58	44.55±0.57
6C	23.31±0.58	29.89±0.65	53.57±0.33	66.92±0.63

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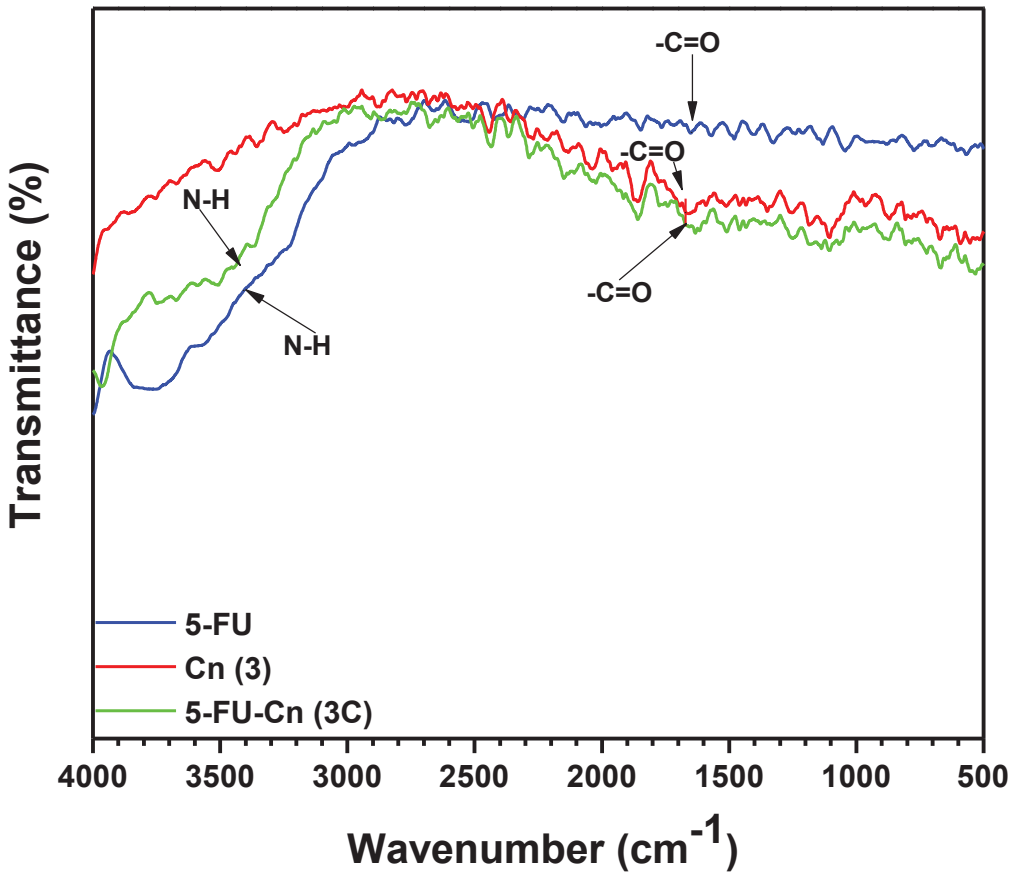

Figure 1. IR spectra of 5-FU, Cn (Cinnamic acid) co-former and 5-FU-Cn co-crystals.

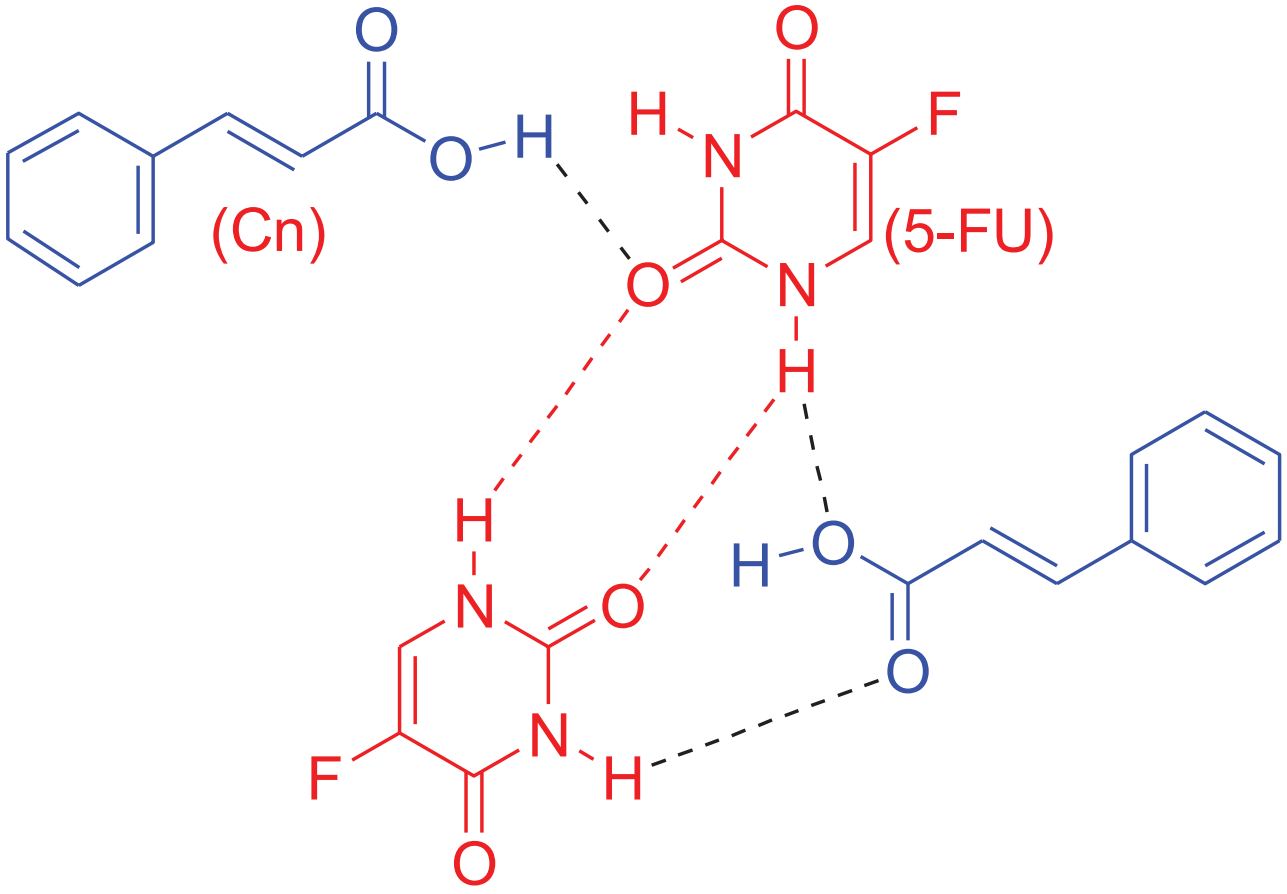

Figure 2. Proposed supramolecular interactions in 5-FU-Cn co-crystals.

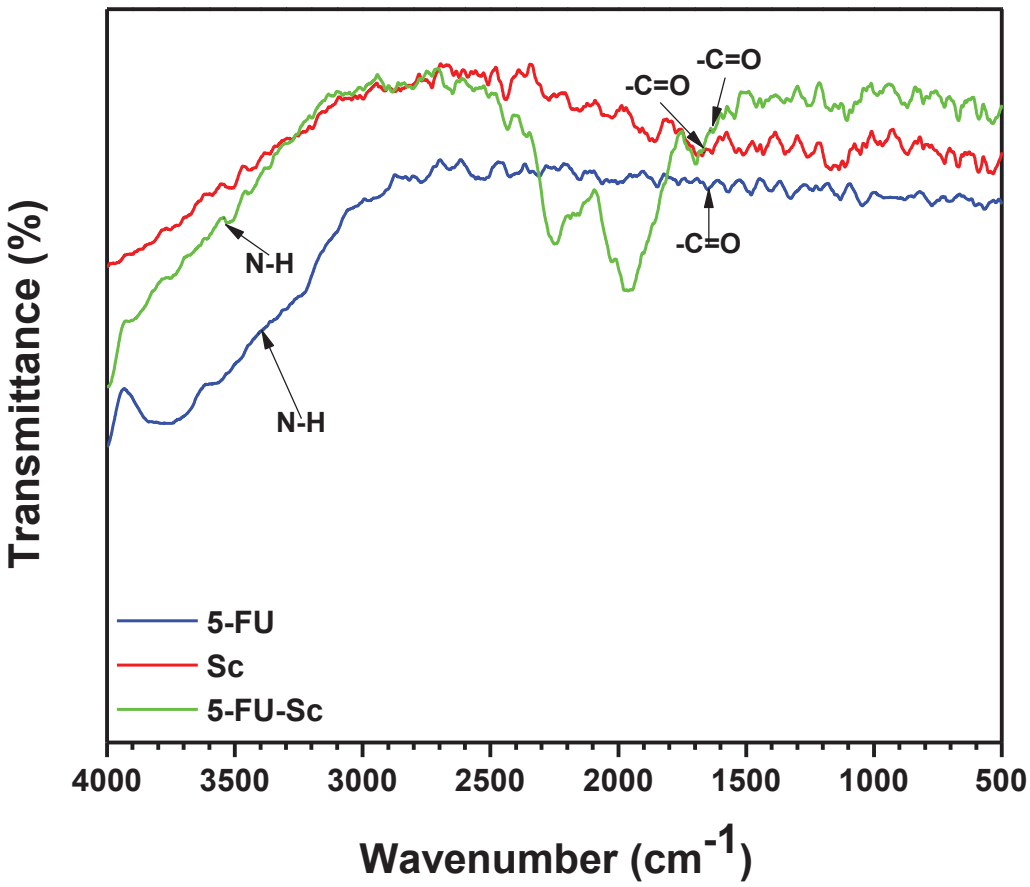

Figure 3. IR spectra of 5-FU, Sc (Succinic acid) co-former and 5-FU-Sc co-crystals.

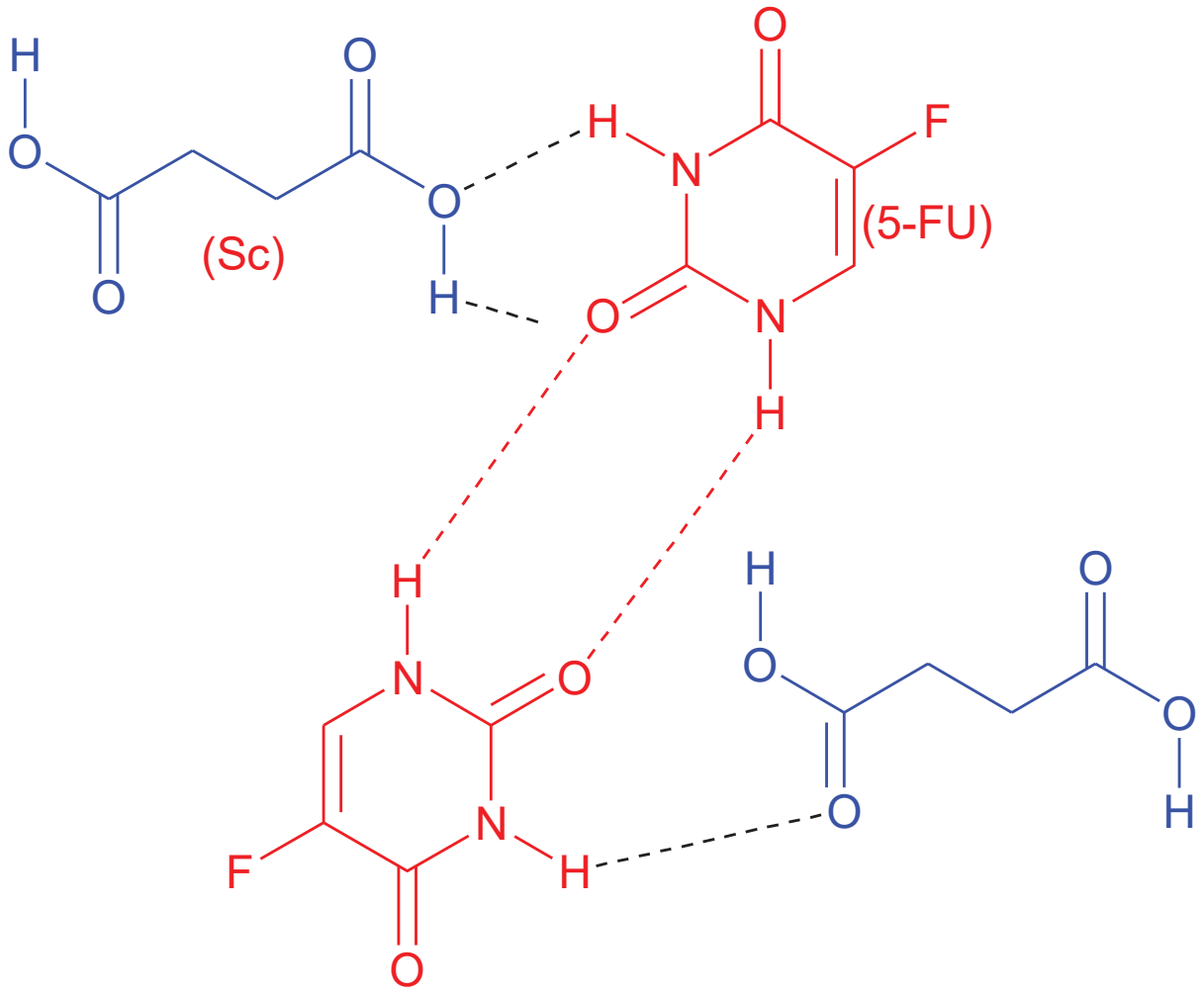

Figure 4. Proposed supramolecular interactions in 5-FU-Sc co-crystals.

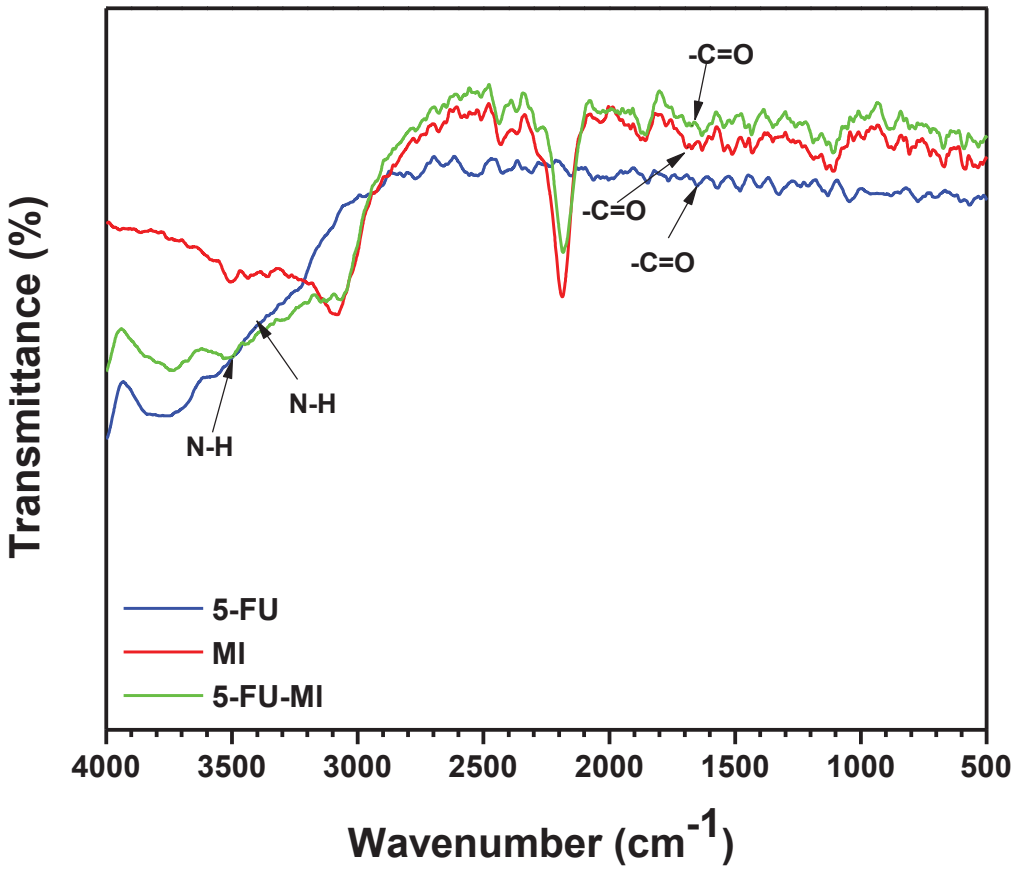

Figure 5. IR spectra of 5-FU, Ml (Malic acid) co-former and 5-FU-Ml co-crystals.

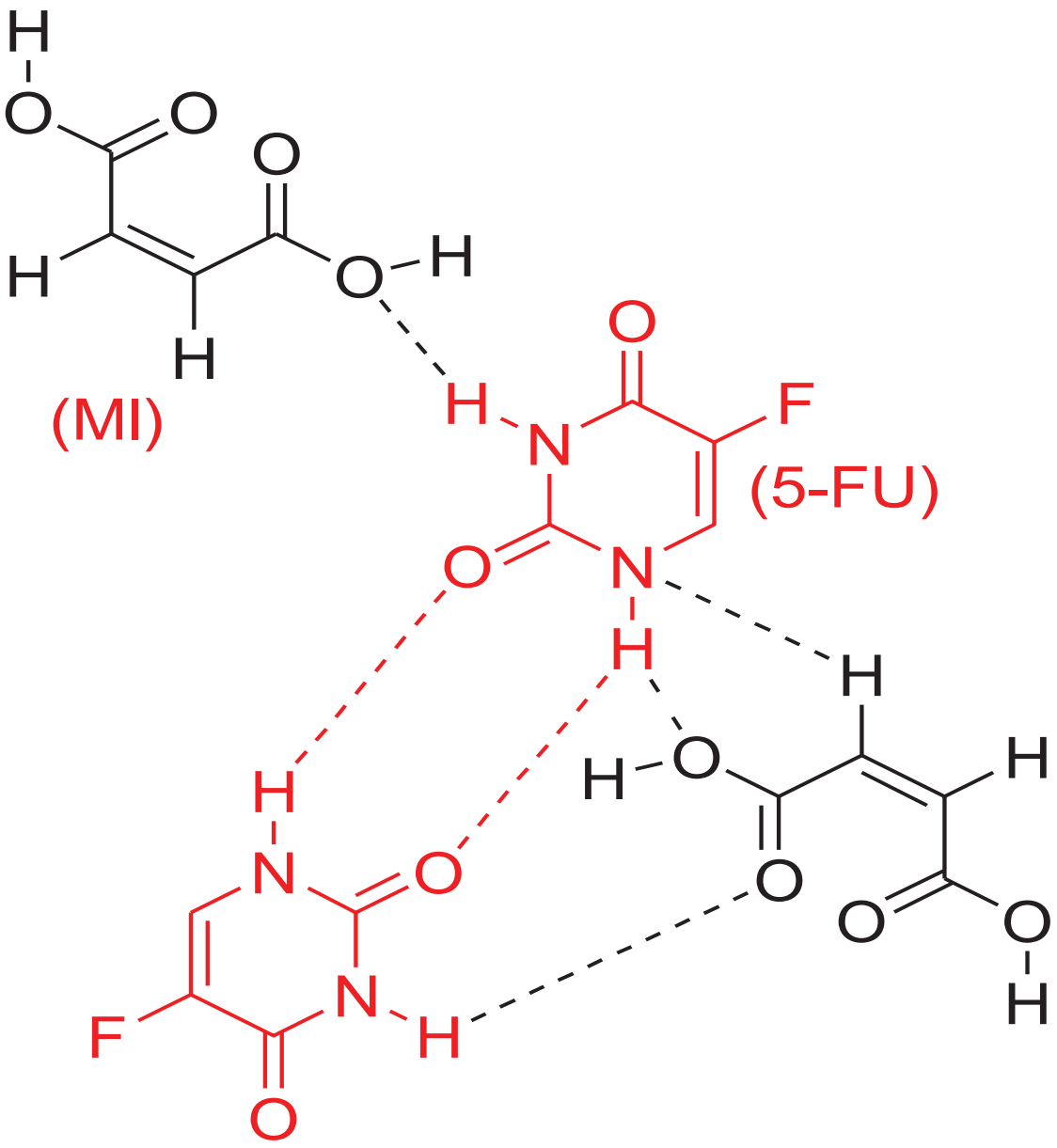

Figure 6. Proposed supramolecular interactions in 5-FU-Ml co-crystals.

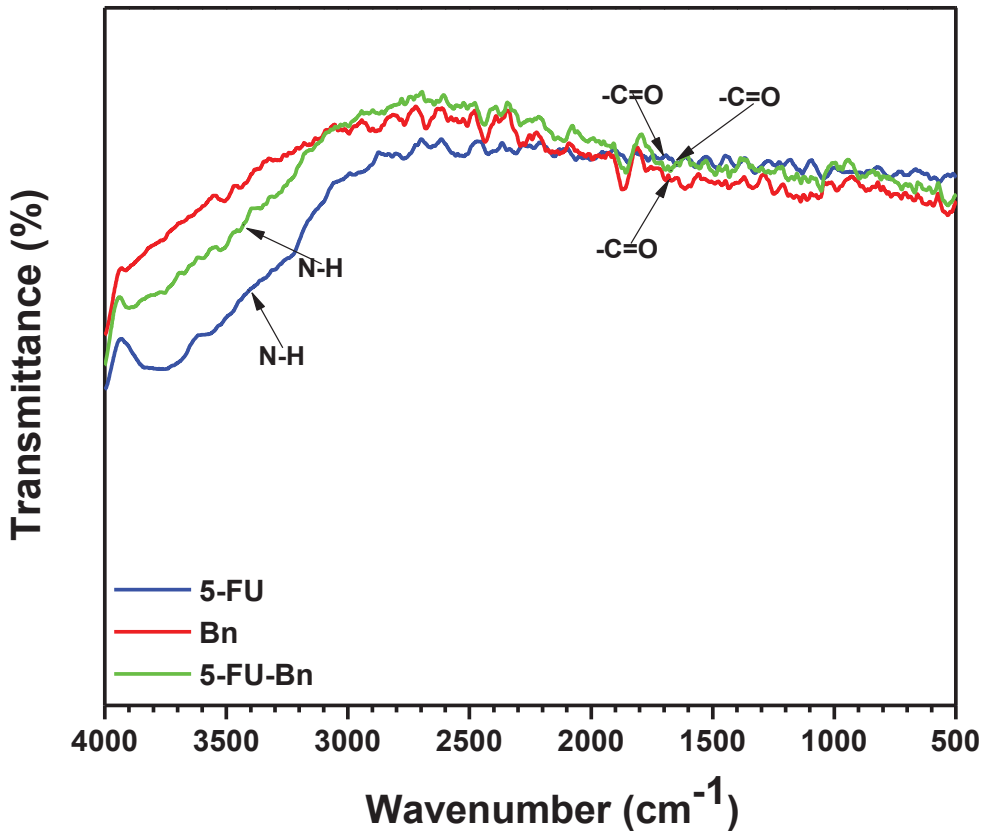

Figure 7. IR spectra of 5-FU, Bn (Benzoic acid) co-former and 5-FU-Bn co-crystals.

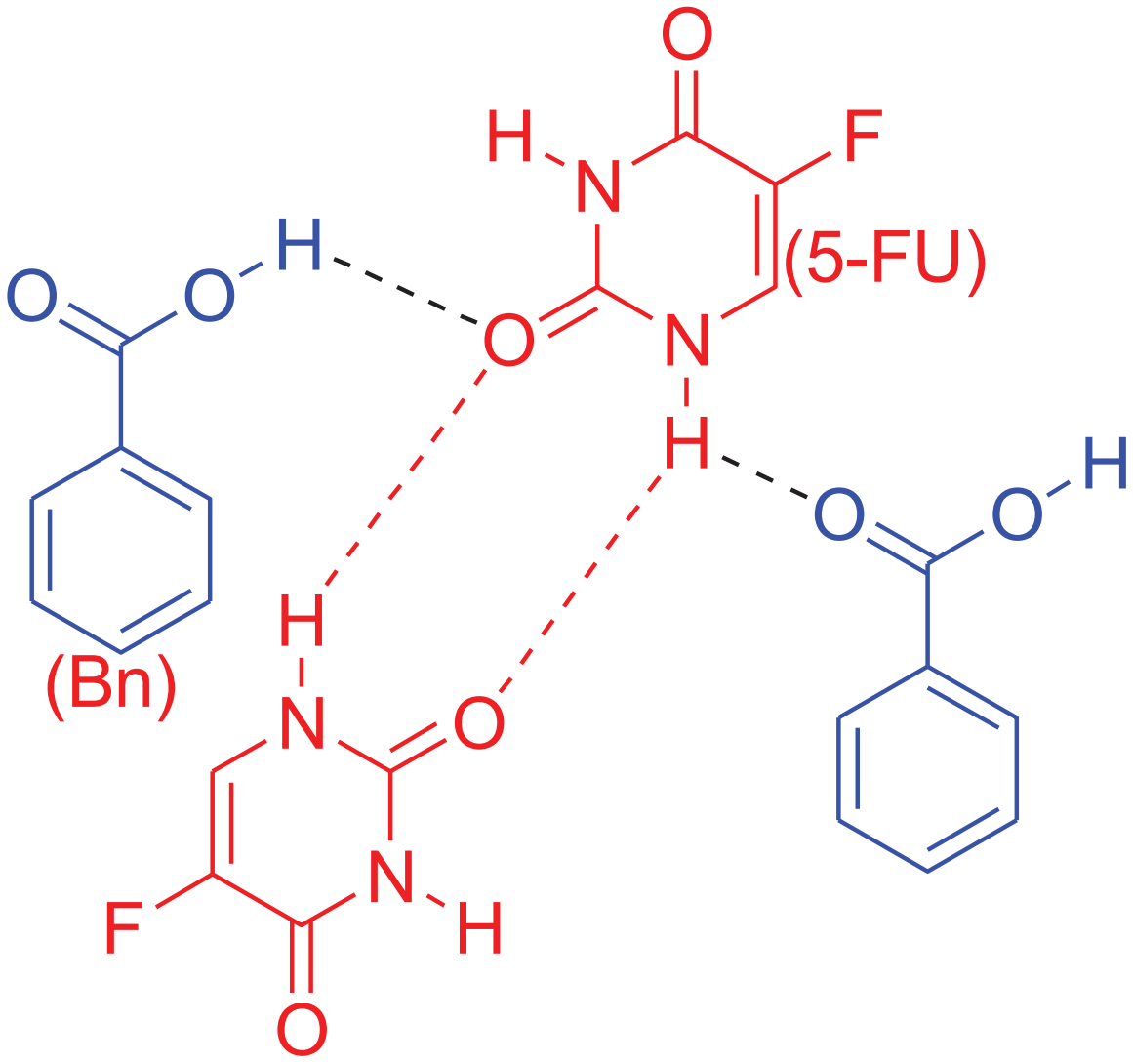

Figure 8. Proposed supramolecular interactions in 5-FU-Bn co-crystals.

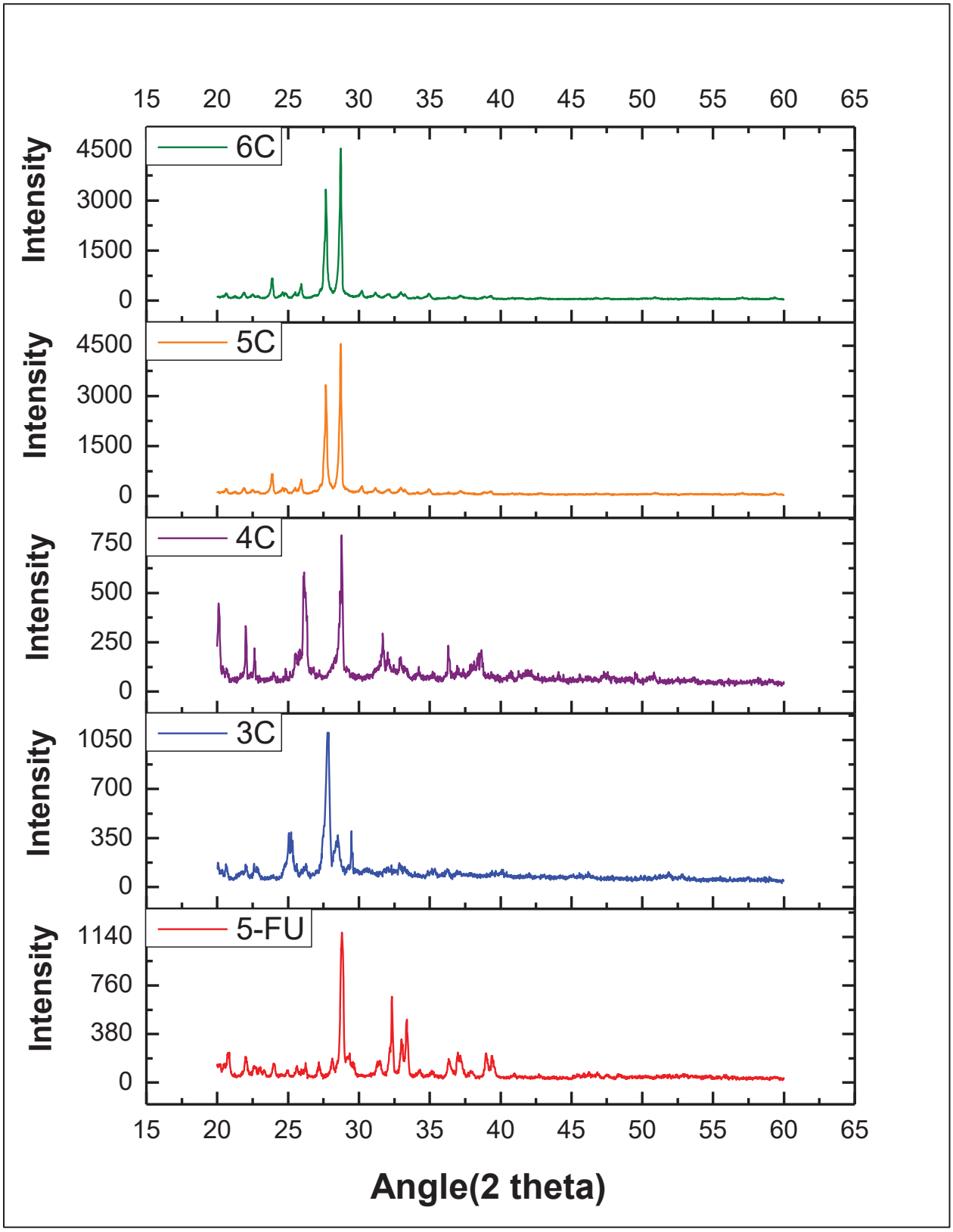

Figure 9. PXRD spectra of API and co-formers.

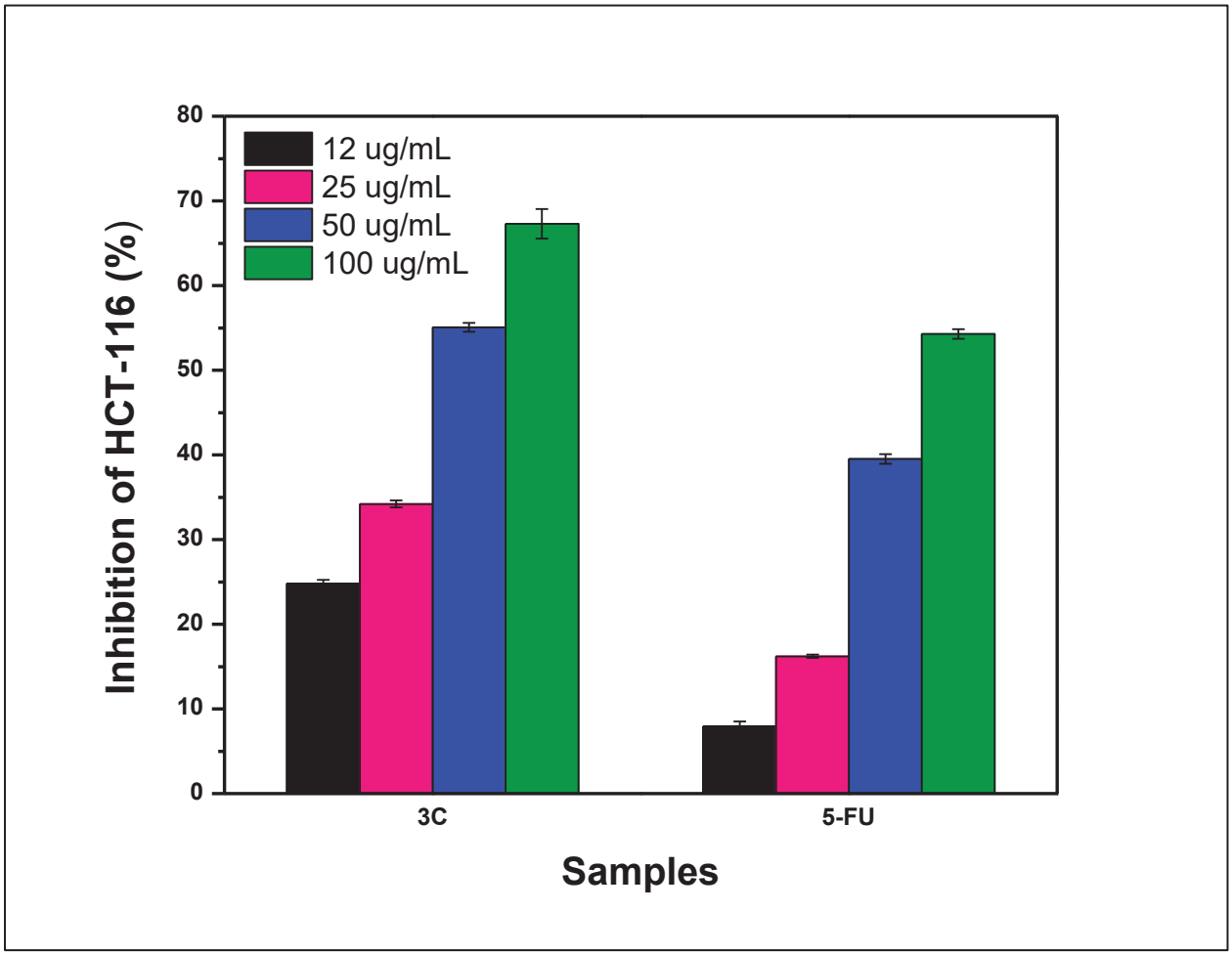

Figure 10. Comparison of percentage growth inhibition of 5-FU-Cn co-crystals with 5-FU alone at varying concentrations of API and synthesized co-crystals against HCT-116 colorectal cell lines.

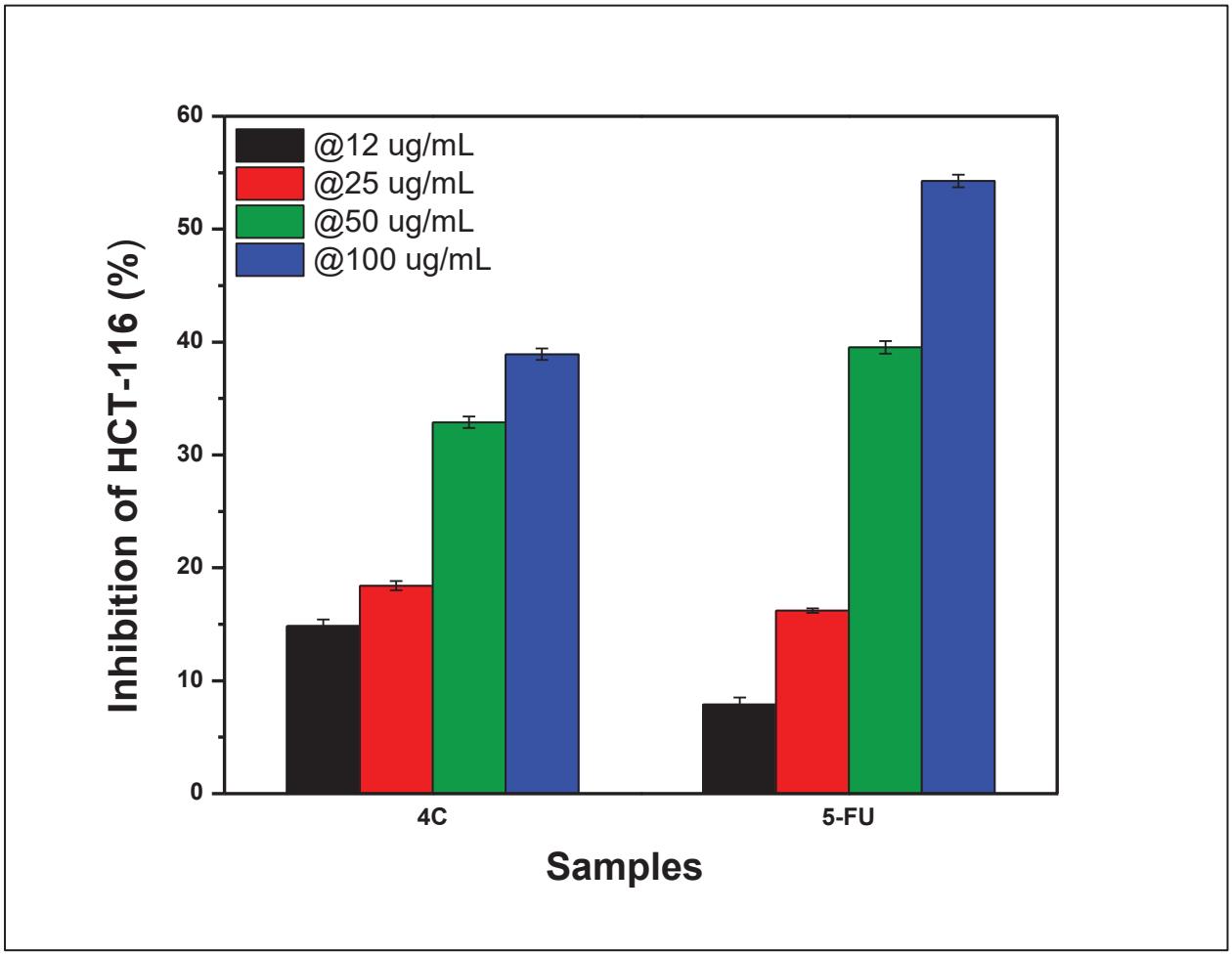

Figure 11. Comparison of percentage growth inhibition of 5-FU-Sc co-crystals with 5-FU alone at varying concentrations of API and synthesized co-crystals against HCT-116 colorectal cell lines.

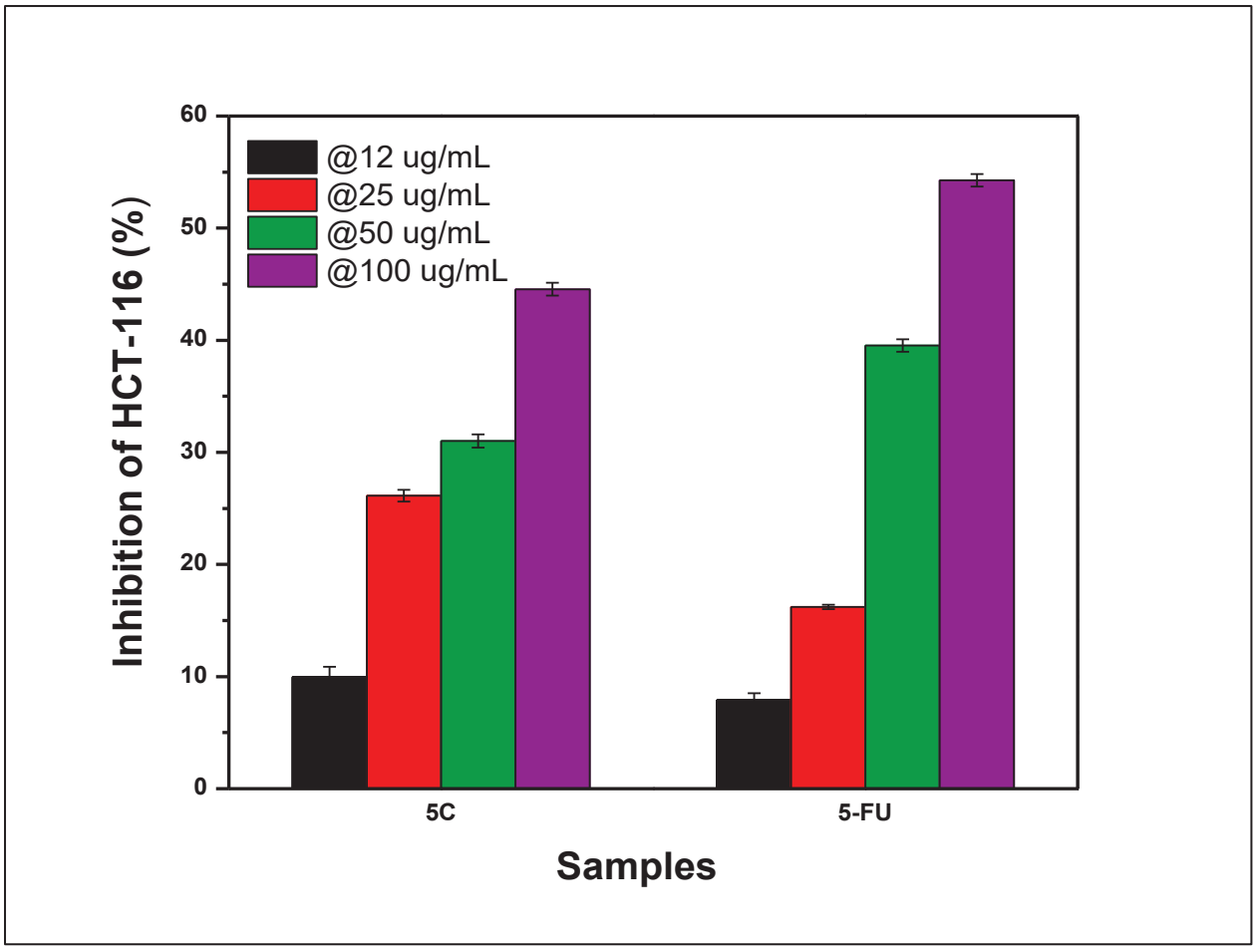

Figure 12. Comparison of percentage growth inhibition of 5-FU-Ml co-crystals with 5-FU alone at varying concentrations of API and synthesized co-crystals against HCT-116 colorectal cell lines.

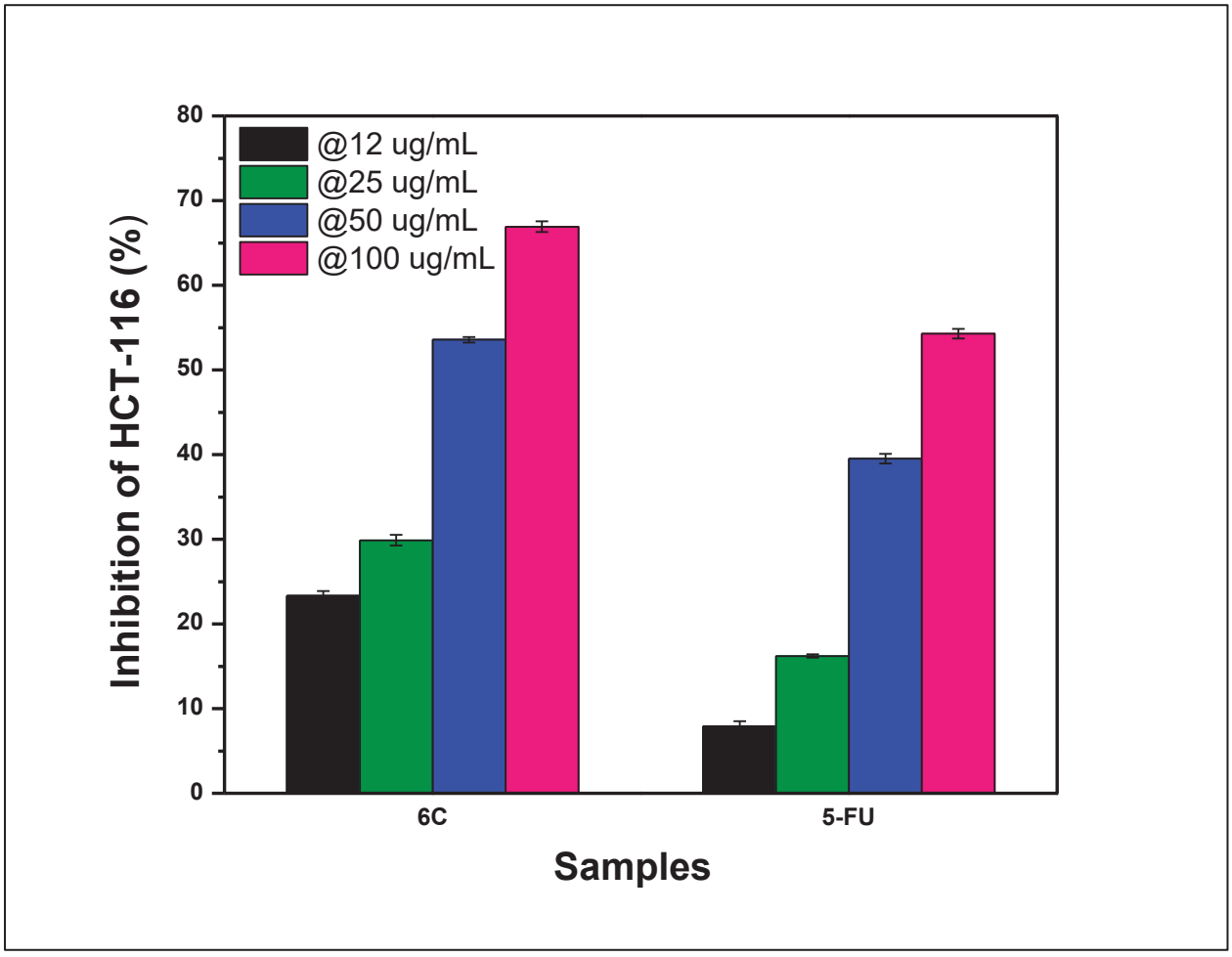

Figure 13. Comparison of percentage growth inhibition of 5-FU-Bn co-crystals with 5-FU alone at varying concentrations of API and synthesized co-crystals against HCT-116 colorectal cell lines.

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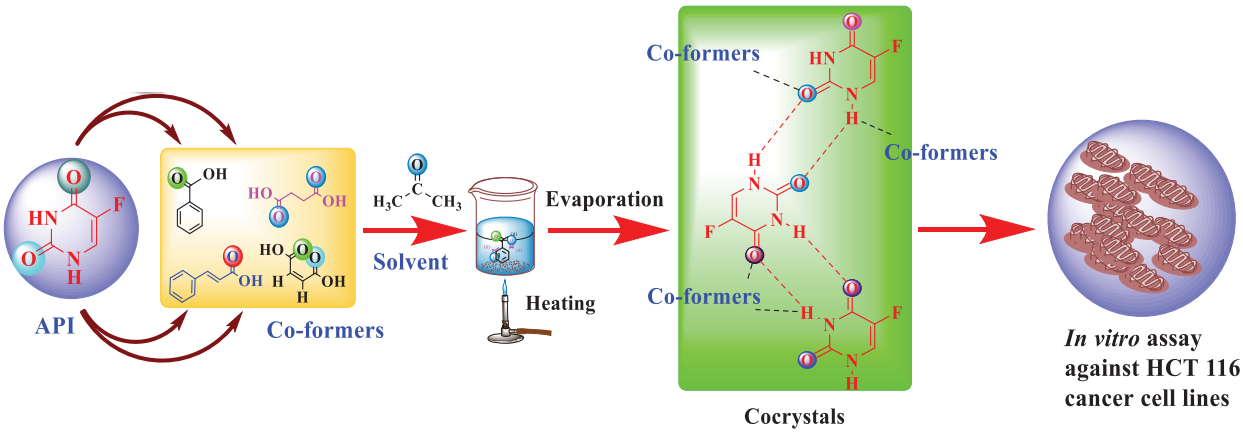
Synthesis of 5-fluorouracil co-crystals with novel organic acid as co-formers and its anticancer evaluation against HCT-116 colorectal cell lines

Farhat Jubeen, Aisha Liaqat, Fiza Amjad, Misbah Sultan, Sania Zafar Iqbal, Imran Sajid, Muhammad Bilal Khan Niazi, Farooq Sher



Co-crystallization technique to develop prodrugs of 5-Flourouracil with four organic acids: Succinic acid, cinnamic acid, malic acid and benzoic acid was adopted. Characterization and anticancer potential of the synthesized crystalline prodrugs were accomplished through FTIR, PXRD and MTT assay against HCT-116 colorectal cell lines. All interactions in synthesized prodrugs developed following vander walls interactions and exhibited greater cytotoxicity than 5-Flourouracil.